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BIOCHEMICAL AND GENETIC MARKERS OF MINERAL BONE DISEASE IN SOUTH AFRICAN PATIENTS WITH CHRONIC KIDNEY DISEASE

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

By

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Student number: 1111800

Johannesburg, 2017

DECLARATION

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

10th October, 2017

DEDICATION

To patients with Chronic Kidney Disease

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS RESEARCH

Waziri B, Duarte R, Naicker S. Biochemical markers of mineral bone disorder in South African patients on maintenance haemodialysis. Afri Health Sci. 2017;17(2): 445-45

Waziri B, Duarte R, Naicker S. High serum alkaline phosphatase, hypercalcaemia, race and mortality in South African maintenance haemodialysis patients. Int J Nephrol. 2017; 2795432. doi: 10.1155/2017/2795432. Epub 2017 Jan 12.

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Waziri B, Duarte R, Naicker S. Association between biochemical markers of mineral bone disease and mortality in maintenance haemodialysis patients. Poster presentation at the World Conference of Nephrology, Mexico. April 24- 27, 2017. Winner of the 3rd prize of the ISN Fellowship Awards 2017.

ABSTRACT

Background

Abnormalities of mineral bone disease have been consistently associated with adverse clinical outcomes in patients with chronic kidney disease (CKD). The consequences of these changes have also been shown to differ across races. However, in Africa the impact of derangements of CKD -mineral and bone disorder (CKD-MBD) on patients with CKD is largely unknown. In addition, studies from the USA have reported racial variations in markers of CKD and it remains unclear whether genetic factors may explain this discrepancy in the levels of biochemical markers of CKD-MBD across ethnic groups. Therefore, this study has been conducted to determine the existence of racial differences in the levels of fibroblast growth factor 23(FGF23) and traditional markers of mineral bone metabolism in a heterogeneous African CKD population, and to provide important insights into the pattern and genetic variability of CKD-MBD in sub-Saharan Africa.

Methods

This was a cross sectional multicenter study carried out from April 2015 to May 2016, involving two hundred and ninety three CKD patients from three renal units in Johannesburg, South Africa. The retrospective arm of this study involved two hundred and thirteen patients undergoing maintenance haemodialysis (MHD) from two dialysis centers in Johannesburg between January 2009 and March 2016. The first part of this study described the pattern of CKD-MBD in MHD patients using traditional markers of CKD-MBD. The second part of the study looked into the spectrum of CKD-MBD and racial variations in markers of CKD-MBD in pre dialysis and dialysis patients. This was followed by the genetic aspect of the study that examined the influence of vitamin D receptor polymorphisms on biochemical markers of mineral bone disorders. Lastly, the study also evaluated the association between markers of CKD-MBD and mortality in MHD patients.

Results

The prevalence of hyperparathyroidism (iPTH>150 pg/mL), hyperphosphataemia, hypocalcaemia and 25-hydroxyvitamin D deficiency (<30 ng/mL) was 73.4%, 57.0%, 20.3% and 80.7 % respectively in our MHD patients. The combination of markers of bone turnover (iPTH>150 pg/mL and total alkaline phosphatase > 112 U/L) suggestive of high turnover bone disease, was present in 47.3 % of the study population. The odds ratios for developing secondary hyperparathyroidism with hypocalcaemia and hyperphosphataemia were 5.32 (95% CI 1.10 - 25.9, P =0.03) and 3.06 (95 % CI 1.15 - 8.10, P =0.02) respectively.

The 293 CKD patients (208 blacks, 85 whites) had an overall mean age of 51.1±13.6 years, and black patients were significantly younger than the white patients (48.4 ±.13.6 versus 57.1±15.5 years; p<0.001). In comparison to whites, blacks had higher median iPTH (498 [37-1084] versus 274[131-595] pg/ml; P=0.03), alkaline phosphatase (122[89-192] versus 103[74-144] U/L; P=0.03) and mean 25- hydroxyvitamin D (26.8±12.7 versus 22.7 ±12.2 ng/ml, P=0.01) levels, while their median FGF23 (100 [34-639] versus 233[80-1370] pg/ml; P=0.002) and mean serum phosphate (1.3±0.5 versus 1.5±0.5, P =0.001) levels were significantly lower.

With the exception of vitamin D receptor (VDR) *Taq I* polymorphism, the distribution of the VDR polymorphisms differs significantly between blacks and whites. In hemodialysis patients, the *BsmI* Bb genotype was significantly associated with moderate secondary hyperparathyroidism (OR, 3.88; 95 CI 1.13-13.25, P=0.03) and severe hyperparathyroidism (OR, 2.54; 95 CI 1.08-5.96, P=0.03).

Patients with high total alkaline phosphatase (TAP) had significantly higher risk of death compared to patients with TAP <112 U/L (hazard ratio, 2.50; 95% CI 1.24–5.01, P = 0.01). Similarly, serum calcium >2.75 mmol/L was associated with increased risk of death compared to patients within levels of 2.10–2.37 mmol/L (HR 6.34, 95% CI 1.40–28.76; P = 0.02). The HR for death in white patients compared to black patients was 6.88; 95% CI 1.82–25.88; P = 0.004.

Conclusions

Secondary hyperparathyroidism and 25–hydroxyvitamin D deficiency were common in our haemodialysis patients. The study also highlighted the existence of racial differences in the circulating markers of mineral bone disorders in our African CKD population. In addition, the study showed that both moderate and severe secondary hyperparathyroidism are predicted

by the *BsmI* Bb genotype, and the over expression of this genotype in black patients may partly explain the ethnic variations in the severity of secondary hyperparathyroidism in the CKD population. High levels of serum alkaline phosphatase, hypercalcaemia, and white race are associated with increased risk of death in MHD patients.

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I also wish to thank my wife Hauwa Muhammad Mayaki and my mum Aisha Musa, for their patience and support.

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LIST OF ABBREVIATIONS

b- ALP: Bone specific alkaline phosphatase

BSP: Bone Sialoprotein

CaSRs: Calcium sensing receptors

CKD: Chronic kidney disease

CKD-MBDCKD-MBD: Chronic kidney disease- mineral and bone disorder

CTX: C-terminal telopeptide of type I collagen.

DEQAS: Vitamin D external quality assurance scheme

DM: Diabetes mellitus

DNA: Deoxyribonucleic acid

DOPPS: Dialysis Outcomes and Practice Patterns

ECLIA: electrochemiluminescence immunoassay

EDTA: Ethylene diamine tetra acetic

eGFR : Estimated glomerular filtration rate

ESAs: Erythropoiesis-stimulating agents

ESKD: End stage kidney disease

FGF23: Fibroblast Growth Factor 23

HERO: Handling erythropoietin resistance with oxypentifylline

HPLC: High performance liquid chromatography

Hazard ratio: HR

IFCC: International Federation of Clinical Chemistry and Laboratory Medicine

IOF: International Osteoporosis Foundation

iPTH: Intact Parathyroid hormone

KDIGO: Kidney Disease -Improving Global Outcomes

K/DOQI: Kidney Disease Outcomes Quality Initiative

MHD: Maintenance haemodialysis

MDRD: Modified Diet Renal Disease

MESA: Multi ethnic study of atherosclerosis

NHANES: National Health and Nutrition Examination Survey

NKF: National Kidney Foundation

1,25 $(OH)_2D_3$: 1, 25-dihydroxyvitamin D

25(OH) D: 25- hydroxyvitamin D

OR: Odds ratio

OC: Osteocalcin

PCR: Polymerase chain reaction

PINP: Procollagen type I N propeptide

PICP: Carboxy (C-) terminal propeptide

ROD: Renal osteodystrophy

SNPs: Single nucleotide polymorphisms

TAP: Total alkaline phosphatase

TRAP 5b: Tartrate resistant acid phosphatase

UK: United Kingdom

USA: United States of America

VDR: Vitamin D receptor

PREFACE

Despite the existence of regional and global guidelines to curtail the adverse clinical outcomes associated with CKD-MBD, the majority of CKD patients are still affected by the consequences of abnormalities of CKD-MBD. In Africa, the spectrum and burden of CKD-MBD is largely unknown. In addition, studies from the USA have shown racial variations in markers of CKD-MBD, and survival advantage associated with black patients on haemodialysis. However, due to variations in practice pattern, geographic location, and genetic factors between Africa and the USA, it remains unclear if data obtained from the USA CKD population could be extrapolated to African CKD patients. Furthermore, in 2013 the KDIGO working group highlighted the existence of large gaps in knowledge in the field of CKD-MBD and thus recommended the need for further studies to assist in updating the 2009 KDIGO clinical practice guidelines on the diagnosis and treatment of CKD-MBD.

Therefore, based on the above aforementioned reasons, this study was undertaken to determine the spectrum and burden of CKD-MBD in South African patients with CKD, the existence of ethnic variations in markers of CKD-MBD in a heterogeneous African CKD population, and the influence of VDR polymorphisms on secondary hyperparathyroidism. Information obtained from this study will assist us in better understanding of factors associated with mortality and with improving patients' outcomes. It will also provide an insight on candidate genes associated with secondary hyperparathyroidism and likely provide an additional target for the treatment of secondary hyperparathyroidism.

This thesis is presented in an integrated format consisting of seven chapters:

Chapter 1: Literature review

Chapter 2: Overall Methodology

Chapter 3: Manuscript on biochemical markers of mineral bone disorder in South African patients on maintenance haemodialysis. (Published in the African Health Sciences Journal July, 2017).

Chapter 4: Manuscript on high serum alkaline phosphatase, hypercalcaemia, race and mortality in South African maintenance haemodialysis patients. (Published in the International Journal of Nephrology, 12th January, 2017)

Chapter 5: Manuscript on racial variations in the markers of mineral bone disorders in chronic kidney disease patients in South Africa. Undergoing peer review by the Kidney International Reports.

Chapter 6: Manuscript on influence of vitamin D receptor polymorphisms on biochemical markers of mineral bone disorders in South African patients with chronic kidney disease. Accepted for publication in BMC Nephrology. October, 2017.

Chapter 7: Consists of an integrated summary of the study findings, limitations, recommendations and conclusions

Candidate's contributions

I was responsible for the conceptualization of this project, writing up the protocol and collection of data. All the manuscripts were drafted by me. I conducted all the statistical analysis. The laboratory work was carried out by me under the supervision of my laboratory collaborators.

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Chronic kidney disease (CKD) is a worldwide health problem affecting 5–10% of the world's population (1, 2) and the majority of these patients are at an increased risk of developing disturbances of bone and mineral metabolism. These disturbances lead to a constellation of bone lesions which was previously referred to as renal osteodystrophy (ROD), with affected patients manifesting with symptoms such as bone pain, muscle tendon rupture, pruritus and high incidence of fractures (3, 4). Subsequently, evidence has shown that patients with ROD are also predisposed to cardiovascular calcification with associated high morbidity and mortality rates (5, 6). Unfortunately, the term ROD does not encompass this important extra skeletal manifestation. Therefore, to address these drawbacks and accommodate the extra skeletal manifestations, the Kidney Disease-Improving Global Outcomes (KDIGO) Foundation initiated a controversies conference with the aim of providing a globally acceptable definition and classification system for renal osteodystrophy. The KDIGO work group recommended a broader term, CKD-mineral and bone disorder (CKD-MBD) for a systemic disorder of mineral and bone metabolism due to CKD and that the term renal osteodystrophy should exclusively be used to describe disorders in bone morphology associated with CKD (6). However, in clinical settings, a bone biopsy is less frequently utilized because it is an invasive and cumbersome procedure and requires highly skilled personnel to interpret the obtained tissue samples. For these reasons, clinicians largely depend on trends in the levels of parathyroid hormone in conjunction with levels of serum phosphate, calcium and alkaline phosphatase as markers of bone turnover which are used to guide the treatment of mineral bone disorder (4). However, the practice of utilizing one biomarker to predict the complex dynamic bone remodelling process has been questioned (6, 7). Therefore, one of the objectives of this study is to further assess other biochemical markers of bone turnover (FGF23, total alkaline phosphatase) and correlate them with parathyroid hormone (PTH).

Furthermore, several studies have shown race to be an important factor that may influence PTH, serum phosphate and vitamin D levels (8-10). These studies showed that compared

with whites, blacks have higher PTH and lower 25-hydroxyvitamin D [25 (OH) D] levels. These comparative studies were largely carried out between Caucasians and Black Americans and little is known about the association in African CKD patients. In addition, the impact of these biochemical abnormalities have been shown to differ across race and thus the need for race-specific target values for these markers of mineral bone disorder (11). This study also seeks to examine the impact of these markers in a heterogeneous African CKD population.

Although the mechanism behind racial variation in the levels of biochemical markers of chronic kidney disease mineral and bone disorders (CKD-MBD) remains unclear, it may be explained partly by genetic factors. Hence, one of the objectives of this study was to examine the influence of VDR polymorphisms on secondary hyperparathyroidism and its association with vitamin D levels in black and white South African study participants.

1.2 Historical perspectives

The association between kidney diseases and bone abnormalities dates back to 1883, when Lucas suggested the term " renal rickets" in patients with albuminuria and bone deformities (12). In 1930, Bauer et al (13) established an association between bone lesions (osteitis fibrosacystica) and the parathyroid gland following a review of 88 patients with endocrine bone disorders. Seven years later, Albright and colleagues postulated that CKD patients with phosphate retention and low levels of calcium are prone to parathyroid gland hyperplasia and renal osteitis fibrosa. Subsequently, in 1940s, the term renal osteodystrophy was coined and used interchangeably with renal rickets (14).

The emergence of the "trade off hypothesis" by Bricker and Slatopolsky (15, 16) provided an insight into the pathogenesis of renal osteodystrophy. The theory states that progressive nephron loss in CKD patients leads to a number of compensatory mechanisms such as elevated PTH in response to retained phosphate.

In the 1960s and 1970s, the two predominant forms of renal osteodystrophy in patients with end stage kidney disease (ESKD) were osteitis fibrosa and mixed uraemic osteodystrophy with a minority of patients presenting with osteomalacia prior to dialysis (17). However, osteomalacia became a major problem following initiation of dialysis secondary to aluminum intoxication in some centers; the two most affected dialysis centers (Ottawa and Newcastle)

had high concentrations of aluminum and fluoride in their tap water. This entity of renal osteodystrophy (osteomalacia) was characterized by microcytic anaemia and encephalopathy (18). However, adynamic bone disease was not only peculiar to aluminum contamination of tap water used for dialysis but also associated with the use of large amounts of aluminum containing phosphate binders and active vitamin D therapy (19). Subsequently, there was a rapid decline in the occurrence of this disease entity with improvement in water purification systems and reduced prescription of aluminum-containing phosphate binders.

1. 3 Definitions and Guidelines

1.3.1 Definitions

In 2003, the National Kidney Foundation proposed that renal osteodystrophy should be defined as a constellation of bone disorders present or exacerbated by CKD that lead to bone fragility and fractures, abnormal mineral metabolism, and extra skeletal manifestations (20). Despite incorporating a triad of abnormal mineral metabolism, skeletal and extra skeletal manifestations this definition failed to be acceptable globally. Therefore, to ensure a widely acceptable definition, the second KDIGO controversies conference in 2005 came up with a broader term CKD-MBD. The conference participants agreed that CKD-MBD should be defined "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, parathyroid hormone (PTH), or vitamin D metabolism; (ii) abnormalities in bone turnover, mineralization, volume, linear growth, or strength; or (iii) vascular or other soft tissue calcification" (6). This internationally acceptable definition has led to ease of valid comparison of studies in the field of CKD-MBD.

1.3.2 Guidelines

In an ongoing effort to reduce the adverse clinical events associated with CKD-MBD, several global and regional guidelines were proposed to assist clinicians in the management of patients with CKD-MBD. These guidelines provided recommended target reference values for intact PTH, alkaline phosphatase and serum calcium. However, comparison of these guidelines has shown lack of harmonization with the existence of relevant clinical differences in the target values (21). For example in 2003, the Kidney Disease Outcomes Quality

Initiative (K/DOQI) clinical practice guidelines recommended maintaining serum phosphate between 3.5 and 5.5mg/dL (1.13–1.78 mmol/L) for CKD stage 5D, serum parathyroid hormone (PTH) between 150 and 300 pg/mL(16.5–33.0 pmol/L) and corrected serum calcium in the range of 8.4 and 9.5 mg/dL (2.10–2.37 mmol/L) (20), while in 2009, the KDIGO clinical practice guidelines recommended maintaining serum calcium and phosphate within the normal range and plasma PTH in the range of two to nine times the upper normal limit of the assay (22). One of the major drawbacks of the KDIGO guideline, which has drawn criticism, is the vagueness of some of the non-numerically defined recommendations. Despite these drawbacks, it is still preferred to the narrow target levels of calcium, PTH and phosphorous, as recommended by the 2003 KDOQI guidelines.

Table 1. 1: Recommended guidelines by different professional groups

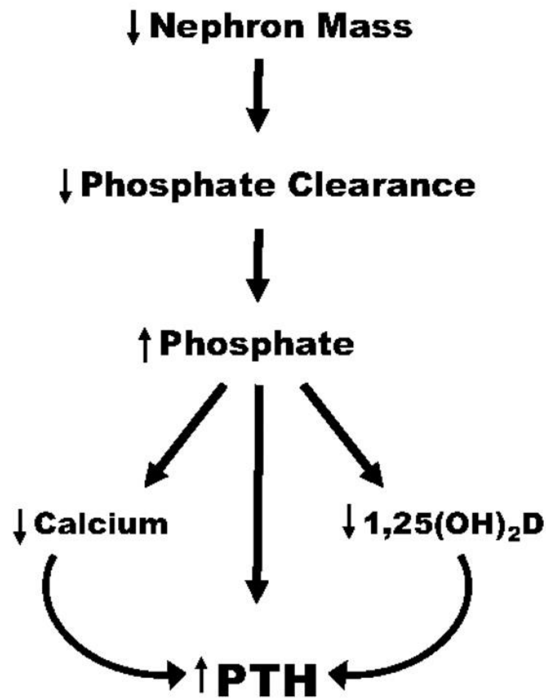
Group	Year	Recommended levels		
		Corrected calcium (mg/dl)	Phosphorous (mg/dl)	PTH (pg/ml)
UK Renal Association (23)	2002	8.8-10.4	< 5.6	< 4× upper normal range
K/DOQI (20)	2003	8.4-9.5	3.5-5.5	150-300
Canadian Society of Nephrology (24)	2006	Within normal range	Within normal range	100-500
Japanese Society for Dialysis Therapy (25)	2008	8.4-10.0	3.5-6.0	60-240
KDIGO (22)	2009	Within normal range	Within normal range	2- 9× upper limit of normal

KDIGO= Kidney Disease -Improving Global Outcomes, K/DOQI= Kidney Disease Outcomes Quality Initiative, UK= United Kingdom, PTH= Parathyroid hormone.

1.3 Pathogenesis of CKD-MBD

Classically, prior to the discovery of Fibroblast Growth Factor 23 (FGF23), phosphate retention due to a decline in renal function had been considered as the main trigger of secondary hyperparathyroidism (26). The retained phosphate leads to a triad of hyperphosphataemia, low 1,25(OH)2D3 and hypocalcaemia which are well-known stimuli for PTH secretion that in turn enhances phosphate excretion and development of secondary hyperparathyroidism in advanced CKD. However, what mitigates this process in the early

stages of CKD continued to be a point of discussion. Some authors have observed that calcitriol deficiency occurred earlier than hyperphosphatemia and hypocalcemia suggesting that it may be the main initiator of secondary hyperparathyroidism. Therefore, the pathophysiology of secondary hyperparathyroidism is a complex process that involves an interaction between several factors. In the classic hypothesis, the trade-off of PTH for normalization of calcium and phosphate levels is the development of secondary hyperparathyroidism (15, 27). The role of phosphate in the pathogenesis of secondary hyperparathyroidism was further supported by studies that demonstrated an association between high phosphate diets and parathyroid hyperplasia (28, 29). However, the pathophysiology of secondary hyperparathyroidism has evolved with new discoveries (4). For example, the emergence of FGF23 has revolutionized the understanding of the mechanisms underlying the development of secondary hyperparathyroidism, leading to an updated trade-off hypothesis. Plasma FGF23 levels become elevated with progressively worsening renal function, likely to occur before observed changes in the levels of phosphate and PTH (30).



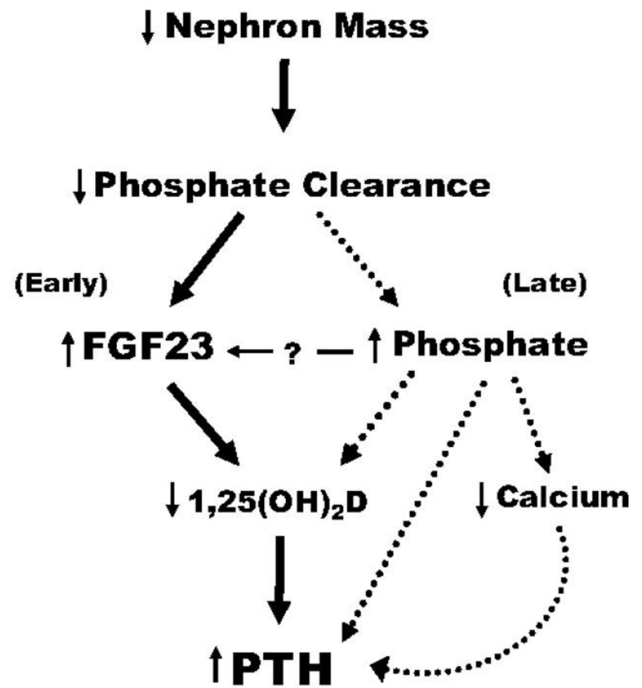


Figure 1. 1: **Classic versus updated hypothesis for the evolution of CKD MBD** (27)

1.4.1 Role of FGF23 in the pathogenesis of secondary hyperparathyroidism

Fibroblast growth factor 23 (FGF23) is derived from osteocytes and plays a vital role in vitamin D and phosphate metabolism. It requires Klotho (a transmembrane protein) to enable it to bind to the FGF receptor (FGFR) in classic target organs such as kidneys and parathyroid glands (31). Klotho is a transmembrane protein that confers tissue specificity to FGF23. The importance of this co-receptor was demonstrated in *kloth*o null mice showing a phenotype similar to that of FGF23 null mice, with features of premature aging, vascular calcification, altered calcium/phosphate metabolism with hyperphosphataemia, and shortened lifespan (32, 33). Fibroblast growth factor 23 enhances phosphate excretion in the proximal renal tubule by decreasing the expression of luminal sodium-dependent phosphate transporters, and may also decrease intestinal phosphate absorption by inhibiting NaPi cotransporter activity (34). In addition, it reduces synthesis of 1, 25-dihydroxyvitamin D [1, 25(OH) $_{2D3}$] by down-

regulating the activity of 1α-hydroxylase and accentuating the activity of 24-hydroxylase (35, 36). In early stages of CKD, high levels of FGF23 attenuate hyperphosphataemia at the expense of 1, 25(OH) $_2$ vitamin D suppression, thus initiating the development of secondary hyperparathyroidism (36). The decrease in serum 1, 25 (OH) $_2$D3 leads to decreased intestinal calcium absorption. The triad of low levels of calcium, calcitriol and hyperphosphataemia further enhances excessive PTH secretion. This excess PTH leads to mobilization of calcium from the bone and osteitis fibrosa. Other consequences of progressive worsening of kidney function include hypo responsiveness of the vitamin D receptor (VDR) on the parathyroid gland with further enhancement of PTH production and reduced expression of the calcium sensing receptor on the parathyroid gland leading to parathyroid gland hyperplasia. In some subset of patients, the parathyroid gland undergoes hypertrophy and becomes autonomous (37).

1.5 Diagnosis of CKD-MBD

In 1983, Sherald et al. (38) proposed a classification for renal oesteodystrophy based on bone histomorphometry findings namely: high turnover disease, low turnover and mixed uraemic osteodystrophy. The emphasis on this classification was on bone turnover; however, since bone biopsy is not routinely used for monitoring patients there is a need for reliable biomarkers for assessing and monitoring patients with CKD-MBD. Therefore, the KDIGO guidelines recommended the use of serum PTH in conjunction with total or bone specific alkaline phosphatase (b-ALP) since high or low levels of these markers correlate with underlying bone turnover. In addition, due to the dynamic nature and complexity of bone homeostasis, it is difficult to rely on one biochemical marker as a surrogate test of bone formation (39). Therefore, utilizing both PTH and b-ALP as recommended by the KDIGO group may be necessary. Furthermore, in an attempt to address the diagnostic utility of various biochemical markers of mineral bone disorders, the KDIGO group conducted one of the largest bone biopsy studies involving 492 dialysis patients. In their multivariate analysis, both intact PTH and whole PTH were found to remain significantly predictive in differentiating high from non-high bone turnover. In addition to PTH, they also assessed the additive value of bone- specific alkaline phosphatase and the amino – terminal propeptide of type 1 procollagen (PINP) in providing diagnostic accuracy. Surprisingly, the inclusion of specific b-ALP level added only non-statistically significant value to PTH while PINP did not

(40). However, due to limited serum samples they could not assess the diagnostic utility of FGF23, 25 (OH) D and other newer biomarkers of CKD-MBD.

1.5.1 Parathyroid hormone

The parathyroid gland plays a vital role in the regulation of mineral bone homeostasis through a complex interaction with the bone and kidney. Alterations in extracellular levels of calcium ion are perceived by the parathyroid calcium sensing receptors (CaSRs) which further regulate the production and secretion of PTH (41). Parathyroid hormone exerts its effects on the bone to enhance mobilization of calcium and phosphate, while on the kidneys it inhibits urinary excretion of calcium and phosphate reabsorption. It also enhances the synthesis of 1, 25(OH) $_2$D3. The first generation PTH assays were the radioimmunoassays (RIAs) that utilized an antibody to locate an epitope in the c-terminal or mid portions of the PTH molecule. The first generation assays were later found to be associated with some drawbacks such as cross reactivity with other fragments of PTH (mid and carboxyl terminal). These fragments are produced by the liver and excreted by the kidneys. Therefore, as a result of cross reactivity, levels of PTH measured in CKD patients by these assays will be markedly elevated (42). Subsequently, in the 1980s, the two site immunoradiometric assays were launched to address the inadequacies associated with the first generation assays (43). The second generation assay which is being used in our study specifically measures the full length PTH (Intact PTH). Although, more recently, it was believed that the second generation assays may also recognize other fragments such as PTH (7-84) (44), it still remains the most widely used assay. The third generation assays which are now believed to be specific for PTH (1-84) are also available (45). However, the improved diagnostic value of the third generation as compared to second generation assays has not been established (46). In 2001, Monier et al. (47) assessed whether the use of the plasma PTH (1-84)/C-PTH fragment ratio could predict bone turnover better than individual PTH levels measured with second or third generation assays. Their results showed that the PTH-(1-84)/C-PTH fragment ratio was the best predictor of bone turnover, with a ratio> 1 predicting high or normal bone turnover (sensitivity 100%), while a ratio <1 indicated a high probability (sensitivity 87.5%) of low bone turnover. However, subsequent studies did not find any advantage in assessment of bone turnover with this ratio compared to a single value of PTH. Therefore, the KDIGO group is of the opinion that both second and third generation assays are comparable, with not

enough evidence to recommend switching to the third generation assay. Similarly, the use of both assays to arrive at a ratio will lead to a considerable increase in costs of evaluating CKD-MBD. Despite the limitations associated with PTH measurement, it still remains the recommended marker for monitoring CKD-MBD. The choice of PTH as a marker for monitoring secondary hyperparathyroidism has been supported by studies that correlated elevated levels of these hormones with poor clinical outcomes. The US Renal Data System revealed reduction in fracture risk by 32 % post parathyroidectomy after adjusting for confounding variables (48). In another study, independent of age and diabetes status, elevated levels of PTH were associated with a history of heart failure and myocardial infarction (49). Additionally, in a large randomized trial, decreased levels of PTH with paricalcitol therapy was significantly associated with decreased cardiovascular hospitalization (50). Furthermore, bone remodeling is a dynamic process with an average remodeling cycle of 3-6 months for an area of bone. Therefore, the use of multiple bone biopsies as a gold standard for diagnosing and monitoring renal osteodystrophy is impracticable. In clinical settings, an ideal biomarker for monitoring the management of CKD-MBD should be noninvasive and can be repeatedly measured

1.6 Classification of markers of bone turnover

The complex and dynamic processes of bone remodeling are under the influence of osteoclasts (bone resorption), osteoblasts (bone formation) and osteocytes (bone maintenance) (51). In healthy individuals, these complex interactions ensure that the amount of bone removed is replaced with newly formed bone (51). In CKD patients, there is dysregulation of this balance which is reflected by abnormal levels of markers of bone turnover. These markers can broadly be categorized into markers of bone formation and bone resorption as shown in Table 1.2.

1.6.1 Total alkaline phosphatase (TAP)

Prior to the availability of commercial intact parathyroid hormone (PTH) assays, serum total alkaline phosphatase (TAP) measurements were used as one of the surrogate markers of high bone turnover that was utilized in the management of CKD-MBD (52). Alkaline phosphatases are membrane-bound tetrameric enzymes that extract phosphate from proteins and nucleotides at alkaline PH (4, 53). TAP is produced by various organs such as intestine,

liver, kidney, and bone. However, in healthy adults, the TAP activity in serum is derived mainly from the bone and the liver (51). They are involved in osteoid formation and mineralization. Although not as specific as bone specific alkaline phosphatase, serum TAP is the most widely utilized marker of bone formation because it is readily available and inexpensive. In addition, studies have reported a good correlation between bone specific alkaline phosphatase and TAP (51).

1.6.2 Specific bone alkaline phosphatase (b-ALP)

Specific b-ALP originates from osteoblasts and is involved in osteoid formation and mineralization. Studies have revealed a significant correlation between bone formation rate and bone specific alkaline phosphatase (54, 55). Studies relating to its superiority to PTH in identifying low versus high bone turnover have been inconsistent. For example, Couttenye et al. (56) showed that bone specific b-ALP has a better positive predictive value than PTH in identifying adynamic bone disease in haemodialysis patients, while Lehmann et al. (57) demonstrated that the predictive ability for high versus low bone turnover status was similar for TAP, b-ALP, tartrate resistant acid phosphatase (TRAP 5b) and parathyroid hormone in CKD Stage 5 patients.

1.6.3 Osteocalcin (OC)

Osteocalcin (OC) which is also called bone-Gla protein, is exclusively produced by osteoblasts, odontoblasts and chondrocytes and thus considered a marker osteoblast function (51). A histomorphometry study has reported a good correlation between serum levels of OC and bone formation rate (58). Osteocalcin is unstable in serum and rapidly degrades into OC fragments and intact peptide. The various assays used to measure serum OC have been shown to detect fragments of various sizes, thus limiting their application in clinical practice (51).

1.6.4 Procollagen Type I Propeptides

The procollagen type I propeptides are synthesized from collagen type I, which is the predominant form of collagen found in bone (51). Type I collagen could also be found in other tissues such as skin, cornea, blood vessels, tendons and cartilages. This collagen is produced mainly by osteoblasts in the form of a procollagen molecule during bone formation. This procollagen molecule consists of short terminal peptides namely the carboxy (C-)

terminal propeptide (PICP) and the amino (N-) terminal propeptide (PINP (59). In 2011, the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) made a recommendation that a marker of bone formation (serum procollagen type I N propeptide, s-PINP) and a marker of bone resorption (serum C-terminal telopeptide of type I collagen, s-CTX) should be used as reference analytes for bone turnover markers in clinical studies, particularly for monitoring osteoporosis (59). However, it is noteworthy that the KDIGO guidelines did not recommend routine measurements of markers of collagen synthesis such as CTX in patients with CKD stages 3-5D. The reason for this recommendation was based on the evidence that the levels of these markers did not appear to be more superior at predicting bone histology than serum PTH or specific bone alkaline phosphatase (22).

1.6.5 Tartrate – resistant acid phosphatase (TRAP-5B)

This is another marker of bone resorption which is produced by osteoclasts (60). Compared to PTH and PINP, TRAP- 5B has been shown to correlate more strongly with histological indices of osteoclasts, and found to be very stable and not affected by kidney function or fasting status of the patients (61, 62).

Other markers of bone turnover are summarized in Table 1.2.

Table 1. 2: **Markers of bone turnover** (51)

Marker	Tissue of origin	Specimen	Comments
Bone formation			
Specific bone alkaline phosphatase (b-ALP)	Bone	Serum	Originate from osteoblasts
Osteocalcin (OC)	Bone, Platelets	Serum	Predominantly from osteoblasts
C-terminal propeptide of type I procollagen (PICP)	Bone, soft tissue, skin	Serum	Arises from proliferating osteoblasts and fibroblasts
N-terminal propeptide of type I procollagen (PINP)	Bone, soft tissue, skin	Serum	Arises from proliferating osteoblast and fibroblasts
Bone resorption			
Hydroxyproline	Bone, cartilage, soft tissue, skin	Urine	Present in both the newly synthesized and the mature collagen.
Hydroxylysine glycosides	Bone, soft tissue, skin, serum & complement	Urine serum	Hydroxylysine in collagen is glycosylated to varying degrees, depending on tissue type
Carboxyterminal crosslinked telopeptide of type I collagen (CTX-I)	All tissues containing type I collagen	Urine (a-/ s) Serum (s only)	Collagen type I, predominantly from the bone. Isomerisation of aspartyl to s-aspartyl occurs with ageing of collagen molecule.
Aminoterminal crosslinked telopeptide of type I collagen (NTX-I)	All tissues containing type I collagen	Urine Serum	Collagen type I, predominantly from the bone.
Bone Sialoprotein (BSP)	Bone, Dentin, hypertrophic cartilage	Serum	Synthesized by osteoblasts and osteoclastic cells.
Tartrate-resistant acid phosphatase	Bone Blood	Plasma Serum	Six isoenzymes are present in the human tissues and b and 5b predominates in the bone (Osteoclasts).
Cathepsins (K,L)	Osteoclasts Macrophage, Osteoclasts	Plasma, Serum	Cathepsin K plays an essential role in osteoclast-mediated bone matrix

Dual-energy X-ray absorptiometry (DXA) is a non invasive radiological technique used in the assessment of mineral bone density, although largely utilized in predicting bone fracture in healthy population, the findings in evaluating risk fracture using DXA in patients with CKD 3-5D have been inconsistent and limited by cross sectional study design (63).

Furthermore, the use DXA to identify osteoporosis in the healthy population which is characterized by low bone mineral density may be inappropriate in patients with advanced CKD, since abnormal markers of CKD-MBD will lead to poor bone quality even in the setting of normal mineral or high mineral bone content (22, 63). Thus the 2009 KIDIGO guidelines made no recommendations for the routine use of DXA in patients with CKD 3-5D (22). However, four recent prospective studies consistently reported that hip BMD predicted bone fractures in CKD patients and based on these findings, the recently 2017 updated guidelines recommended assessment of BMD using DXA in high risk patients (64).

1.7 Prevalence of secondary hyperparathyroidism

The pattern of CKD-MBD has evolved over time due to various factors such as change in practice patterns, underlying aetiology of CKD and duration on dialysis (65). For example, in 1996, Coen et al. (66) reported the following biopsy findings in 76 Italian CKD patients not on bone-related medications: adyanamic bone disease in 9 patients, mixed osteodystrophy in 26, advanced mixed osteodystrophy in 22, predominant hyperparathyroidism in 2, predominant osteomalacia in 7 and normal features in 10. Two other studies were contradictory in relation to the prevalence of adyanamic bone disease in predialysis CKD patients. Dhal et al. (67) reported that osteomalacia was rare in predialysis CKD, while Mora Palma et al. (68) reported that osteomalacia was a common finding in predialysis CKD patients with chronic tubulointerstitial nephritis. However, comparison of the prevalence of adyanamic bone disease across studies is difficult due to a lack of standardized definition.

In a review of 1209 bone biopsies for patients on dialysis in several Latin American countries, Jorgetti et al. (69) reported that osteomalacia and mixed forms of bone disease due to aluminum deposition were more prevalent in Brazil, Argentina and Uruguay, while in Portugal and Spain, high bone turnover due to secondary hyperparathyroidism were the predominant forms.

Subsequently in the late 1990s to date, most studies on the spectrum of CKD-MBD were based on biochemical parameters of bone turnover, likely due to ease of measurement, minimally invasive and significant association between levels of these markers and adverse clinical outcomes (70). In addition, the availability of global guidelines has allowed comparisons of biochemical parameters across studies.

Data from the Dialysis Outcomes and Practice Patterns (DOPPS) study revealed a wide variation in the abnormal markers of bone disease across different countries. Based on KDOQI recommended PTH values (150 to 300pg/ml), the proportion of patients with PTH > 300 pg/ml were 30.3 % for the United States, 26.9 % for European countries, and 19.0 % for Japan. The factors positively associated with hyperparathyroidism (>300 pg/dL) were black race, duration of ESKD and vitamin D therapy (71).

In the CORES study involving six Latin American countries, the proportion of patients with PTH levels above 300 pg/ml was 30.9 %; 26.2 % were within the KDOQI recommended targets, and 42.8 % were below 150 pg/ml (72).

Studies from India have reported a high prevalence of secondary hyperparathyroidism in haemodialysis populations. In a cross sectional study involving 150 predialysis and dialysis patients, high prevalence of secondary hyperparathyroidism was reported in both CKD stage 4 and CKD stage 5D (84.62% versus 88.29%) (73). The use of a PTH cut off value of 69 pg/mL for the advanced stages of CKD might have contributed to the high prevalence. However, in another study from India, a similar trend was reported with higher cut off values, 50 (72%) patients had PTH levels greater than twice the upper range of normal and 45 (61%) patients had PTH >300 pg/ml (74).

In Africa, few studies have described the pattern of CKD-MBD based on biochemical parameters.

In a cross sectional study involving 103 MHD patients from Libya, patients were categorized according to PTH into three categories: hyperparathyroid bone disease (iPTH> 450 pg/ml), adynamic bone disease (iPTH< 60 pg/ml), normal bone (iPTH 60 to 450 pg/ml). Based on this categorization, 29 of the patients (28.1%) had biochemical evidence of hyperparathyroid bone disease (iPTH>450 pg/ml) and 28 patients (27%) had low turnover adynamic bone disease (iPTH<60 pg/ml) and overall prevalence of ROD was 55.3% (75). Another study from Senegal reported a very high prevalence of secondary hyperparathyroidism in 57 of the 118 patients (76). These studies were limited by smaller sample sizes and non-availability of data on vitamin D status. In addition, the exact cut off value of PTH used in the Senegal study was not stated. The present study aimed to improve on these limitations by enrolling larger number of patients, assessing vitamin D and FGF23 levels.

Patterns of CKD-MBD in Diabetes Mellitus

Diabetes mellitus (DM) has been shown to alter bone modelling by modulating the functions of both osteoblasts and osteoclasts (77, 78). Type 2 Diabetes mellitus is associated with decreased bone remodeling. Type 2 diabetic patients were reported to have 20-50% lower PTH levels than the controls, despite reduced GFR, which is indicative of reduced PTH secretion in diabetic patients (79). Similarly, other bone markers of bone turnover such as osteocalcin, PINP and β-C-terminal telopeptide of type collagen (β-CTX) were lower in patients with type 2 DM as compared to non-diabetic patients (80). These findings were further supported by studies that utilized bone biopsy that reported low bone formation with reduced mineralization in diabetic patients (81, 82).The mechanism behind this low bone formation is largely attributed to increased accumulation of advanced glycation end products (AGEs) in bone matrix (83). The accumulated AGES in the bone matrix ultimately distort the activity of both osteoclasts and osteoblasts leading low bone formation in this group of patients.

1.8 Clinical impact of CKD-MBD

Several observational studies have shown an association between deranged markers of MBD and poor clinical outcomes in both predialysis and dialysis patients. For example, elevated levels of phosphate, calcium and PTH have been shown to be associated with cardiovascular-specific mortality in patients with CKD. In a large, prospective, multicenter, cohort study (Netherlands Cooperative Study on the Adequacy of Dialysis) involving 1,629 haemodialysis and peritoneal dialysis patients, a significant increase in hazard ratio (HR) of 1.57 (1.07–2.30) in patients with the highest quartile of phosphate using both baseline and time-dependent values was reported (84). Similarly, Block et al. (85) reported an increased risk of death with increasing levels of phosphate, RR 1.07, 1.25, 1.43, 1.67, and 2.02 for serum phosphorus levels of 5.0 to 6.0, 6.0 to 7.0, 7.0 to 8.0, 8.0 to 9.0, and >9.0 mg/dL respectively in 40,538 patients on maintenance haemodialysis. The consistent association of hyperphosphataemia with increased mortality has been linked to its direct calcifying effect on coronary vessels and cardiac valves.

Studies relating to PTH have shown a “U” shaped association with increased risk of death at extreme values of PTH. The highest value of PTH that is associated with increased risk of

death varies across studies from >400 pg/mL (86) to >500 pg/mL (87) and >600 pg/mL (85). On the lower end of PTH, some studies have associated PTH below the K/DOQI recommended lower threshold (<150 pg/mL) with increased death risk (86, 88).

Despite the differences in the cut-off points utilized by various studies, hypercalcaemia has been consistently associated with increased risk of mortality in MHD patients (89, 90). In the three phases of the dialysis outcomes and practice patterns study (DOPPSI, II and III) with 25,588 HD patients, calcium levels greater than 10.0 mg/dL (>2.5 mmol/L) were significantly associated with greater risk of all cause and cardiovascular mortality in both baseline and time dependent models (90). The reasons for this consistent association could be linked to acceleration of arterial calcification by hypercalcaemia (91, 92).

A triad of high calcium, elevated phosphate levels and high or low PTH levels was associated with increased mortality in MHD patients (93). Alkaline phosphatase which is one of the markers of high bone turnover was recommended to be measured annually by the KDIGO. This relatively cheap diagnostic test has been consistently associated with increased mortality in both predialysis and dialysis populations (94-96). For example, a USA multicenter observational study of haemodialysis patients reported that higher levels of alkaline phosphatase were associated with increased risk of hospitalization and death (94). Bhedu et al. (96) reported a similar association with higher levels of alkaline phosphatase in patients with CKD stages 3 and 4. Their findings suggested that independent of confounding variables such as liver function, serum phosphate and calcium, serum alkaline phosphatase was associated with increased risk of death in predialysis CKD patients. The role of high levels of alkaline phosphatase in the pathogenesis of vascular calcification was supported by a longitudinal study involving 134 stage 4 and 5 CKD patients (88). This 2 year prospective study revealed that higher levels of serum alkaline phosphatase were significantly associated with progressive vascular calcification (97). This relationship was independent of levels of serum fetuin –A, calcium, C – reactive protein and PTH.

1.8.1 FGF23 and CKD progression

Studies have shown a strong correlation between serum FGF23 levels and eGFR. As renal function declines FGF23 levels increase. In end-stage renal disease, FGF23 levels can be up to 1000-fold above the normal range, likely due to retained phosphate or decreased renal

clearance (98). In a prospective study involving 177 non-diabetic patients with CKD stages 1-5, with a median follow up period of 53 months, both serum intact FGF23 (iFGF23) and c-terminal FGF23 levels (cFGF) above optimal cut-off levels predicted a doubling of serum creatinine and/or the need for renal replacement therapy, independent of eGFR, proteinuria, and other indices of mineral metabolism, such as calcium, phosphate and parathyroid hormone (99).

Similarly, in a Brazilian prospective study comprising type 2 diabetes mellitus patients with macroalbuminuric nephropathy, iFGF23 was an independent predictor of the composite primary outcome defined as death, doubling of baseline serum creatinine and/or need for dialysis, even after adjustment for creatinine clearance and intact parathyroid hormone (100). One of the limitations of the two aforementioned studies was the relatively small sample size. However, this finding was confirmed in a large multicenter prospective study CRIC (Chronic Renal Insufficiency Cohort) involving 3879 CKD stages 2–4 patients with a median follow-up of 3.5 years, high cFGF23 levels were independently associated with poor renal outcome (101). These studies were largely carried out in Caucasians or African Americans. Few if any studies have investigated the role of FGF23 in African CKD patients.

1.8.2 FGF23 and cardiovascular outcome

In the HOST (Homocysteine study), compared to patients with the lowest FGF-23 levels, patients in the highest quartile of baseline FGF23 had a hazard ratio of 2.58 for future cardiovascular events in univariate analysis. High levels of FGF23 remained a significant predictor of cardiovascular outcome, while vitamin D, calcitriol and PTH did not. Regarding the composite cardiovascular endpoint, elevated FGF23 was an independent predictor of myocardial infarction and lower extremity amputation (102). These studies have also shown an association between elevated levels of FGF23 in CKD and left ventricular hypertrophy, vascular calcification and mortality (101, 102).

1.9 Vitamin D and CKD

Vitamin D is mainly derived from dietary sources or synthesized by the skin from UVB light. It is first converted to 25(OH) D in the liver and subsequently activated to its active form known as 1, 25(OH) 2D3 (103). Until recently, the final step of activation, 1α-hydroxylation

was thought to occur primarily in the kidney (104). However, emerging evidence continued to reveal that in addition to the kidney pathway for activation of 25 (OH) D, a peripheral autocrine (non-renal) pathway is also involved in calcitriol synthesis (105). In fact, the hypothesis of extra renal 1α-hydroxylase had been proposed several years before the availability of calcitriol and its analogues. For example, studies have demonstrated that there were occasional anephric patients who had measurable blood levels of vitamin D metabolites (1α, 25-dihydroxyvitamin D3) following administration of vitamin D or 25 (OH) D3 (106, 107). Furthermore, recent epidemiological data and studies of the vitamin D receptor (VDR) knockout mouse have shown that the role of vitamin D is not solely restricted to its classical function of regulating calcium and phosphate homeostasis (108). Vitamin D, in the form of calcitriol, mediates a wide variety of cell differentiation around the body. The non-classical role of vitamin D includes regulation of the renin angiotensin system and the nuclear factor - kappa B pathway which are commonly implicated in various disease pathological processes (109, 110). Furthermore, the emergence of the non-classical pathway has also given insights into the mechanisms behind the significant association between vitamin D deficiency and multiple chronic disease conditions such as cardiovascular disease, diabetes, malignancies and CKD (111). This has led to a special interest in the importance of addressing vitamin D deficiency particularly in CKD patients who are more susceptible to vitamin D deficiency. Therefore, it now advocated that having corrected the abnormal calcium/phosphate axis of the CKD patient that the nephrologist should also mitigate the wider effects of vitamin D insufficiency (105).

1.9.1 Prevalence of vitamin D deficiency

The comparison of epidemiological data on the prevalence of vitamin D deficiency is somewhat hampered by the lack of consensus on how to define vitamin D deficiency (112). However, the most widely acceptable cut off value for defining vitamin D deficiency is serum 25 (OH) vitamin D levels below 20 ng/ml, while patients with 25 (OH) D levels between 20 and 30 ng/ml are considered to be vitamin D insufficient (113). Based on these cut off values vitamin D deficiency or insufficiency has been considered a global health problem affecting approximately 1 billion people worldwide (114). Studies have reported that 40 -100 % of USA and European elderly men and women living in the community are deficient in vitamin D (111). Vitamin D deficiency is also common in countries with

abundant sunshine, when there is limited skin exposure to UVB sunlight. For example, studies from Saudi Arabia, India, and Lebanon, have reported that 30 to 50% of children and adults had 25 (OH) D levels under 20 ng/ml (115-117). In Africa, although nationally representative data are sparse, a systematic review by Prentice et al. (118) revealed a collective prevalence of 25 (OH) D <25 nmol/l (10 ng/ml) between 5–20%. Most of the African studies were conducted on healthy children and children with Ricketts, while a few were on healthy adults, pregnant and lactating women (119-121). In addition, various test methodologies were used in these studies and only three papers reported the use of the Vitamin D External Quality Assessment Scheme (DEQAS) which monitors 25 (OH) D assay performance. (118).

Patients with CKD are more likely to have 25 (OH) D deficiency due to a reduction in the level of 1-α-hydroxylase available for the conversion of 25 (OH) D to active vitamin D. Other causes of vitamin D deficiency in CKD patients include urinary loss of vitamin D binding protein, reduced dietary intake due to uraemia, and increased levels of FGF23.

Several studies have shown high prevalence of vitamin D deficiency ranging from 60- 80 % in patients with CKD. In a cross sectional study from the US involving patients with CKD stages 3 and 4, La Clair et al (122) reported that only 29 and 17% of patients with CKD stage 3 and 4 respectively had adequate vitamin D status. They used much lower 25 (OH) D levels of < 10 ng/ml to define vitamin D deficiency and levels of 10-30 ng/ml as insufficiency. In another multicenter cross sectional study from the United States, Wolf et al reported that 78 % of the study cohort had vitamin D deficiency defined as serum 25 (OH) D <30 ng ml−1 and 18 % of the study population was severely deficient (serum 25 (OH) D <10 ng ml−1) (123). The reported high prevalence of 25 (OH) D deficiency in these studies is in contrast with the Canadian study to evaluate early kidney disease (SEEK) which found that only 12 % of the participants with estimated GFR <20ml/min/1.73 m2 had vitamin D deficiency. The reason for this lower prevalence rate may be attributable to dietary habits in Canada and vitamin D supplementation of food. Furthermore, the high variations in the proportion of vitamin D deficiency across studies may be due to differences in ethnic distribution, degree of sun exposure, and utilization of different cut off values. In addition, differences in test methodologies may have also accounted for the observed variations. For example, significant 25 (OH) D intra and inter variations have been reported with different auto

analyzers (124). In our study HPLC, which is considered to be the gold standard for vitamin D measurement (125), was used to specifically measure 25 (OH) D3, and is less affected by vitamin D supplementation.

In Africa, data on prevalence of vitamin D status in CKD population is sparse. In a recent cross sectional study from Senegal involving 46 haemodialysis patients, Seck et al. (126) reported that prevalence of vitamin D deficiency was 32.6% and 28 patients (60.8%) had vitamin D levels between 15 µg/l and 30 µg/l. This study was limited by small sample size which reduced the significance of their conclusion.

1.9.2 Vitamin D levels and patients outcome in CKD

In the Third National Health and Nutrition Examination Survey (NHANES III) involving 15,068 participants, de Boer et al. (127) reported a stepwise increase in the prevalence of albuminuria (8.9%, 11.5%, 13.7%, and 15.8%; $P < 0.001$) with decreasing quartiles of 25(OH)D .

In another prospective study involving clinically stable patients with stages 2–5 CKD, 25 (OH) D independently predicted both time to death and progression to ESKD (128). Other well established clinical correlates of vitamin D deficiency are insulin resistance, cardiomyopathy and dysregulation of the immune system (128-131). Therefore, co-existence of these conditions in patients with CKD who are already at risk of cardiovascular events is monumental.

The relationship between vitamin D deficiency and increased mortality in ESKD was further supported by several observational studies that implicated the use of paracalcitol and 1, 25 D and 1α-hydroxylated-VD with better survival in CKD patients (132-134). These studies were limited by the observational study design and thus the need for randomized controlled trials (RCTs) to further clarify the survival benefit from active and/or pre-active vitamin D. Interestingly, an association has also been established between active vitamin D analogues and reduction in proteinuria, likely through an interaction with the renin angiotensin aldosterone system (135).

However, despite all the above aforementioned associated adverse clinical outcomes with vitamin D deficiency, little is known on vitamin D status in African CKD populations.

Therefore, one of our study objectives was to determine the vitamin D status in our patients and further explore its relationship with secondary hyperparathyroidism.

1.10 Ethnic variations in biochemical markers of CKD-MBD

The mechanism behind the existence of racial differences in the regulation calcium/ PTH/ calcitriol axis is largely unknown. However, it was proposed that blacks are susceptible to lower levels of 25 (OH) D, leading to increased parathyroid gland mass and secondary hyperparathyroidism. In addition, despite lower levels of 25 (OH) D, blacks have higher levels of 1, 25(OH) $_{2D3}$ and lower urinary calcium than whites. In a retrospective study involving 1367 haemodialysis patients, black race was independently associated with severe hyperparathyroidism. This study was limited by non-availability of data on vitamin D levels. Subsequently, other studies have consistently reported higher levels of PTH and lower levels of 25 (OH) D in blacks than whites. The consequences of these abnormal markers of CKD have also been shown to differ across races and thus the need for race specific target values for these markers of MBD (11). For example, in a multi ethnic study of atherosclerosis (MESA) involving 6436 participants, 25 (OH) D deficiency was associated with increased risk of coronary heart disease in white but not in black Americans (136). A similar trend was found in the National Health and Nutrition Examination Survey (NHANES III), where low 25 (OH) D was associated with a higher risk of all-cause mortality in white compared to black participants (137). Furthermore, FGF23, which is now being considered as the principal mediator of secondary hyperparathyroidism, has also been shown to differ across races (8, 138).

While there are considerable number of studies from developed countries that have looked at ethnic variations in markers of CKD-MBD among healthy population, there are only few studies from South Africa that have conducted a head to head comparison between White and Black populations. A previous study that dates back to 1997 reported no ethnic differences in serum intact PTH and calcitonin levels; however, Black women had lower serum 25 (OH)D compared to White women (19.3 vs 26.3 ng/ml, $p = 0.0001$)(139).

The unestablished molecular basis for these racial variations in the markers of CKD-MBD highlighted a gap in knowledge and the need for further research in this field. Therefore, the

current study was embarked on, with the aim of exploring the extent of these differences in our patients and to assess whether genetic factors could partly explain these differences.

1.11 Genetic basis for CKD-MBD

1.11.1 VDR polymorphisms

The VDR gene that encodes VDR is found on chromosome 12q12.14 and is made up of eight protein-coding exons (exons 2–9) and six untranslated exons (1a–1f). Single nucleotide polymorphisms (SNPs) and point mutations occur in the introns or the 3′ untranslated region (UTR) of the VDR gene (140). Alterations in these regions would lead to abnormal production of VDR. The four common forms of VDR polymorphisms are (*ApaI* G>T [rs7975232], *TaqI* C>T [rs731236] and *BsmI* A>G [rs1544410]) which reside within an area between exons 8 and 9 with unknown function and in a unique linkage disequilibrium (LD) block spanning the VDR exons 3–9, while *FokI* T>C (rs10735810) resides in the non-coding exon (140).

The VDR plays a vital role in mediating the effects of the biological active form of vitamin D (1, 25(OH) 2D3, therefore it is biologically plausible that variations in these receptors will modulate the consequences associated with vitamin D deficiency (141). In 1994, Morrison et al. (142) were the first to report an association between VDR polymorphisms and bone metabolism. This report showed that the common allelic variants in the VDR encoding genes can predict differences in bone density in healthy individuals (142). Subsequently, several researchers have explored this relationship in CKD populations with emphasis on the calcium/ PTH/ calcitriol axis (143, 144). The *BsmI* polymorphism (BB genotype) has been associated with slower progression of secondary hyperparathyroidism and normal levels of calcitriol in predialysis CKD patients, and lower levels of PTH in haemodialysis, and a better reduction in PTH levels in response to a single bolus of calcitriol therapy compared to patients with bb genotype (144, 145). However, contrary to earlier studies, findings from subsequent studies on the associations between VDR polymorphisms and markers of mineral bone disease have been inconsistent. For instance, some studies reported no difference in PTH levels between the various *BsmI* genotypes (146, 147), while Chudek et al. revealed significantly lower levels of calcitriol in patients with the BB genotype (148). Similarly, some studies have linked other VDR polymorphisms to mineral bone metabolism in

haemodialysis patients. The VDR *FokI* polymorphism (FF genotype) was reported to be associated with higher PTH levels (149).

Furthermore, the existence of racial disparities in abnormal markers of CKD-MBD and the better survival paradox in African Americans compared to white dialysis patients may be explained partly by the racial differences in the distribution of VDR polymorphisms and VDR receptor activation therapy. Most of these studies were conducted on European, Asian and American CKD populations, while studies from Africa were largely on non-CKD populations. Therefore, in line with ongoing efforts to better understand the mechanisms behind racial disparities in markers of CKD-MBD, we aimed to explore the variations in the VDR polymorphisms between black and white African CKD patients and its relationship with biochemical markers of mineral bone disorders.

1.12 Aims

1. To determine the spectrum of CKD-MBD in South African CKD patients.
2. To determine the association between biochemical markers of CKD-MBD and mortality in maintenance haemodialysis patients.
3. To compare markers of mineral bone disease between black and white South African patients with CKD.
4. To compare the association between FGF23 and traditional markers of CKD-MBD.
5. To evaluate the relationship between VDR polymorphisms and biochemical markers of CKD-MBD.

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CHAPTER 2: MATERIALS AND METHODS

2.1 Study design, population and sites

This was a cross -sectional multicenter study carried out from April 2015 to May 2016, involving two hundred and ninety three CKD patients from three renal units in Johannesburg, South Africa. The retrospective arm of this study involved two hundred and thirteen patients undergoing MHD from two dialysis centers in Johannesburg between January 2009 and March 2016. Patients on maintenance haemodialysis were contacted on the days of their dialysis and, when confirmed eligible for enrolment, were given study information sheets and consent forms before recruitment. The pre dialysis patients were contacted on their clinic days. A comparative control arm of the study involved ninety apparently healthy participants without kidney disease.

2.1.1 Sample size estimation

Minimal sample size was determined using Fisher's statistical formula.

$$\text{Sample size (N)} = \frac{Z^2PQ}{D^2}$$

Z = 1.96, that is normal standard deviation at 95% confidence interval

P = Prevalence rate of CKD Mineral bone disorder

Q = 1-P D = 0.05 Precision

The prevalence (P) of CKD Mineral bone disease is 88.29% based on the findings of Gosh B et al(1) . Hence, a prevalence of 88.29 % was used to calculate the sample size.

$$N = \frac{(1.96)^2 \times 0.883 \times 0.117}{(0.05)^2} = 158$$

Therefore, a minimum of 158 patients with CKD will be enrolled. The ratio of study population to control was 2: 1. Thus a total of 80 healthy controls will be required for comparison.

2.1.2 Study sites

Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), Wits Donald Gordon Medical Center and Glynwood Hospital, South Africa

2.1.3 Inclusion Criteria

Patients with established CKD stages 3-5D, aged ≥ 18 years who gave informed consent were enrolled.

2.1.4 Exclusion Criteria

Patients with active or chronic liver disease, on treatment with steroids or bisphosphonates, and having malignancies were excluded. In addition, we excluded Indian and mixed races to allow for a proper comparison between black and white patients.

2.1.5 Ethical consideration

The research protocol was approved by the Health Research and Ethics committee (HREC) of the University of the Witwatersrand; clearance certificate number M141016. Written informed consent was obtained from each patient before enrolment into the study.

2.2 Screening and evaluation protocol

2.2.1 A structured questionnaire was used to obtain patients' demographic characteristics, blood pressure measurements, co-morbid disease, underlying aetiology of CKD and medication history related to CKD-MBD. Determination of race was based on self-report by the participants.

2.2.1 Blood collection and preparation

Whole blood samples were collected into plain separator vacutainer tubes (for serum), and ethylene diamine tetra acetic tubes (for plasma). Samples were left to clot and then centrifuged at 5000 rpm at 4 ^{o}C for 10 minutes. Both serum and plasma were aliquoted into 1.5ml micro centrifuge tubes and stored at -80 ^{o}C. Whole blood collected for VDR genotyping was stored at −20 ^{o}C.

2.3 Laboratory measurements

2.3.1 Plasma intact PTH

Plasma intact PTH was measured by an electrochemiluminescence immunoassay (ECLIA) run on a Cobas 6000 auto analyzer (Roche Diagnostics, Mannheim, Germany). The test methodology entails adding 50 µL of sample to a biotinylated monoclonal PTH-specific antibody, and monoclonal PTH-specific antibody labeled with a ruthenium. Addition of streptavidin-coated micro particles enhance the complex bound to the solid phase, and the unbound substances are washed off. A voltage is then applied to the electrode to generate chemiluminescent emission. There is a direct relationship between the amount of PTH in the sample and the generated chemiluminescent emission which is measured by a photomultiplier.

2.3.2 Fibroblast growth factor 23

Fibroblast growth factor 23 was measured using a sandwich based enzyme-linked immunosorbent assay kit from EMD Millipore Corporation (Billerica, MA, USA); assay lower limit of detection was 3.2 pg/ml. 50 µL of plasma samples were added to the appropriate wells in addition to 100 µL of conjugate antibody. The plate was covered with a sealer and allowed to incubate for 2 hours on a microliter plate shaker. The solutions were then decanted from the wells and washed three times with a diluted wash buffer. 100 µL of detection antibody was then added to all wells and further incubated for 1 hour on a plate shaker. The solutions were decanted from the wells and washed three times with a diluted wash buffer. The washing step was repeated after adding 100 µL of enzyme solution with 30 minutes incubation period. Finally, 100 µL of substrate solution was added to each well to stop the reaction. Optical density at 450 nm was measured on an ELx800 microplate reader (BioTek, Winooski, VT, USA).

2.3.3 Plasma 25 (OH) vitamin D

Plasma 25(OH) D was measured using the high performance liquid chromatography (HPLC) kit (Recipe, Munich, Germany). HPLC was used to selectively measure 25-(OH) vitamin D2 and 25-(OH) vitamin D3 at a wave length of 264nm. The intra and inter assay coefficients of

variation (CVs) were < 5%. Our institutional laboratory is a participating member in the vitamin D external quality assurance scheme (DEQAS). In this study, 25(OH) D3 was used as a marker of vitamin D status to avoid confounding of the results from exogenous vitamin D supplementation.

2.3.4 Determination of serum calcium, phosphate and alkaline phosphatase

Serum calcium, phosphate and alkaline phosphatase were measured using the ADVIA 1800 centaur auto analyzer (Siemens Diagnostics, Tarrytown, USA), and their test principles were as follows:

In alkaline medium calcium ions reacts with 0-cresolpthalein complex one to form a violet colour that is measured at 545/658nm.

In an acidic medium created by sulphuric acid, inorganic phosphate reacts with ammonium molybdate to form phosphomolybdate complex which is measured at 340/658nm.

2.3.5 Determination of serum creatinine and glomerular filtration rate

Creatinine was measured by a modified Jaffe reaction method which is based on a colour formation from a reaction of creatinine with picric acid alkaline medium. The creatinine assay is traceable to isotope dilution mass spectrometry (IDMS). The glomerular filtration rate (GFR) was estimated using the four- variable Modified Diet Renal Disease (MDRD) equation(2) : GFR (in mL/min per 1.73 m^2) = 175 × SCr (exp[−1.154]) × Age (exp[−0.203]) × (0.742 if female) × (1.21 if black).

The rationale behind the use of MDRD in this study is based on the fact that our study population are patients with already established CKD with GFR < 60mls/min per 1.73 m^2, and MDRD study equation is appropriate for patients with CKD stages 3 and 4(3). One of the limitations of MDRD is in underestimation of GFR in early stages of CKD. Although the proposed CKD- Epidemiology Collaboration (CKD- EPI) equation accounts for this limitation, it performs better in healthy population (3). Therefore, the MDRD and CKD-EPI equations perform equivalently in CKD stages 3-5.

2.4 Genotyping

DNA was extracted from whole blood using the Maxwell DNA purification kit (Promega AS1010, USA). The DNA concentrations and purity were determined using NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA) at A260 and A260/A280 ratios. Salting out method was used to re extract DNA from samples with low yield (<10 ng/ul).

Briefly, the salting out method involved suspending buffy coats of nucleated cells in 15 mls polypropylene tubes containing 3mls of nucleic lysis buffer (1% SDS; 2 mM EDTA) and 2 mg/ml proteinase K. The cell lysate was treated overnight with proteinase K at 37^0C. The tubes were vigorously shaken following addition of sodium chloride and subsequently incubated on ice for five minutes. The pellets were washed with 70 % cold ethanol and spun at 800 rpm for 20 minutes to remove the excess salt used in precipitating the DNA. The DNA pellets were finally, dissolved in 100 mL of 10 mM TrisHCl, pH 8 prior to quantification.

The thermocycling condition involved initial denaturation step at 95 °C for 3minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds , annealing at 65°C (67 °C for *Apa I*) for 20 seconds and a final extension at 72 °C for 1 minute.

DNA products were amplified using appropriate primers as shown in Table 2.1.

Table2. 1: Primers, restriction enzymes and annealing temperatures

Gene	Primer	Enzymes	Allele	Annealing temperature
ApaI	F:5CAGAGCATGGACAGGGAGCAAG 3’ R:5’GCAACTCCTCATGGCTGAGGTCTCA 3’	Apa I (New England Biolabs)	AA(TT): 740 bp. Aa(TG):740 bp, 520bp and 220bp aa(GG) 520bp, 220bp.	65 ^{0}C
BsmI	F:5’CAACCAAGACTACAAGTACCGCGTCAGTGA 3’ R:5’AACCAGCGGGAAGAGGTCAAGGG 3 ‘	*BsmI* (New England Biolabs)	BB (AA): 825bp. Bb (AG):825bp, 650 bp and 175 bp. bb(GG):650 bp, 175bp	65 ^{0}C
FokI	F:5’AGCTGGCCCTGGCACTGACTCTTGCTCT 3’ R:5’ATGGAAACACCTTGCTTCTTCTCCCTC 3’	*FokI* (New England Biolabs)	FF(CC): 265 bp Ff(CT) 265bp, 196 bp and 69 bp. ff(TT):196bp,69 bp	67 ^{0}C
TaqI	F:5’CAGAGCATGGACAGGGAGCAAG 3’ R:5’GCAACTCCTCATGGCTGAGGTCTCA 3’	*Taq*I (New England Biolabs	TT(TT): 495bp, 245bp Tt(TC): 495bp, 290bp,245bp, and 205 bp. Tt(CC):290bp,245bp, and 205 bp	65 ^{0}C

The PCR products were then digested with enzymes *ApaI*, *BsmI*, *FokI*, and *Taq*I (New England Biolabs, Beverly, MA, USA) according to the supplier’s protocol. Digestions for *BsmI* and *TaqI* were at 65 ^{0}C left overnight, and 3hrs at 25 ^{0}C for *ApaI*, while *FokI* was incubated at 37 ^{0}C for 3 hrs. Restricted products were electrophoresed on either 10% polyacrylamide or 1.5% agarose gels and then visualized by the Gel Doc TM EZ imager (Bio-Rad systems, USA). Genotypes were scored based on the presence or absence of a restriction site for the enzymes *BsmI*, *ApaI*, and *Taq*I at the 3′ untranslated region and *FokI* at the N-terminal region of the gene.

2.5 Statistical analysis

Detailed description of statistical methods are provided in the in the methods section of each manuscript.

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CHAPTER 3: MANUSCRIPT 1

Biochemical markers of mineral bone disorder in South African patients on maintenance haemodialysis

ABSTRACT

Background: Despite the high mortality and morbidity associated with abnormalities in mineral and bone metabolism in haemodialysis patients, there are limited data on the pattern of mineral bone disorder in African CKD populations. Therefore, the purpose of this study was to describe the pattern of mineral bone disease by evaluating biochemical parameters in patients on maintenance haemodialysis (MHD).

Methods: We evaluated the serum/plasma intact parathyroid hormone (iPTH), corrected calcium, phosphate, total alkaline phosphatase (TAP) and 25 (OH) D levels of two hundred and seven patients undergoing MHD at two dialysis centers in Johannesburg.

Results: The MHD patients (133 men, 74 women) had a mean age of 54.5±15.6 years with a median dialysis vintage of 24 months (IQR, 12-48) and a mean kt/V of 1.45±0.28. The prevalence of hyperparathyroidism (iPTH>150 pg/mL),hyperphosphataemia, hypocalcaemia and 25(OH) D deficiency (<30 ng/mL) was 73.4%, 57.0%, 20.3% and 80.7 % respectively. The combination of markers of bone turnover (iPTH>150pg/mL and TAP> 112 U/L) suggestive of high turnover bone disease, was present in 47.3 % of the study population. In multiple regression analysis, the odds ratio for developing secondary hyperparathyroidism with hypocalcaemia and hyperphosphataemia were 5.32 (95% CI 1.10 - 25.9, P <0.05) and 3.06 (95 % CI 1.15 - 8.10, P <0.05) respectively. 47.3 % of MHD patients had iPTH within the recommended KDIGO guidelines.

Conclusion: Secondary hyperparathyroidism and 25 (OH) D deficiency were common in our haemodialysis patients. Hypocalcaemia and hyperphosphataemia were strong predictors for developing secondary hyperparathyroidism.

Keywords: Biochemical markers, guide lines, mineral bone disorder, haemodialysis

3.1 Introduction

Chronic kidney disease-mineral bone disorder (CKD-MBD) is now defined 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, parathyroid hormone (PTH), or vitamin D metabolism; (ii) abnormalities in bone turnover, mineralization, volume, linear growth, or strength; or (iii) vascular or other soft tissue calcification and that the term renal osteodystrophy should exclusively be used to describe disorders in bone morphology associated with CKD"(1, 2). Although bone biopsy is the gold standard for adequately describing the spectrum of CKD-MBD, it is less frequently utilized in clinical settings because of associated constraints. It is an invasive and cumbersome procedure that requires highly skilled personnel to interpret the obtained tissue samples. Therefore, clinicians largely depend on the biochemical parameters for monitoring and management of this important clinical entity that is associated with adverse clinical outcomes in CKD patients. In addition, the above aforementioned internationally acceptable definition has led to the ease of diagnosing CKD-MBD and allows valid comparison of studies in this field.

In 2009, through extensive review of the literature the KDIGO (Kidney Disease Improving Global Outcomes) guideline work group came up with guidelines to assist clinicians in the management of patients with CKD-MBD(2). The guidelines were largely based on studies conducted on Asian, European and American populations. These guidelines were adopted by many African countries despite existence of racial differences in PTH, vitamin D and phosphorus as demonstrated by several studies (3-5). The lack of use of literature from Africa is likely due non availability of robust data from these regions. Therefore, information obtained from this present study will add to the existing paucity of data on African patients with CKD-MBD.

3.2 Materials and Methods

3.2.1 Participants and study design

This was a cross sectional descriptive study involving two hundred and seven patients undergoing MHD (from June 2009 to April 2016) at two dialysis centers in Johannesburg, aged ≥ 18 years with complete data for analysis. Exclusion criteria included patients with active malignancy, active liver disease, and patients on medications such as bisphosphonates or warfarin. Demographic and clinical data collected were age, gender, history of medications, underlying aetiology of CKD, duration of haemodialysis and blood pressure measurements. The research protocol was approved by the Health Research and Ethics committee (HREC) of the University of the Witwatersrand.

3.2.2 Laboratory measurements

Plasma intact PTH was measured by an electrochemiluminescence immunoassay (ECLIA) run on a Cobas 6000auto analyzer (Roche Diagnostics, Mannheim, Germany; reference range 10-65 pg/mL).

Serum 25 (OH) D was measured by a chemiluminescentmicro particleimmunoassay (CMIA) technique (Abbott Laboratories, Abbott Park, Illinois, US). Reference ranges: < 10 ng/mL as severe deficiency, 10-29 ng/mL as moderate deficiency, 30-100 ng/ mL as sufficiency and > 100 ng/ mL as toxic(6).

Calcium, Phosphate and Alkaline phosphatase were measured using the ARCHITECT C8000 auto analyzer (Abbot Laboratories, Abbott Park, Illinois, US). Other biochemical parameters were determined using routine laboratory techniques.

Laboratory reference range for calcium and phosphate were as follows:

Calcium (2.12-2.50 mmol/L)

Phosphate (0.79-1.45mmol/L).

Based on the above reference values and KDIGO recommendations the following definitions were employed in this study:

Hyperparathyroidism and hypoparathyroidism were defined as intact PTH >150 pg/ml and PTH < 15 pg/ml respectively.

Hyperphosphataemia and hypophosphataemia were defined as phosphate levels >1.45 mmol/L and <0.80mmol/L respectively.

Hypercalcaemia and hypocalcaemia were defined as calcium levels >2.50mmol/l and <2.12mmol/l respectively.

3.2. 3 Statistical analysis

Patients' demographic and baseline characteristics are presented as means and standard deviations (SD) or medians (Interquartile ranges) depending on the distribution of the variable, while categorical data were presented as proportions or percentages. The means of biochemical parameters were compared between diabetic and non-diabetic patients using an independent t–test and Pearson's or Fisher's exact test was utilized for proportion comparisons. Associations between log transformed PTH and other biochemical parameters were assessed by multiple linear regression analyses following significant associations obtained from univariate regression analyses. A logistic regression model was used to evaluate the effect of other biochemical parameters on the odds of developing secondary hyperparathyroidism. A p-value of less than 0.05 was considered statistically significant at the 95% confidence interval. All analyses were performed using STATA version 12 (STATA Corp., TX, and USA).

3.3 Results

Two hundred and seven MHD patients (133 men, 74 women) were enrolled. Their mean age was 54.5±15.6 years with a median dialysis vintage of 24months (IQR, 12-48) and a mean Kt/V of1.45±0.28. Fifty six (27.1%) of the study population were diabetic. Medications received included calcium carbonate 154 (74.4%), alfacalcidol 132 (63.8%), cholecalciferol 45(21.7%) and 12 (5.8%) were on cinacalcet (Table 1). The majority of the patients were on three times weekly 4 hr sessions of haemodialysis.

Table 3.2 shows a comparison of biochemical parameters and distribution of CKD-MBD abnormalities between diabetic (n=56) and non-diabetic (n=151) patients. Diabetic patients

had significantly lower mean 25(OH) D as compared to non-diabetic patients. Other biochemical parameters were comparable between the groups.

The overall prevalence of hyperparathyroidism (iPTH>150pg/mL), hyperphosphataemia, hypocalcaemia and 25 (OH) D deficiency (<30 ng/mL) was 73.4%, 57.0%, 20.3% and 80.7 % respectively. The combination of markers of bone turnover (iPTH>150pg/mL and TAP > 112 IU/L) suggestive of high turnover bone disease, was present in 47.3 % of the study population (Table 3.2).

Patients within KDIGO-recommended targets for calcium, phosphate and PTH were 63.8%, 37.7 %, and 47.3% respectively (Table 3.3).

In logistic regression analysis, hypocalcaemia and hyperphosphatasemia were identified as predictors of hyperparathyroidism (p <0.05) (Table 3.4).

Univariate linear regression revealed a significant association between log transformed PTH and phosphate (r^2=0.03, p=0.007), calcium (r^2=0.02, p=0.03), TAP (r^2=0.05, p=0.006), 25 – OH vitamin D (r^2=0.05, p=0.005). When including all these parameters in a multiple regression analysis, only phosphate and 25 (OH) D remained significantly correlated with log PTH; p= 0.02 and 0.04 respectively.

3.4 Discussion

In agreement with previous studies from Africa and developed countries (7-9), this study has revealed a high prevalence of derangements of biochemical markers of mineral bone disorder. Based on the cut-off values utilized by this study, hyperparathyroidism (PTH>150) was present in 73.4% of the patients and 37% with a stricter cut-off value of 585 ng/ml (9 times the upper normal of our laboratory assay). However, because of the dynamic nature and complexity of bone homeostasis it is difficult to rely on one biochemical marker as a surrogate test of bone formation (10). Therefore, utilizing both PTH and TAP, though not as specific as bone specific alkaline phosphatase, almost half of our study population was identified as having high bone turn over. In addition, the 2009 Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommended measurement of TAP every 12 months

in CKD 4-5D (2), and the additional cost in routinely utilizing bone specific alkaline phosphatase to monitor the management of CKD-MBD has not been justified.

Comparison of epidemiological data on prevalence of vitamin D deficiency is being hampered by lack of consensus on how to define this deficiency. However, the widely accepted definition includes serum 25 (OH) D level below 20 ng/ml while patients with 25 (OH) D levels between 20 and 30 ng/ml are considered to be vitamin D insufficient(6). One of the striking results of our study was the high prevalence (80.7%, N=161) of inadequate vitamin D status in our study population and of which 29.2 % were severely vitamin D deficient (<15 ng/m/). These findings are consistent with previous cross sectional studies in Americans (11-13). In contrast to our finding regarding severe deficiency, Jabbar et al(14) reported vitamin D deficiency (<15ng/ml) in 90 % of the patients and attributed it to the low vitamin D content in the traditional (vegetarian) Indian diet. Diabetic patients had significantly lower mean levels of 25(OH) D and inadequate vitamin D compared to non-diabetic patients. This finding is in line with previous studies from Europe and Asia (15, 16) in which a high prevalence of vitamin D deficiency was demonstrated in > 90% of the diabetic patients. This remarkably high prevalence could have been due to proteinuria, which is more common in diabetic nephropathy and is associated with heavy urinary loss of vitamin D binding protein, as proposed by previous studies (16, 17). However, in general, factors that could account for vitamin D deficiency in patients with CKD include reduced dietary intake, loss of vitamin D binding protein in urine and increased levels of FGF23 in CKD (16).

In an attempt to reduce the adverse clinical outcomes associated with CKD-MBD several guidelines were proposed by various regional and global bodies (2, 18, 19). Based on the recommended target ranges by the widely adopted KDIGO guidelines,(2) more than half of our study population had serum phosphate levels above the target level (Phosphate >1.45mmol/L). This is similar to other large observational multicenter studies [Dialysis outcomes and practice patterns (DOPPS I) (20)and a study from Italy (21)] that both reported serum phosphate of >5.5mg/dL(1.78mmol/L) in 51.6% of the patients. Despite the advances in haemodialysis, with the invention of more effective dialysis membranes and the use of ultrapure dialysate, the removal of phosphate is still inadequate. The conventional HD (3 times per week, 4 h session) removes approximately 2.3-2.6g/wk compared to 4.5-4.9g by the nocturnal HD (8hrs/day) (22). The majority of our patients are on conventional HD with a

very few of them (9.2%) on nocturnal dialysis. Therefore, one of the reasons that could have accounted for the high prevalence of hyperphosphataemia is the ineffective removal of phosphate by conventional haemodialysis. In addition, calcium carbonate which is used as phosphate binder in most of our patients has lower phosphate binding capacity compared to non-calcium based phosphate binders. Other notable patient factors are the adherence to both medications and dietary restriction.

In this study, according to the KDIGO recommendation that PTH should be maintained between 2-9 times the upper limit of normal range (i.e 130-585 pg/ml), the proportion of patients within the target guideline level was higher than that reported by previous studies (7, 20, 23). Their reported lower values were likely due to utilization of KDOQI guidelines with narrower recommended ranges of PTH (150-300pg/ml).

Interestingly, a significant percentage of our patients had their corrected calcium within the KDIGO recommended guidelines which is higher than the report from DOPPS II 42.5 % (20). Re analyzing our data based on KDOQI the number still remains slightly higher (54.1%). Overall, the discrepancy between the current study and other previous studies may likely be due to differences in the use of phosphate binders, dialysate calcium concentration and dietary phosphate intake.

In keeping with previous studies (24), logistic regression analysis revealed hypocalcaemia and hyperphosphateamia as predictors of hyperparathyroidism. This further supports the classical role of hyperphospataemia and hypocalcaemia in the pathogenesis of CKD-MBD. In addition, we found phosphataemia to have remained closely related to log-transformed PTH in multivariate linear regression.

The strength of our study is the larger sample size compared to other previous studies from Africa (7, 9, 25).

The limitations of our study include: first, the lack of bone biopsy to definitively describe the patterns of CKD-MBD in our patients. However, studies have shown a good association between biochemical markers and histological findings (26, 27). Second, this was a cross sectional study so we could not establish the temporal relationships between the biochemical markers and phosphate binders. Thus there is a need for a longitudinal study to assess these

relationships. Nonetheless, findings from this study have provided us with important insights on the spectrum of CKD-MBD in African MHD patients.

In conclusion, abnormalities of biochemical markers of mineral bone disorder were common in our MHD patients and moderately large proportion of the patients was outside the KDIGO recommended target levels. However, due to the existence of racial differences in PTH, 25(OH) D and phosphorus levels it is unclear whether these guidelines could be extrapolated to African MHD patients. Therefore, there is a need for large multicenter studies in Africa to support management of CKD-MBD in African patients with CKD.

Conflict of interest: The authors declare they have no conflict of interest.

Table 3. 1: Study population characteristics

Parameters	Results
Age (years)	54.5± 15.6
Gender, n (%)	
Male	133(64.23%)
Female	74(35.75%)
Diabetes, n (%)	56(27.1%)
Race, n (%)	
Black	121(58.5%)
Non Black	86(41.5%)
Serum creatinine(µmmol/L)	695.48± 278.61
Serum calcium(mmol/L)	2.28±0.22
Serum phosphate(mmo/L)	1.59±0.56
Intact PTH(pg/ mL)	451.85±430.38
Plasma 25 -OH vitamin D(ng/ml)	21.16±10.71
Haemoglobin(g/dL)	10.26± 2.0
Albumin(g/L)	31.91± 6.12
Systolic BP(mmHg)	134.18±21.81
Diastolic BP(mmHg)	72.03±13.74
Alkaline phosphatase(UI/L)	143.21± 115.26
Kt/V	1.45±0.28
Dialysate Calcium(mmol/L)	2.22±0.09
Dialysis vintage(months)	24(12-48)
Calcium carbonate use n (%)	154(74.4%)
Cinacalcet n (%)	12(5.8%)
Alfacalcidol n (%)	132(63.8%)
Cholecalciferol n (%)	45(21.7%)

Continuous variables are presented as mean± standard deviation and categorical data as frequencies (percentages). BP= blood pressure

Table 3. 2: Comparison of parameters/distribution of mineral bone disorder between diabetic and non – diabetic patients

Variables	ALL(N=207)	DM(n=56)	Non DM(n=151)	P
Intact PTH	451.9±430.4	439.1±489.5	454.70±413.1	0.83
Corrected Calcium(mmol/L)	2.28±0.22	2.30±0.21	2.275±0.22	0.41
Phosphate(mmol/L)	1.59±0.56	1.52±0.51	1.61±0.58	0.25
Alkaline phosphatase(IU/L)	143.21± 115.26	155.77±131.72	139.15±109.41	0.41
Hyperparathyroidism (>150 pg/ml)	152 (73.4%)	38 (67.9%)	114 (75.5%)	0.84
Hypoparathyroidism (<10 pg/ml)	2 (9.7%)	1(1.79%)	1 (6.6%)	0.44
Hyperphosphataemia(>1.45mmol/l)	118(57.0%)	27 (48.2%)	91 (60.3%)	0.19
Hypophosphataemia(<0.80mmol/L)	11(5.3%)	4 (7.1%)	7 (4.6%)	0.44
Hypercalcaemia (>2.50mmol/L)	26(12.6%)	8(14.3%)	18 (11.9%)	0.53
Hypocalcaemia(<2.12mmol/L)	42 (20.3%)	10 (17.9%)	32 (21.2%)	0.91
ALP (>112IU/L)	103 (49.8%)	33 (58.9%)	70 (46.4%)	0.76
25-Hydroxyvitamin D (N=161)				
25 (OH) D(ng/ml)	21.16±10.71	18.12±12.77	22.21±9.82	0.04
(<30 ng/ml)	130 (80.7%)	35 (92.1%)	95 (77.2%)	0.04
(<15ng/ml)	47 (29.2%)	16 (42.1%)	31 (25.2%)	0.03
PTH>150 & TAP>112	98 (47.3%)	24 (42.7%)	74 (49.0%)	0.58

Continuous variables are presented as mean± standard deviation and categorical data as frequencies (percentages). PTH= Parathyroid hormone, TAP= total alkaline phosphatase,

Table 3. 3: Distribution of patients achieving recommended guideline targets

Guidelines	Below target	Within target	Above target
KDIGO			
PTH(2-9× upper normal range)	44(21.3%)	98(47.3%)	65(31.4%)
Calcium(within normal range)	48(23.1)	132(63.8%)	27(13.0%)
Phosphate(within normal range)	11(5.3%)	78(37.7%)	118(57.0%)
NKF KDOQI			
PTH(150-300 pg/mL)	51(24.6%)	54(26.1%)	102(49.3%)
corrected calcium(8.9-9.5 mg/d L)	32(14.5%)	112(54.1%)	63(30.4%)
Phosphate(3.5-5.5mg/dL))	35(16.9%)	104(50.2%)	65(31.4%)

KDIGO, Kidney Disease Improving Global Outcomes; NKF KDOQI, National Kidney Foundation Kidney Disease Outcome Quality Initiative.

Table 3. 4: Logistic regression analysis for predictors of hyperparathyroidism

Variables	Odds ratio	95 CI	P-values
Phosphate	3.06	1.15-8.10	0.02
Calcium	5.32	1.10-25.9	0.03
Alkaline phosphatise	1.67	0.66-4.29	0.28
Diabetes mellitus	0.60	0.19-1.84	0.37
Age	0.76	0.25-2.33	0.64
25(OH) D	0.39	0.10-1.60	0.20

CI, confidence interval; covariates were by categories, Age≥ 65 years versus<65 years, phosphate >1.45 mmol/l versus≤1.45mmol/l, calcium<2.12mmol/l versus≥2.12mmol/l, Alkaline phosphatase>112 UI/L versus ≤112 UI/L, 25 (OH) D <30ng/ml versus≥ 30ng/ml, Diabetes mellitus versus No diabetes.

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CHAPTER 4: MANUSCRIPT 2

High serum alkaline phosphatase, hypercalcaemia, race and mortality in South African maintenance haemodialysis patients.

ABSTRACT

Background

Studies relating to associations between high alkaline phosphatase, altered markers of mineral bone disorder and increased mortality in maintenance haemodialysis patients (MHD) patients are mainly from Europe, America and Asia. However, little is known on the existence of this association in African MHD patients. Therefore, the aim of this study was to determine the relationship between serum total alkaline phosphatase (TAP) and mortality in African MHD patients.

Patients and Methods

The study enrolled a total of 213 patients on MHD from two dialysis centers in Johannesburg between January 2009 and March 2016. Patients were categorized into a low TAP group (≤112 U/L) versus a high TAP group (>112 U/L) based on median TAP of 112 U/L. Using multivariate Cox regression analysis the impact of serum TAP on the primary outcome (death) was assessed.

Results

The MHD patients (136 men, 76 women) had a mean age of 54.5±15.6 years with a median dialysis vintage of 24 months (IQR, 12-48) and a mean Kt/V (single pool) of 1.44±0.3. During the follow up period of 7 years, there were 55 (25.8%) deaths. After adjusting for cofounders such as age, other markers of bone disorder and co morbidity (diabetes mellitus), patients in the high TAP group had significantly higher risk of death compared to patients in the low TAP group (hazard ratio, 2.50; 95% CI 1.24-5.01, p=0.01). Similarly, serum calcium >2.75 mmol/L was associated with increased risk of death compared to patients within levels

of 2.10-2.37 mmol/L (HR 6.34, 95% CI 1.40-28.76; P=0.02). The HR for death in white patients compared to black patients was 6.88; 95% CI 1.82-25.88; p=0.004.

Conclusion

High levels of serum alkaline phosphatase, hypercalcaemia and white race are associated with increased risk of death in MHD patients.

Key words: Alkaline phosphatase, calcium, death, maintenance haemodialysis, race.

4.1 Introduction

Prior to the availability of commercial intact parathyroid hormone (PTH) assays, serum total alkaline phosphatase (TAP) measurements were used as one of the surrogate markers of high bone turnover that was utilized in the management of chronic kidney disease mineral and bone disorder (CKD-MBD) (1). Subsequently, in 2003 the Kidney Disease Outcome Quality Initiative (KDOQI) guidelines on CKD-MBD made no recommendations regarding the use of alkaline phosphatase and this has made it a less preferred marker to PTH. However, in 2009 the Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommended measurement of TAP every 12 months in CKD 4-5D(2) and more recently evidence continued to emerge on the importance of higher levels alkaline phosphastase in the pathogenesis of vascular calcification via hydrolysis of pyrophosphate which is a potent inhibitor of vascular calcification (3-5). This was further supported by a study that showed elevated levels of alkaline phosphatase, independent of PTH, calcium or phosphorus as predictor of coronary artery calcification in haemodialysis patients (6). Interestingly, in a recent secondary analysis of the handling erythropoietin resistance with oxypentifylline (HERO) trial, high levels of alkaline phosphatase were also associated with erythropoietin stimulating agent hypo responsiveness (7). These findings may likely explain the unclear pathophysiologic link between high serum alkaline phosphatase and mortality in haemodialysis patients (6).

Although the role of racial disparities in adverse clinical outcomes remains controversial and inconclusive, some studies have demonstrated survival benefits attributable to race in patients

undergoing MHD (8, 9). In addition, the impact of these biochemical abnormalities have been shown to differ across race and thus the need for race specific target values for these markers of mineral bone disorder (10, 11).

Therefore, the aim of this study is to determine if there is a link between high serum alkaline phosphatase and mortality in African MHD patients.

4.2 Patients and Methods

This study was a retrospective review of patients undergoing MHD from two dialysis centers in Johannesburg between January 2009 and March 2016. A total of 213 patients aged ≥ 18 years with available baseline line variables of interest were included. Exclusion criteria included patients with missing important data for analysis, being on dialysis for less than three months, having active or chronic liver disease and having malignancies. In addition, we excluded Indian and mixed races to allow for a proper comparison between black and white patients. Retrieved data included patients' demographic characteristics, blood pressure measurements, duration on haemodialysis, co-morbid disease and medication history related to CKD-MBD. Determination of race was based on self-report by the participants.

Patients were categorized into the low TAP group (≤112 U/L) versus the high TAP group (>112 U/L) based on median TAP level of 112 U/L. Secondary analysis involved exploring the relationship between race, other markers of mineral bone disorder, and primary outcome. In line with a previous study (12) total calcium levels were categorized into four categories with the KDOQI target range as the reference category. Based on the KDIGO CKD-MBD guidelines, PTH was divided into three categories.

The primary outcome of this study was death and events other than death were censored and this included kidney transplantation, loss to follow–up, or still undergoing haemodialysis at the end of the study.

Laboratory measurements

Patients' baseline biochemical parameters (within the first three months of initiating dialysis) were assessed. Most of the biochemical markers were measured monthly except for quarterly PTH. Plasma intact PTH was measured by an electrochemiluminescence immunoassay

(ECLIA) run on a Cobas 6000 auto analyzer (Roche Diagnostics, Mannheim, Germany; reference range is 10-65 pg/mL). Serum 25 (OH) D was measured by a chemiluminescent micro particle immunoassay (CMIA) technique run on the ARCHITECT C8000 auto analyzer (Abbott Laboratories, Abbott Park, Illinois, US). Reference ranges: < 10 ng/mL as severe deficiency, 10-29 ng/mL as moderate deficiency, 30-100 ng/ mL as sufficiency and > 100 ng/ mL as toxic.

Serum calcium, phosphate and alkaline phosphatase were measured using the ARCHITECT C8000 auto analyzer (Abbot Laboratories, Abbott Park, Illinois, US). The corrected calcium was determined using the formula: corrected calcium (mmol/L) = calcium measured (mmol/L) + 0.02 [40-albumin (g/L)]. Total alkaline phosphatase reference range is 53-128 U/L.

Plasma albumin was measured by colorimetric (bromocresol green) method on a Cobas 6000 auto analyzer (Roche Diagnostics, Mannheim, Germany; reference range 35-52 g/L).

Other biochemical parameters were determined using routine laboratory techniques.

Blood samples were generally collected predialysis at midweek with the exception of the post dialysis serum urea for kinetic modeling.

Calculation of normalized protein catabolic rate was based on the formula (13), nPCR= $(0.136 \times F) + 0.251$. Where $F = Kt/V \times ([\text{pre dialysis BUN} + \text{post dialysis BUN}] \div 2)$.

Statistical analysis

Pearson's or Fisher's exact test was utilized for proportion comparisons. Continuous variables are presented as means± standard deviations or medians and inter quartile ranges (IQR) as appropriate. Associations between serum alkaline phosphatase and other biochemical parameters were assessed by multiple linear regression analyses. Cox proportional model was used to determine the crude and adjusted hazard ratios of death for different categories of serum alkaline phosphatase, calcium, PTH, phosphate, 25 (OH) D and white versus black patients. Patients' demographic and baseline characteristics were compared between the low and high total alkaline phophatase groups as well as white versus black patients, using an independent t – test and Mann- Whitney-U test for normally

distributed and non-normally distributed variables respectively. One way ANOVA and Kruskal-Wallis tests were used to compare normally and non- normally distributed continuous variables across categories of serum calcium.

A P value of less 0.05 was considered statistically significant at the 95% confidence interval. All analyses were performed using STATA version 12 (STATA Corp., TX, and USA).

4.3 Results

The study included two hundred and thirteen patients (137 men, 76 women) undergoing MHD. The mean (±SD) of age, median dialysis vintage and mean Kt/V were 54.5±15.6 years, 24 months (IQR, 12-48) and 1.44±0.3 respectively. The majority of the patients were on three times weekly, 4 hr sessions of haemodialysis. Most of the patients were dialyzed with a dialysate calcium concentration of 1.50 mmol/L, which is usually modified based on serum levels of calcium. The blood and dialysis flow rates are generally 300-400mls/min and 500 mls/min respectively. However, these values varied according to patient's blood pressure and haemodynamic state. A native arteriovenous fistula was used in more than half of the study population (60.6%). Almost all patients (93.0 %) were on erythropoiesis-stimulating agents (ESAs).

Table 4.1 shows the comparisons of baseline clinical characteristics between patients in high TAP and low TAP groups. The low alkaline phosphatase group had significantly higher mean age than the high TAP group. Other parameters were comparable between the groups. For the management of CKD-MBD, 76.9 % of the patients were on calcium carbonate and 64.3% on alfacalcidol with a similar distribution of drug usage across the groups. The study population included 120 (56.3%) black and 93(43.7%) white patients. The mean age, hemoglobin concentration, albumin and phosphate were significantly higher in white compared to black patients. Fifty six (26.3%) of the study population had diabetes and the proportion was higher in black patients (30.0% versus 21.5 %, P=0.02) (Table 4.2).

The characteristics of the patients across different categories of serum calcium levels are shown in Table 4.3. Patients in the highest category of calcium levels had significantly lower

mean serum creatinine, and a fewer of them were on calcium carbonate and alfacalcidol. No significant differences were found in other parameters of the patients across the serum calcium categories. The overall mean dialysate calcium was 1.65±0.24 mmol/l and patients with higher levels of calcium are more likely to be dialyzed with lower dialysate calcium concentration. To further explore our practice pattern regarding treatment of hypecalcaemia, available data revealed that only 5 patients in the highest category of calcium had parathyroidectomy while the majority of them were dialyzed with 1.25 mmol/l of dialysate calcium concentration and had their calcium carbonate and alfacalcidol discontinued.

During a follow up period of 7 years there were 57 (26.8%) deaths. After adjusting for cofounders such as age, other markers of bone disorder (calcium, phosphate, and PTH), serum alanine transaminase, 25 (OH) D and co-morbidity (diabetes mellitus), patients in the high TAP group had significantly higher risk of death compared to patients in the low TAP group (hazard ratio, 2.5; 95% CI 1.24-5.01, log rank P=0.01).

Patients in the highest category of corrected calcium (>2.75 mmol/L) had more than a six fold increased risk of death compared to patients with normal calcium (HR 6.34, 95% CI 1.40-28.76; P=0.02). Similarly, we found a significant association between race and mortality, in which white patients had an accentuated six fold increase in adjusted hazard ratio for death compared to black patients (HR 6.88,95% CI 1.82-25.88; P=0.004) (Table4). Figures 4.1, 4.2, and 4. 3 show Kaplan Meier Survival curves for TAP, race and calcium levels respectively.

Univariate linear regression analysis revealed a significant association between TAP and age (r^2 = 0.04, P=0.008), corrected calcium (r^2=0.03, P=0.04), and PTH (r^2=0.04, P=0.006). In multivariate regression analyses PTH and calcium remained significantly correlated with TAP, P=0.006 and 0.04 respectively.

4.4 Discussion

Several studies from Europe, America and Asia have consistently shown a linear relationship between high serum alkaline phosphate and mortality in the haemodialysis population (14-

17), while results relating to other markers of mineral metabolism revealed non- linear (U or J patterns) associations (12, 18, 19). Such data relating to the impact of markers of CKD-MBD on mortality in African MHD patients are lacking. In this present study, higher levels of TAP, hypercalcaemia and white race were associated with increased risk of death. These findings are consistent with other large studies where higher levels of TAP were independently associated with higher risk of mortality (14, 17).

Interestingly, this association was also reported in CKD patients as well as in the general population (20, 21). The National Health and Nutrition Examination Survey (NHANES) data revealed an independent association between elevated levels of TAP and mortality in the general population (21). This further supports the notion that TAP is more than a marker of high bone turnover and may be a reliable predictor of mortality.

The mechanisms for this association have been linked to enhanced vascular calcification by high levels of serum TAP through hydrolysis of pyrophosphate or activation of apatite crystal formation (22). In addition to vascular calcification, elevated levels of TAP have been associated with high C reactive protein, insulin resistance and 25 (OH) D deficie (23-26). In contrast to our study, we found no significant difference in the mean levels of 25(OH) D between patients with high TAP and low TAP.

Despite the variations in the cut-off points for defining hypercalcaemia by various studies, hypercalcaemia has been consistently associated with increased risk for mortality in haemodialysis patients (12, 18, 27). Consistent with our finding, a linear relationship was observed between higher calcium categories and increased risk of death (12, 18). In a large global representation of HD patients including the three phases of the dialysis outcomes and practice patterns study (DOPPSI,II and III) with 25,588 HD patients, calcium levels greater than 10.0 mg/dl (>2.5mmol/l) were significantly associated with greater risk of all cause and cardiovascular mortality in both baseline and time dependent models (27). The reasons for this consistent association could be linked to acceleration of arterial calcification by hypercalcaemia (28, 29). Besides vascular calcification, high levels of calcium, but not high PTH have been associated with poor mental health in MHD patients (30). In contrast, studies relating to hypocalcaemia and risk of death have yielded contradictory reports. Lowrie and Lew (31) were the first to establish the association of increased mortality with calcium levels <9.0 mg in over 12,000 HD patients, while in another large study from the US involving 40,

538 HD patients, the mortality risk with low serum calcium levels was attenuated after adjusting for confounding variables (18). In the dialysis outcomes and practice patterns study (32), serum calcium levels < 7.8 were associated with lower mortality risk. In agreement with DOPPS, we found a similar trend, though not statistical significant, with serum calcium levels below 2.12 mmol/L.

Hypercalcemia is an undesirable effect associated with the use of calcium based phosphate binders and vitamin D analogues in controlling secondary hyperparathyroidism. This may likely have accounted for the lower levels of PTH seen in our category of patients with calcium levels above 2.75 mmol/L. Although cinacalcet which is one of the newer drugs that effectively lowers PTH without raising serum calcium levels recently became available in South Africa, it is quite expensive, thus limiting its use to a few of our patients. In addition, the higher mean phosphate level in this group of patients is likely due to the concomitant use of alfacalcidol that enhances intestinal absorption of calcium and phosphate.

A notable finding in the current study is that white patients have poor survival compared to black patients. This finding is consistent with recent emerging data from the USA that reported better survival in black patients compared with white patients on MHD (10). The reasons underlying this racial survival benefit remain unclear, and several studies have proposed explanations for the better survival of black MHD patients compared to whites. A large USA observational study reported that the widely perceived survival advantage for black dialysis patients applies only to older adults, with a reversal of the higher risk of death in the younger age group (<50 years) (33). This is contrary to several studies including the current study, where the risk persisted after adjusting for the significantly higher mean age in the white patients (34, 35). Indeed, the better survival in black patients persisted in a study that comprehensively adjusted for demographics and dialysis modality among several other cofounding variables (34).

Another important observation we made in this study was that white patients had significantly higher levels of serum albumin. We expected this to give white patients a survival benefit. However, the reason for this reversal could likely be explained by a finding from a previous study where markers of worse nutritional status (hypoalbuminemia), or smaller muscle mass and increased body fat in African American patients correlated less strongly with mortality than in whites (36). Additionally, studies have criticized the use of

serum albumin in CKD patients as a marker of nutritional status as inappropriate (37, 38). In fact, the hazard ratio becomes accentuated after adjusting for serum albumin suggesting that the effect of race on mortality is likely to be through other mechanisms besides nutritional status.

In line with previous studies (33, 39), black patients had higher median intact PTH though this was not statistically significant. Some studies have reported survival benefit with active D therapy and that black patients are more likely to receive active vitamin agents due to higher PTH compared to white patients (40, 41). However, it is unlikely that treatment with vitamin D alone may explain the racial survival paradox that has existed for several years. Additionally, reports relating to PTH levels have been controversial and studies are divided on which levels are associated with increased mortality. Similar to earlier (17, 18, 42) and more recent studies (10, 43), we did not find significant association of mortality with severe hyperparathyroidism. On the other hand, studies that have shown significant associations are not unified on what levels of PTH are associated with increased mortality. Therefore, randomized control trials are needed to show the effect of treatment on PTH levels that are associated with favorable clinical outcomes.

Our findings should be considered in the context of the following limitations. Firstly, the retrospective nature of this study could not allow us to make causal associations between markers of mineral bone disease and study outcome (death). In addition, the use of a single baseline laboratory measurement precludes the performance of time dependent Cox analysis to account for variations in the biochemical markers on the impact of death over a period of time. However, few studies have shown no significant difference between the baseline and time dependent Cox analysis (12).

Secondly, the relatively small sample size precludes generalizability of our findings to African HD patients. Thus, there is a need for multicenter studies in Africa, to provide robust data on this important clinical entity (CKD-MBD) in African HD patients.

Thirdly, similar to several observational studies we could not account for residual confounding variables. For instance, aside from diabetes mellitus, other co morbid conditions could not be ascertained. However, part of the exclusion criteria was to avoid patients with some co-existing conditions that are known as potential confounders.

The strengths of this study lie in the heterogeneous nature of our study population (black and white patients) in an African setting which has allowed comparisons of data not only for Black Africans with Black Americans, but also between whites in Africa and USA/Europe. To our knowledge, this the first study in sub Saharan Africa that has given important insights regarding the impact of alkaline phosphatase, calcium and race on mortality in African MHD patients.

In summary, high TAP, hypercalcaemia and white race are associated with increased risk of death in MHD patients, thus, reaffirming the need to pay more attention to the two modifiable risk factors (calcium and TAP) in the management of CKD-MBD.

Conflict of interest: The authors declare they have no conflict of interest.

Ethical approval: All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The research protocol was approved by the Health Research and Ethics committee (HREC) of the University of the Witwatersrand; clearance certificate number M141016.

Table 4. 1: Comparisons of baseline characteristics between patients in high TAP and low TAP groups

Characteristic	All(N=213)	TAP≤112(n=98)	TAP>112(n=115)	P value
Age(years)	54.53±15.62	57.3±15.5	51.1±15.1	0.008
Female, n (%)	76(35.7%)	35(35.7%)	41(35.7%)	0.25
Diabetes, n (%)	56(26.3%)	27(27.6%)	29(25.2%)	0.76
Weight (Kg)	71±9.6	70±9.5	69±9.6	0.53
BMI (Kg/m^2)	24.7±0.9	24.9±1.0	24.5±1.6	0.83
Dialysis vintage (months)	24(12-48)	36(12-60)	36(12-48)	0.55
Systolic BP(mmHg)	134±21.8	135.5±19.6	133.5±24.4	0.38
Diastolic BP (mmHg)	72.0±13.73	70.7±12.0	74.1±13.8	0.86
Haemoglobin(g/dl)	10.3±2.0	10.2±1.9	9.9±2.1	0.10
Potassium(mmol/l)	4.62±0.8	4.6±0.9	4.6±0.8	0.55
Calcium (mmol/l)	2.25±0.14	2.32±0.30	2.34±0.29	0.58
Corrected calcium(mmol/l)	2.40 ±0.25	2.50±0.22	2.50±0.21	0.42
iPTH(pg/ml)	307(148-656)	246(137-527)	325(152-693)	0.09
Phosphate(mmol/l)	1.59±0.6	1.60±0.6	1.40±0.6	0.07
25-OH vitamin D(ng/ml)	21.16±10.71	20.4±8.8	22.2±12.9	0.83
Alkaline phosphatase(U/L)	112(74-163)	74(62-96)	163(130-223)	<0.001
Albumin(g/L)	31.9±6.0	32.6±5.4	30.3±6.5	0.98
Type of vascular access				
Arteriovenous fistula	129 (60.6%)	65 (66.3%)	64 (55.7%)	0.23
Graft	39 (18.3%)	23 (23.5%)	26 (22.6%)	0.88
Catheter	45 (21.1%)	21(21.4%)	24(20.9%)	0.97
Alanine transaminase (U/L)	21.1±8.9	17.6±8.7	22.9±8.8	0.20
Kt/V	1.44±0.28	1.4±0.3	1.4±0.2	0.72
n PCR (g/kg/day)	1.10±0.24	1.02±0.30	1.08±0.27	0.56
T.cholesterol (mmol/l)	4.18±0.91	4.3±0.9	4.1±0.9	0.14
Medications				
Calcium carbonate, n(%)	163(76.5%)	77(78.6%)	86(74.7%)	0.74
Alfacalcidol, n (%)	137(64.3%)	61(62.2%)	76(66.1%)	0.55
ESA n (%)	198 (93.0%)	94(95.9)	104 (90.4%)	0.50
ESA dose (U/week)	13373±4205	13714±4768	12957±3457	0.53

Continuous variables are presented as means± standard deviations or median (interquartile range) and categorical data as frequencies(percentages) , BP= blood pressure , i PTH= intact parathyroid hormone, TAP= total alkaline phosphatase., ESA=erythropoietin stimulating agent, n PCR= normalized protein catabolic rate, BMI= body mass index.

Table 4. 2: Baseline characteristics of study population by race

Parameters	All (n=213)	Black (n=120)	White (n=93)	P value
Age (years)	54.53±15.62	51.0±14.6	58.7±15.9	<0.001
Haemoglobin (g/dl)	10.3±2.00	9.9±1.98	10.7±1.94	0.004
Systolic Bp (mmHg)	134±21.8	130±20.3	139±22.8	0.98
PTH (pg/ml)	307(148-656)	327(137-658)	290(149-618)	0.97
Calcium (mmol/l)	2.28±0.22	2.26±0.22	2.30±0.21	0.94
Phosphate (mmol/l)	1.59±0.56	1.49±0.57	1.71±0.53	0.004
Albumin (g/l)	31.9±6.0	30.8±6.5	33.04±5.5	0.03
25(OH) vitamin D D(ng/ml)	21.16±10.71	20.57±9.79	21.80±11.67	0.77
TAP (U/L)	112 (74-163)	110 (75-151)	115(71-164)	0.33
T.cholesterol (mmol/l)	4.2±0.8	4.0±0.9	4.1±0.9	0.05
Diabetes, n (%)	56(26.3%)	36(30.0%)	20(21.5%)	0.02
Male, n (%)	137(64.3%)	72(60.0%)	65(69.9%)	0.07
Kt/V	1.44±0.3	1.41±0.3	1.46±0.30	0.40

Continuous variables are presented as means± standard deviations or median (interquartile range) and categorical data as frequencies (percentages). BP= blood pressure, TAP=total alkaline phosphatase, PTH = parathyroid hormone

Table 4. 3: Patient characteristics by serum calcium categories

Parameters	<2.10mmol/l (n=31)	2.10-2.37mmol/l (n=92)	2.38-2.75mmol/l (n=57)	>2.75mmol/l (n=33)	P-value
Age (years)	50.9±15.0	52.9±15.0	58.3±16.4	56.5±26.1	0.09
Systolic Bp (mmHg)	130.9±18.6	138.8±21.5	139.8±30.7	138.9±21.5	0.18
Diastolic Bp (mmHg)	71.2±15.3	71.7±11.2	76.4±18.9	71.2±11.1	0.38
Haemoglobin (g/dl)	10.8±2.4	10.2±1.9	10.1±1.9	8.15±1.9	0.20
Albumin g/L	32.0±5.2	32.7±6.0	30.5±6.6	29.5±5.0	0.26
T.chol (mmol/l)	4.3±1.0	4.2±0.9	4.2±0.9	4.1±0.9	0.97
25(OH) D (ng/ml)	22.8±9.1	22.0±10.4	18.1±8.1	15.8±3.5	0.11
PTH (pg/ml)	568.8±334.8	458.64±424.4	366.2±405.1	254.0±103.2	0.01
Phosphate (mmol/l)	1.5±0.6	1.6±0.6	1.5±0.5	1.6±0.5	0.66
Creatinine(μmol/l)	822.5±261.0	734.4±283.2	592.5±245.5	489.5±355.7	0.002
Kt/V	1.4±0.2	1.5±0.3	1.4±0.3	1.4±0.4	0.33
Vintage (months)	31.3±23.0	34.2±23.0	30.9±21.1	30.0±8.9	0.80
Dialysate Ca (mmol/l)	1.65±0.24	1.63±0.14	1.63±0.14	1.54±0.24	0.50
DM, n	13	15	17	11	0.40
Medications					
Calcium carbonate n (%)	30 (96.8%)	79(85.7%)	41(71.9%)	13(39.4%)	<0.001
Alfacalcidol n (%)	28 (90.3%)	63 (68.4%)	35 (61.4%)	11(33.3%)	<0.001

Continuous variables are presented as means± standard deviations or median (interquartile range) and categorical data as frequencies (percentages). BP= blood pressure, PTH= parathyroid hormone, P values derived by one way ANOVA and Kruskal –Wallis tests for continuous variables and Chi squared for categorical variables. Serum categories based on KDOQI reference range.Ca= Calcium

Table 4. 4: Crude and adjusted hazard ratio (95%CI) of primary outcome by baseline characteristics

Parameter	Crude HR	95%CI	P	Adjusted HR	95%CI	P
TAP >112 U/L	2.20	1.12-4.32	0.02	2.50	1.24-5.01	0.01
Calcium (mmol/l)						
<2.10	0.66	0.32-1.35	0.26	0.97	0.22-4.26	0.97
≥2.10-≤2.37	1.00	Reference				
>2.37-≤2.75	2.31	1.20-4.44	0.02	1.54	0.57-4.18	0.39
>2.75	6.82	1.55-30.1	0.01	6.34	1.40-28.76	0.02
PTH(pg/ml)						
<130	1.00	Reference				
≥130-≤585	1.26	0.57-2.79	0.56	2.77	0.61-12.58	0.19
≥585	1.05	0.44-2.49	0.92	2.22	0.42-11.65	0.35
Phosphate >1.50mmol/l	1.09	0.61-1.95	0.77	1.43	0.47-4.40	0.53
25 OH vitamin D ≤30 ng/ml	2.21	0.66-7.35	0.19	1.07	0.23-4.79	0.92
White race	1.69	0.95-3.04	0.08	6.88	1.82-25.88	0.004

HR=hazard ratio, CI=confidence interval, TAP= total alkaline phosphate, PTH intact parathyroid hormone. Adjusted for age, phosphate, calcium, PTH, TAP, diabetes, Systolic BP, 25-OH vitamin D, alanine transaminase and albumin, serum calcium categories based on KDOQI reference range .

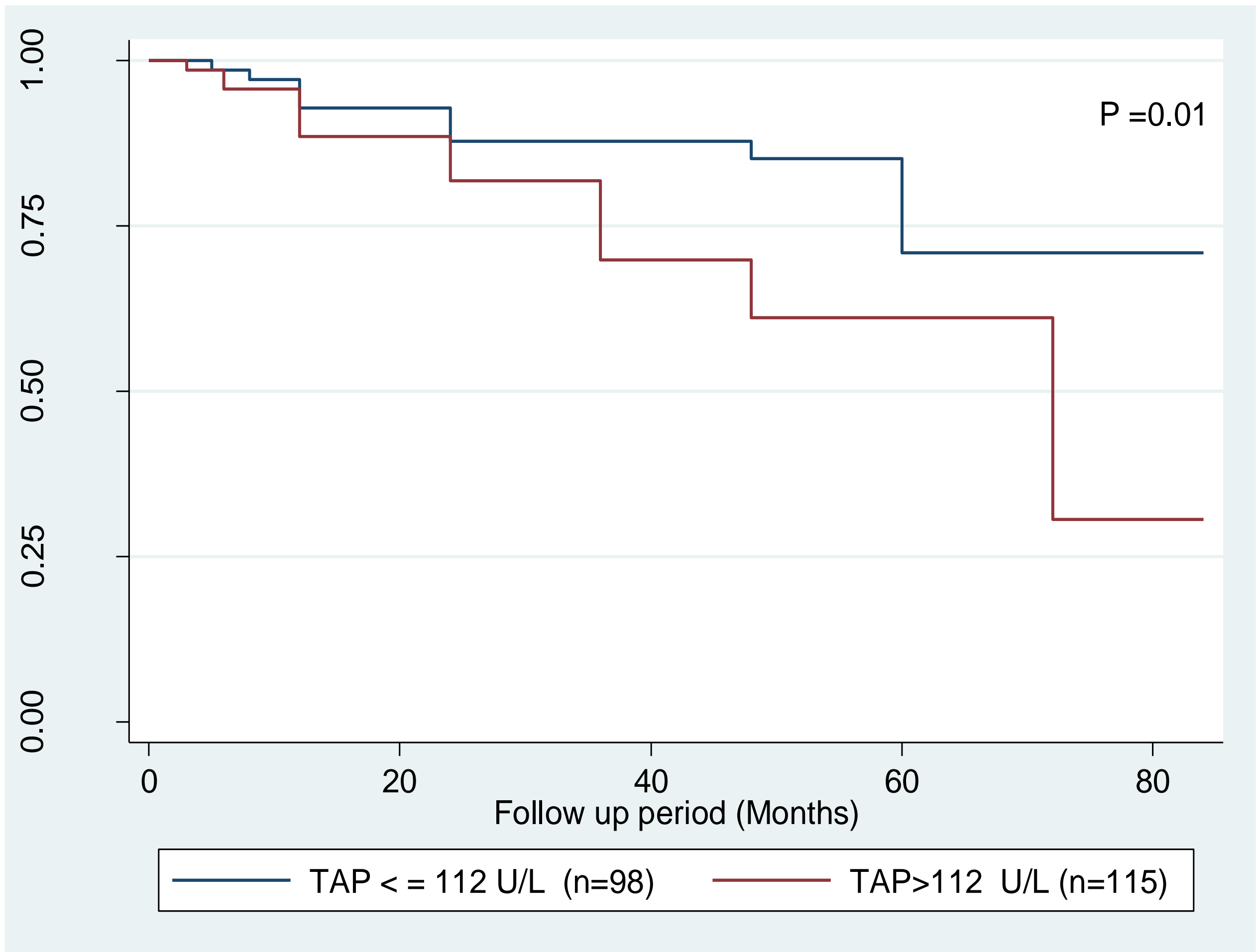

Figure 4. 1: Kaplan Meier curve comparing patients in the high alkaline phosphatase to low alkaline phosphatase group

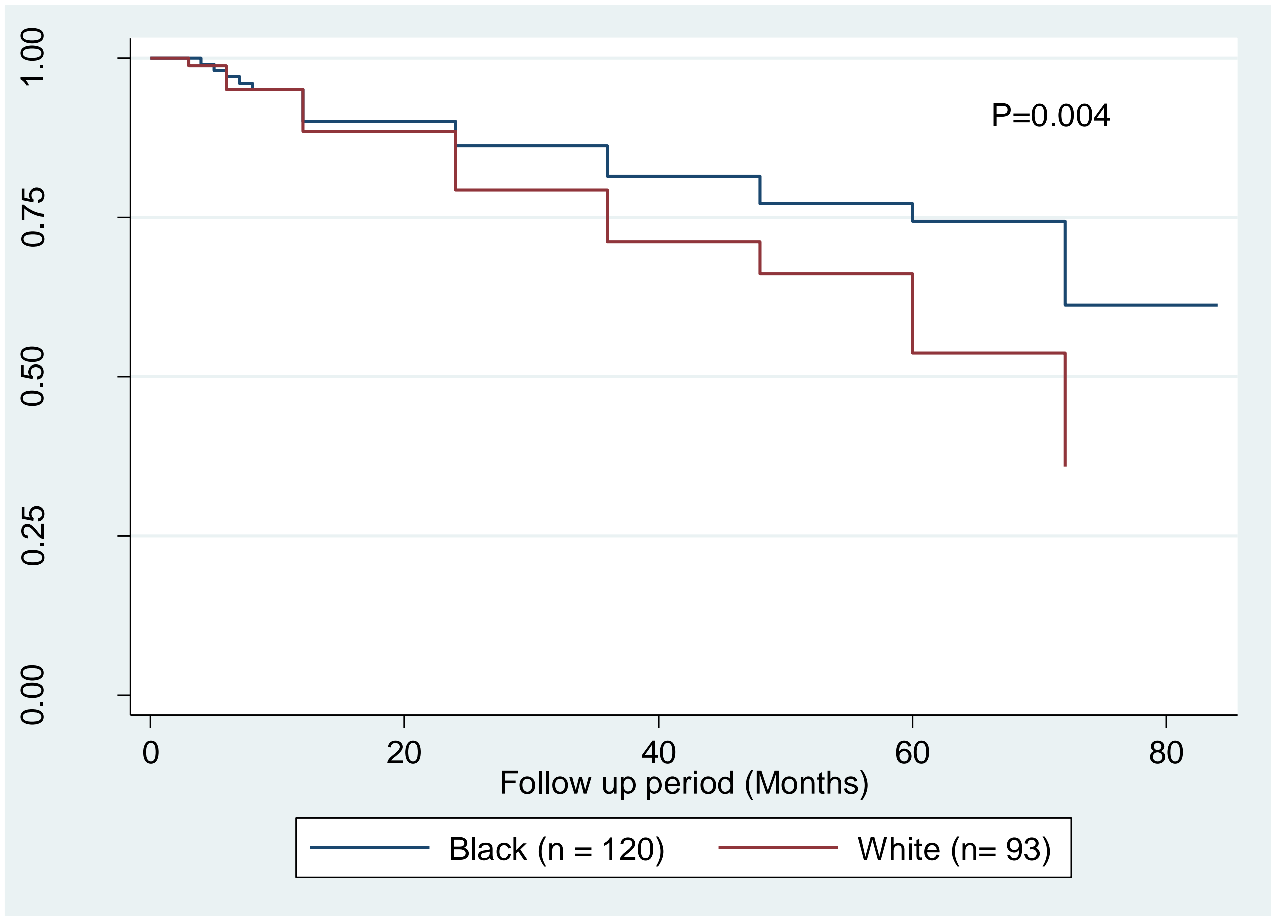

Figure 4. 2: Kaplan Meier survival curve between black and white

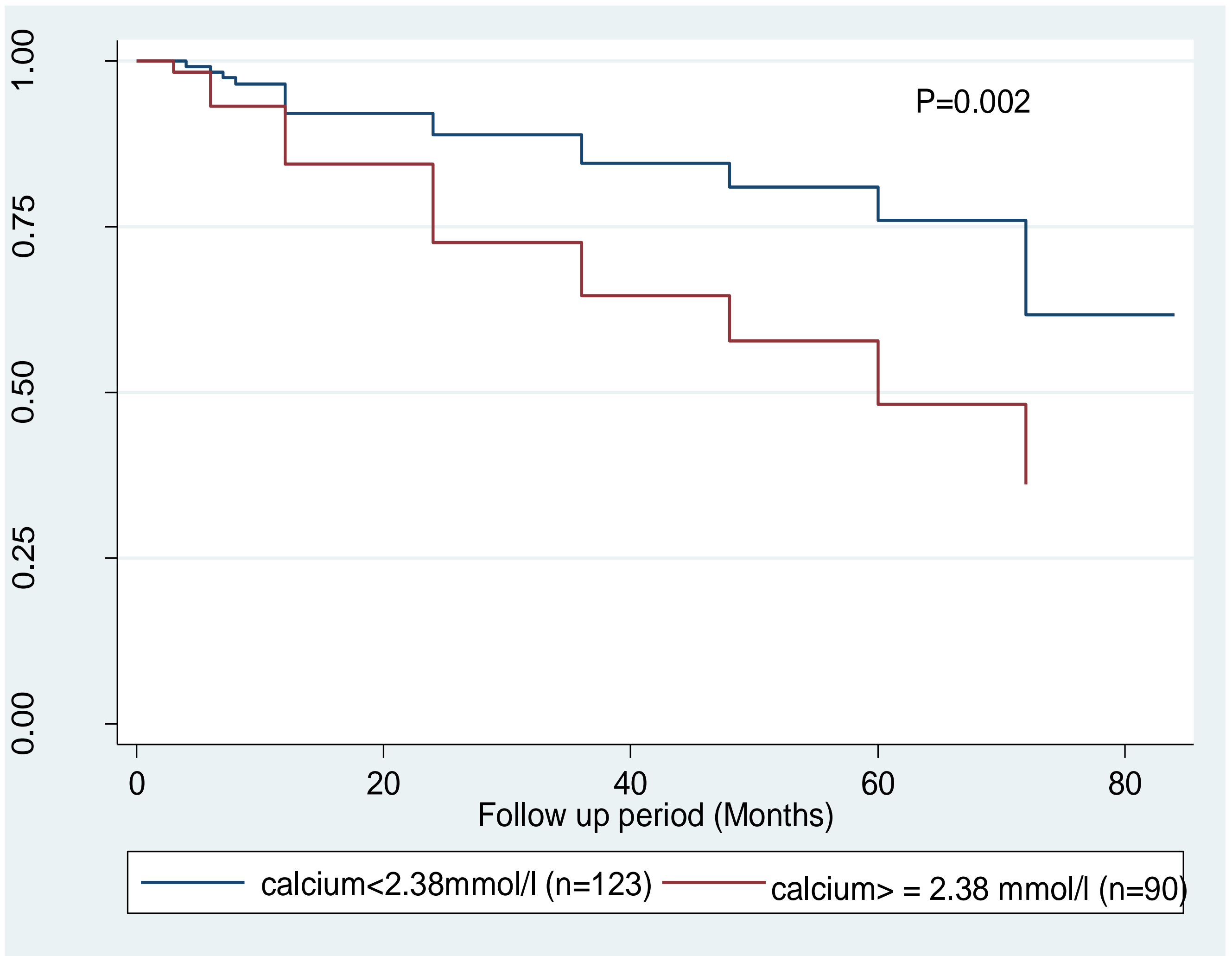

Figure 4. 3: Kaplan Meier survival curves for different categories of calcium

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CHAPTER 5: MANUSCRIPT 3

Racial variations in the markers of mineral bone disorders in chronic kidney disease patients in South Africa

ABSTRACT

Introduction

Several studies have shown that serum intact parathyroid hormone (PTH), phosphate and vitamin D levels differ across races. These comparative studies were largely carried out between Caucasians and Black Americans. However, little is known of the existence of this association in an African chronic kidney disease (CKD) population.

Patients and Methods

A cross –sectional multicenter study involving two hundred and ninety three CKD patients from three renal units in Johannesburg, South Africa.

Results

The 293 CKD patients (208 blacks, 85 whites) had an overall mean age of 51.1±13.6 years, and black patients were significantly younger than the white patients (48.4 ±.13.6 versus 57.1±15.5 years; p<0.001). Compared to whites, blacks had higher median intact PTH (498 [37-1084] versus 274[131-595] pg/ml; p=0.03), alkaline phosphatase (122[89-192] versus 103[74-144] U/L; p=0.03) and mean 25 OH vitamin D_3 (26.8±12.7 versus 22.7 ±12.2 ng/ml, p=0.01) levels , while their median FGF23 (100 [34-639] versus 233[80-1370] pg/ml; p=0.002) and mean serum phosphate (1.3±0.5 versus 1.5±0.5, p=0.001) levels were significantly lower. In multivariable analyses, black race was independently associated with increased log PTH (β =0.488, p=0.01) and decreased log FGF23(β=-0.636, p= 0.02). Similarly, blacks had a 3.08 times higher likelihood (95 % CI, 1.51-6.30, p =0.002) of developing severe hyperparathyroidism than whites.

Conclusion

This study highlighted the existence of racial differences in the circulating markers of mineral bone disorders in an African CKD population.

Keywords: Race, Fibroblast growth factor- 23, Chronic Kidney Disease, Mineral Bone Disorder.

5.1 Introduction

Modifiable abnormalities of markers of mineral bone disease (MBD) have been consistently associated with adverse clinical outcomes in patients with chronic kidney disease (CKD) (1-3). Addressing these adverse outcomes has led to recommendations by various global and regional societies to assist physicians in the management of CKD-MBD (4-6). However, the consequences of these biochemical abnormalities have been shown to differ across different races and thus there is the need to establish race specific target values for these markers of MBD (7). For example, in the multi ethnic study of atherosclerosis (MESA) involving 6436 participants, 25-hydroxyvitamin D (25-(OH) vitamin D) deficiency was associated with increased risk of coronary heart disease in white but not in black Americans (8). A similar trend was found in the National Health and Nutrition Examination Survey (NHANES III) , where low 25 (OH) vitamin D was associated with a higher risk of all-cause mortality compared to black participants (9). Furthermore, fibroblast growth factor (FGF) 23, which is now being considered as the principal mediator of secondary hyperparathyroidism has also been shown to differ across races (10, 11). In general, these comparative studies, largely from American populations reported that compared to whites, blacks have lower levels of 25 (OH) vitamin D and FGF23 with higher parathyroid hormone (PTH) and alkaline phosphate levels, while results relating to phosphate levels are inconsistent (7, 10-12).

The existence of these differences in a heterogeneous African CKD population is largely unknown. Therefore, the aim of this study was to examine racial differences in the levels of FGF23 and traditional markers of mineral bone metabolism in a South African CKD population.

5.2 Materials and Methods

This was a cross sectional multicenter study involving two hundred and ninety three CKD patients from three renal units in Johannesburg, South Africa. Patients enrolled were aged ≥ 18 years with established CKD. We excluded patients with active malignancy, acute kidney injury and a history of parathyroidectomy. In addition, we excluded patients of Indian and mixed race origin due to their negligible numbers. A structured questionnaire was used to obtain patients' demographic characteristics, blood pressure measurements, co-morbid disease and medication history related to CKD-MBD. Determination of race was based on self-report by the participants.

Laboratory measurements

Plasma intact PTH was measured by an electrochemiluminescence immunoassay (ECLIA) run on a Cobas 6000 auto analyzer (Roche Diagnostics, Mannheim, Germany).

FGF23 was measured using a sandwich based enzyme-linked immunosorbent assay kit from EMD Millipore Corporation (Billerica, MA, USA); assay lower limit of detection was 3.2 pg/mL. Plasma 25(OH) D was measured using the high performance liquid chromatography (HPLC) kit (Recipe, Munich, Germany). HPLC was used to selectively measure 25-(OH) D2 and 25-(OH) D3 at a wave length of 264nm. The intra and inter assay coefficients of variation (CVs) were < 5%. Our institutional laboratory is a participating member in the vitamin D external quality assurance scheme (DEQAS). In this study, 25-(OH) D3 was used as a marker of vitamin D status to avoid confounding of the results from exogenous vitamin D supplementation. Serum calcium, phosphate and alkaline phosphatase were measured using the ADVIA 1800 centaur auto analyzer (Siemens Diagnostics, Tarrytown, USA).

Creatinine was measured by a modified Jaffe reaction and glomerular filtration rate (GFR) estimated using the four- variable Modified Diet Renal Disease (MDRD) equation (13): GFR (in mL/min per 1.73 m^2) = 175 × SCr (exp[−1.154]) × Age (exp [−0.203]) × (0.742 if female) × (1.21 if black).

Other biochemical parameters were determined as part of standard of care using routine laboratory techniques.

Operative definitions and laboratory reference values

The reference ranges were 2.12- 2.50 mmol/l for calcium, 0.79-1.45 mmol/l for phosphate, 53-128 U/L for total alkaline phosphatase and 10-65 pg/mL for intact PTH.

25-(OH)vitamin D reference ranges: < 10 ng/mL as severe deficiency, 10-29 ng/mL as moderate deficiency, 30-100 ng/ mL as sufficiency and > 100 ng/ mL as toxic.

Based on the above reference values and KDIGO recommendations (5), hyperparathyroidism was defined as PTH> 130 pg/ml (2 times the upper limit of normal) and severe hyperparathyroidism as PTH > 585pg/ml (9 times the upper limit of normal).

Hyperphosphataemia and hypocalcaemia were defined as serum phosphate > 1.45mmol/l and calcium < 2.12 mmol/l respectively.

Ethical approval: All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The research protocol was approved by the Health Research and Ethics committee (HREC) of the University of the Witwatersrand; clearance certificate number M141016. Written informed consent was obtained from each patient before enrolment into the study.

Statistical analysis

Continuous variables are presented as means± standard deviations or as medians and inter quartile ranges (IQR) as appropriate, while categorical data are reported as a percentage. An independent t-test or Wilcoxon rank -sum test compared continuous variables between blacks and whites while the Pearson's or Fisher's exact test was utilized for proportion comparisons.

PTH and FGF23 data were log transformed due to the skewed nature of these variables. Multiple linear regression models were employed to determine the effect of independent predictors on log transformed PTH and FGF23.

Logistic regression analysis was used to determine the predictors of severity of hyperparathyroidism. Variables with $p < 0.10$ on univariate analyses were eligible for

inclusion in the multivariate analysis. Spearman correlations were used to determine the correlation between FGF23, phosphate, PTH, 25-(OH) vitamin D3 and estimated GFR.

A p-value of less 0.05 was considered statistically significant at the 95% confidence interval. All analyses were performed using STATA version 12 (STATA Corp., TX, and USA).

5.3 Results

Patient characteristics

The 293 CKD patients comprised 208 black and 85 white patients with overall mean age of 51.1±13.6 years. The clinical characteristics of the patients by race are summarized in table 5. 1. Blacks were significantly younger, had higher blood pressure, median iPTH, alkaline phosphatase and mean 25 (OH) D3, but lower levels of median FGF23 and serum phosphate than white patients. Diabetes mellitus was significantly more prevalent in whites. The use of CKD-MBD related medications did not differ by race.

Comparison of markers of CKD -MBD between black and white patients

Racial variations in the levels of PTH, calcium, phosphate and 25-(OH) D3 according to stages of CKD are shown in Table 5.2. Median PTH and mean 25-(OH) D3 were significantly higher in blacks than whites in CKD stage 5. Blacks had higher levels of total alkaline phosphatase (TAP) than whites in stage 4 CKD. Median FGF23 and mean serum phosphate levels increased progressively across stages of CKD and became significantly higher in whites than blacks in CKD stage 5.

Table 5.3 shows comparisons of markers of CKD-MBD between black and white patients with predialysis CKD and End stage renal disease. In predialysis CKD patients, whites had significantly higher levels of FGF23 than blacks (55ng/ml [31-81] versus 32[22-57], p=0.01), and higher levels of calcium (2.33±0.11 versus 2.24±0.14, p=0.005). Other parameters were comparable between the two groups. In patients with ESRD, levels of FGF23 and phosphate were significantly higher in whites than blacks (881[187-3634] versus (329[105-2557], p=0.03) and (1.70±0.48 versus 1.44±0.56, p=0.004) respectively. Compared to whites, blacks had higher levels of PTH (758[360-1350] versus 358[179-814], p=0.0004) and 25-OH D (28.1±13.8 versus 22.0±12.2, p=0.004).

Comparisons between blacks and whites in the prevalence of abnormal levels of calcium, PTH, phosphate and 25-(OH) D3 across stages of CKD are shown in Figure 5.1, 5.2, 5.3 & 5.4. The proportion of patients with hyperphosphataemia (72.7% versus 42.9 %; p<0.001) and vitamin D deficiency (76.7 % versus 57.5 %; p = 0.01) was higher in whites than blacks in CKD stage 5. The prevalence of abnormal levels of other markers of CKD-MBD was similar across the study populations.

Associations between race, clinical characteristics and markers of CKD–MBD

In line with a previous study (14), in a multiple regression analysis adjusted for age, diabetes status, GFR, serum calcium, phosphate and alkaline phosphate levels; black race remained significantly associated with increased log PTH(β =0.488, p=0.01) and decreased log FGF23 (β=-0.636, p= 0.02); Table 5. 4. Similarly, we found a persistent significant association between log FGF23, calcium, phosphate and GFR. In an unadjusted univariate analysis, white race was significantly associated with decreased 25-(OH) D3; however, this was attenuated after adjusting for age, diabetes status, calcium and GFR.

Further exploration of the association between FGF23 and other markers of CKD-MBD, showed FGF23 correlates positively with serum phosphate(r= 0.55, p < 0.001), and PTH (r= 0.40 p <0.001), and inversely with eGFR (r = -0.61, p < 0.001) (Figures 5.5 and 5. 6).

Determinants of secondary hyperparathyroidism

In logistic regression analysis, the independent predictors of severe hyperparathyroidism were black race (OR 3.08; 95% CI: 1.51-6.30, P=0.002) and GFR < 15 mls/min (OR 10.07; 95 % CI: 4.70-21.56, P <0.0001).

5.4 Discussion

The racial disparities in markers of CKD-MBD have been documented in CKD populations in previous studies from America and Europe (14, 15). A few studies from Africa have documented similar findings in healthy populations but not in CKD patients (16, 17). In this present study, with the exception of 25-(OH) vitamin D3 levels, our findings are consistent with those of previous studies (12, 14); we found that PTH and alkaline phosphatase are higher in black than white patients in CKD stage 5 and CKD stage 4 respectively. After

adjusting for the inconsistency between the two groups regarding diabetic status and age, black race still remained significantly associated with increased PTH. Although the mechanisms behind these discrepancies remain largely unclear, several reasons proposed by prior studies included racial differences in skeletal responsiveness to PTH levels, racial variations in sensitivity to the phosphaturic effect of PTH and FGF23 , dietary intake of food rich in phosphate, and underlying genetic differences in bone mineral metabolism (14, 18, 19).

An interesting and unexpected finding was the significantly higher levels of 25(OH) D_3 in CKD stage 5 in blacks compared to whites; this was contrary to most previous studies that reported lower levels of 25-(OH)vitamin D, which was attributed to skin pigmentation (14). It is anticipated that increased skin pigmentation in blacks will lead to decreased synthesis of25-(OH) vitamin D from 7 dehydrocholesterol through exposure to sunlight. One of the limitations of our study was the non-availability of information relating to sun exposure which could have accounted for these differences; it is possible that our black patients with CKD stage 5 spent more time outdoors (outdoor occupations in a sunny climate) leading to more sun exposure compared to white patients. However, some studies have shown that blacks and whites have equal capacities to synthesize vitamin D post exposure to repeated high doses of ultraviolet B light(20, 21). Brazerol et al (21), comparing skin capacity for blacks and whites exposed to similar doses of ultraviolet B rays (280-315nm) twice a week for six weeks, reported similar response to vitamin D synthesis despite the fact that blacks had lower baseline 25 –OH D levels than the white participants. A prior study from South Africa with the aim of assessing vascular calcification in haemodialysis patients found no difference in 25 (OH) vitamin D levels between black and white patients (22). In addition, the inconsistencies that exist in the relationship between vitamin D levels and clinical outcomes in blacks suggest that the mechanism behind the racial disparities is complex and multi-factorial. For example, despite lower levels of 25-(OH) vitamin D demonstrated by some studies, blacks have lower rates of osteoporosis and bone fractures than age and gender matched white participants (23, 24).

Studies relating to the prevalence of 25 (OH) D across stages of CKD revealed conflicting results. Consistent with prior studies, we found no association between vitamin D status and stages of CKD (12). In contrast to our study, the prevalence of 25 deficiency/insufficiency

rose slightly across the stages of CKD in the Nephro Test study (25). However, it is noteworthy that comparisons of vitamin D status across the studies are somewhat hampered by differences in the participants skin pigmentation, latitude, cut off values and assay methods employed between the studies. Additionally, it is suggested that vitamin D deficiency is more profound in diabetic patients likely due to heavy urinary loss of vitamin D binding protein (25, 26). Therefore, the higher prevalence of diabetes in our white participants could have accounted for the racial discrepancy in the levels of 25-(OH) vitamin D. This was further supported by the attenuation of the significant association between white race and 25-(OH)vitamin D3 after adjustment for diabetes status in the linear regression model with 25-(OH)vitamin D3 as the dependent variable.

Consistent with previous studies, phosphate levels increase with worsening of kidney function and the increase was significantly higher in whites than blacks at CKD stage 5. It is possible that white patients are more likely to consume dairy products accounting for the higher phosphate levels; this could not to be ascertained in this study due to the non-availability of dietary history. However, large studies from the US have reported that African Americans had lower consumption of dairy products than whites (27, 28). However, understanding racial differences in serum phosphate levels is intriguing and other factors besides dietary phosphate need to be considered. For example, contrary to our findings some studies have shown surprisingly increased serum phosphate levels in blacks compared to whites despite increased levels of parathyroid hormone and thus attributing these differences to reduced urinary phosphate excretion and lower FGF23 in blacks (10, 11). In line with these studies, our black patients had lower levels of FGF23 compared to whites. It is possible that higher phosphate levels in our white participants could have accounted for the differential levels in FGF23 as a compensatory mechanism. Although this is contrary to the explanation offered by a prior study, that the lower levels of FGF23 in their black participants despite higher levels of phosphate could be as a result of decreased FGF23 expression(10). The complexity in racial disparities with phosphate and FGF23 levels is further compounded by variations in the use of phosphate binders and alfacalcidol. In this study there are no differences between blacks and whites in the use of calcium carbonate and alfacalcidol.

Consistent with previous studies (11, 29), FGF23 correlated positively with phosphate, PTH and inversely with GFR. The directions of these associations are physiologically plausible,

with FGF23 attempting to mitigate the effect of excess phosphate with worsening renal function. It is also noteworthy that significant positive association with white race persisted after adjusting for other confounding variables. In addition, the significant positive association with white race persisted after adjusting for other confounding variables.

The limitations of our study included the cross-sectional study design; therefore we could not determine the longitudinal changes in markers of CKD-MBD, as well as seasonal variation in 25 (OH) vitamin D levels. Information relating to UVB exposure and dietary phosphate are lacking.

The strengths of this study lie in the heterogeneous nature of our study population (black and white patients) in an African setting which has allowed comparisons of data not only for Black Africans with Black Americans, but also between whites in Africa and USA/Europe.

To our knowledge, few studies have compared FGF23 levels across races in developed countries and no such studies have been reported from Africa. Therefore, this is the first study in Africa that has given important insights regarding the associations between FGF23, traditional markers of CKD-MBD and race in an African CKD population. Finally, in this study HPLC which is considered to be the gold standard for vitamin D measurement (30) was used to specifically measure 25-(OH)vitamin D3 that is less affected by vitamin D supplementation. In addition, with the exception of HPLC several test methodologies were shown to demonstrate a considerable variation of individual 25-(OH) vitamin D values as compared with LC-MS/MS defined target concentrations (31).

Conclusion. There was a racial difference in the markers of CKD-MBD; compared with whites, African blacks had higher levels of PTH, alkaline phosphatase and lower levels of FGF23 and serum phosphate. It remains unclear whether the present CKD-MBD management guidelines are appropriate for all races.

DISCLOSURE

SN received research grant support from MRC (SA) and NRF (SA). The remaining authors declared no competing interests.

Table 5. 1: Characteristics of the study population

Parameters	All (n=293)	Black (n=208)	White(n=85)	P
Age(years)	51.1±13.6	48.4±13.6	57.9±15.5	P<0.001
Gender n (%)				
Male	166 (56.7%)	114 (54.8%)	52 (61.2 %)	0.32
Female	127 (43.3 %)	94 (45.2 %)	33(38.8 %)	
Systolic BP(mmHg)	143±25	146±26	135±19	0.007
Diastolic BP(mmHg)	84±20	89±21	71±11	P<0.001
Hb (g/dl)	11.3±2.4	11.3±2.5	11.4±2.2	0.83
Albumin(g/dl)	37.0±7.0	37.0±6.9	37.0±6.7	0.55
Calcium(mmol/l)	2.22±0.24	2.20±0.27	2.30±0.19	0.01
iPTH (pg/ml)	353(133-914)	498(137-1084)	274(131-595)	0.03
FGF23(pg/ml)	130(42-970)	100(34-639)	233(80-1370)	0.002
Phosphate(mmol/l)	1.4±0.5	1.3±0.5	1.5±0.5	0.001
TAP (U/L)	116(83-162)	122(89-192)	103(74-144)	0.03
25-OHD (ng/ml)	25.6±12.7	26.8±12.7	22.7±12.2	0.01
<30 ng/ml	191(91.8%)	128(61.5%)	63 (74.1%)	0.04
<10ng/ml	18(6.1%)	8(3.8%)	10(11.8%)	0.01
Causes of renal disease n(%)				
HTN	188(64.2%)	141(67.8%)	47(55.3%)	0.002
DM	52(17.7%)	25 (12.0%)	27(31.8%)	P<0.001
ADPKD	11(3.8%)	5(2.4%)	6(7.1%)	0.06
Obstructive uropathy	6(2.0%)	3(1.4%)	3(3.5%)	0.50
Unknown	36(12.3%)	34(16.3%)	2(2.4%)	0.05
Medications				
Calcium carbonate	120(41.0%)	85(40.9%)	35(41.2%)	0.77
Alfacalcidol	111(37.9%)	78(37.5%)	33(38.8%)	0.68
Calcium carbonate (mg/day)	3429±694	3375±678	3500±750	0.69
Alfacalcidol (μg/ week)	1.63±0.38	1.86±1.09	1.48±0.87	0.43

Continuous variables are presented as means± standard deviations or median (interquartile range) and categorical data as frequencies (percentages), BP= blood pressure , i PTH= intact parathyroid hormone, TAP= total alkaline phosphatase, DM= diabetes mellitus, FGF = fibroblast growth factor , HTN=hypertension, ADPKD=autosomal dominant polycystic kidney disease

Table 5. 2: Markers of mineral bone metabolism by race and stages of CKD

Variable	CKD stage 3		P	CKD stage 4		P	CKD stage 5		P
	Black(n=34)	White(n=11)		Black(n=40)	White(n=14)		Black(n=134)	White(n=60)	
IPTH(pg/ml)	120(92-218)	86(76-166)	0.21	193(73-373	228(175-329)	0.54	758(360-1350)	358(179-814)	0.0004
FGF23(pg/ml)	30(22-44)	42(31-134)	0.07	35(22-64)	63(34-81)	0.07	329(105-2557)	881(187-3634)	0.03
Calcium(mmol/l)	2.25±0.15	2.31±0.10	0.22	2.23±0.15	2.34±0.13	0.009	2.18±0.31	2.26±0.21	0.08
Phos (mmol/l)	1.04±0.23	1.05±0.14	0.75	1.13±0.25	1.27	0.10	1.44±0.56	1.70±0.48	0.004
25-OHD$_3$ (ng/ml)	24.3±9.3	24.7±11.9	0.91	24.6±11.1	24.3±12.80	0.94	28.1±13.8	22.0±12.2	0.004
TAP (U/L)	98(77-138)	102(78-123)	0.76	114(97-166)	88(74-111)	0.03	123(88-209)	128(73-226)	0.14

Continuous variables are presented as means± standard deviations or median (interquartile range) and categorical data as frequencies (percentages) , IPTH = intact parathyroid hormone , FGF = fibroblast growth factor , i PTH= intact parathyroid hormone, TAP= total alkaline phosphatase, Phos= phosphate

Table 5. 3: **Markers of CKD-MBD by race in pre dialysis and ESKD**

Variable	Pre dialysis		P	End Stage Kidney Disease		P
	Black (n=74)	White (n=25)		Black (n=134)	White(n=60)	
iPTH(pg/ml)	160(84-280)	174(94-253)	0.81	758 (360-1350)	358 (179-814)	0.0004
FGF23 (pg/ml)	32(22-57)	55(31-81)	0.01	329(105-2557)	881(187-3634)	0.03
Calcium(mmol/l)	2.24±0.14	2.33±0.11	0.005	2.18±0.31	2.26±0.21	0.08
Phos (mmol/l)	1.09±0.24	1.18±0.27	0.12	1.44±0.56	1.70±0.48	0.004
25-OHD$_3$ (ng/ml)	24.4±10.3	24.5±12.1	0.99	28.1±13.8	22.0±12.2	0.004
TAP (U/L)	111(89-141)	74(74-114)	0.09	123(88-209)	128(73-226)	0.14
GFR(mls/min /1.73m^2)	30.9±12.7	30.1±12.5	0.77	N/A	N/A	
Kt/V	N/A	N/A		1.41±0.30	1.46±0.30	0.40

Continuous variables are presented as means± standard deviations or median(interquartile range) and categorical data as frequencies(percentages) , BP= blood pressure , i PTH= intact parathyroid hormone, TAP= total alkaline phosphatase,, FGF = fibroblast growth factor, GFR= Glomerular filtration rate, N/A= not applicable

Table 5. 4: Multivariable analysis of determinant of PTH, FGF23 and 25-(OH) D3

Dependent variable (Log PTH)	βcoefficient	P	βCoefficient	p
Independent variable	Unadjusted		Adjusted	
Age	-0.010	0.03	0.013	0.02
Diabetes	-0.390	0.02	-0.112	0.61
Female gender	0.236	0.09	0.247	0.12
Black race	0.413	0.007	0.488	0.01
Calcium	-1.248	<0.001	-1.038	0.001
Phosphate	0.541	<0.001	0.179	0.26
25-(OH) vitamin D3	-0.005	0.35	-0.002	0.77
Alkaline phosphatase	0.002	<0.001	0.002	0.001
GFR	-0.040	<0.001	-0.030	P<0.001
Dependent variable(Log FGF23)				
Independent variable				
Age	-0.033	<0.001	-0.013	0.26
Diabetes	-0.763	0.01	-0.318	0.31
black race	-0.765	0.003	-0.636	0.02
PTH	0.622	<0.001	0.171	0.09
Calcium	0.921	0.05	1.239	0.004
Phosphate	2.077	<0.001	1.041	<0.001
25-(OH) vitamin D3	0.019	0.04	0.009	0.31
Alkaline phosphatase	0.002	0.02	0.001	0.20
GFR	-1.329	<0.001	-0.752	P<0.001
25 (OH)D (Dependent variable)				
Independent variable				
Age	-0.215	<0.001	-0.207	<0.001
Diabetes	-8.745	<0.001	-4.610	0.03
white race	-4.063	0.01	-1.471	0.41
Albumin	0.385	0.001	0.267	0.02
Calcium	9.214	0.002	11.461	<0.001

GFR= Glomerular filtration rate, PTH= Parathyroid hormone

Table5. 5: Predictors of severe hyperparathyroidism (PTH>585 ng/ml)

Variable	OR	95% (CI)	p
Age < 65 years	0.77	0.32-1.84	0.56
Black race	3.08	1.51-6.30)	0.002
Diabetes	0.57	0.25-1.31	0.19
25 (OH) D (<30ng/ml)	1.68	0.90-3.13	0.10
GFR<15mls/min	10.07	4.70-21.56	P<0.001
Female gender	0.82	0.47-1.43	0.48
Hyperphosphataemia(>1.45mmol/l)	1.39	0.75-2.58	0.29
Hypocalcaemia(<2.10mmol/l	1.46	0.74-2.92	0.28

GFR= Glomerular filtration rate

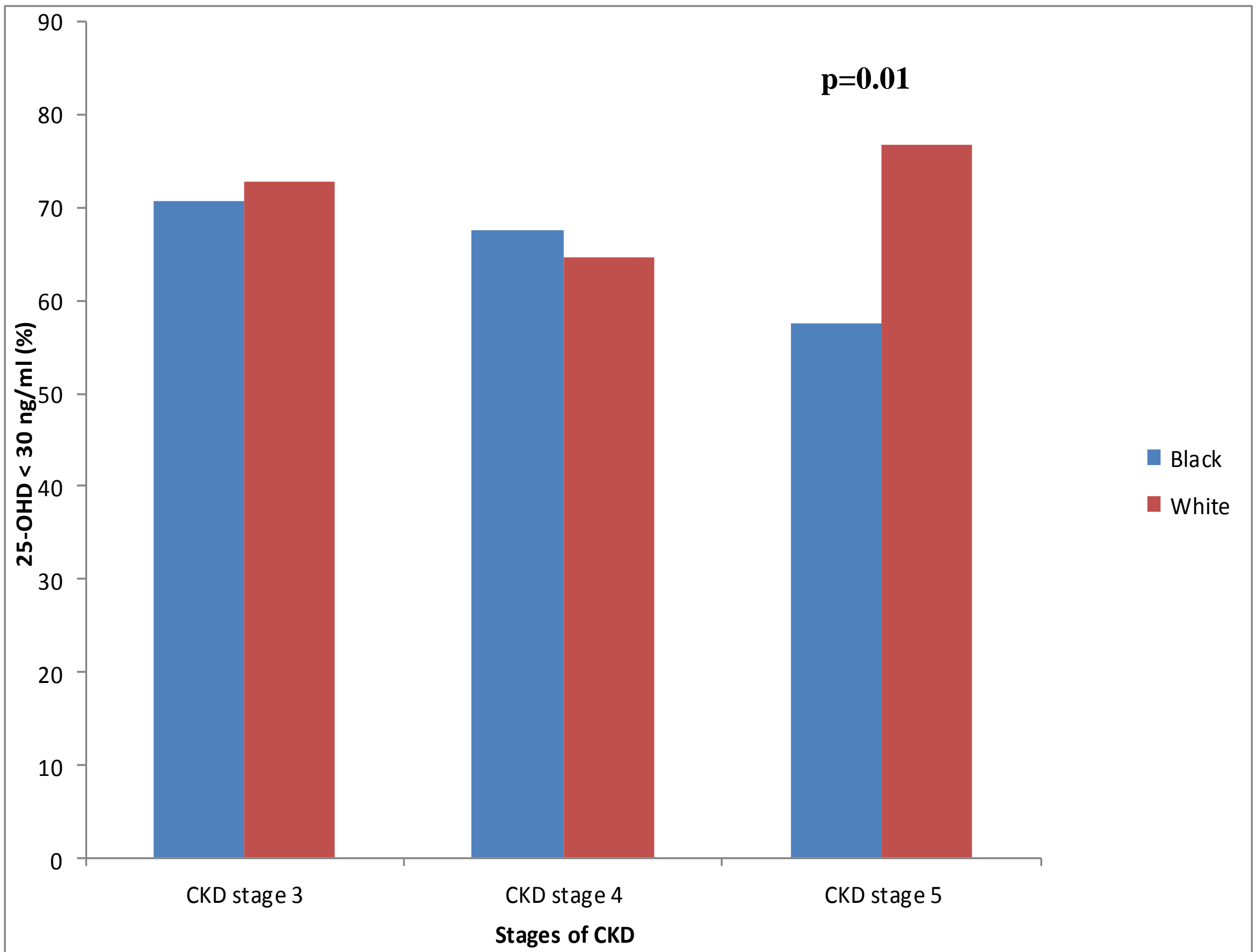

Figure 5. 1: Prevalence of 25 (OH) D3 deficiency by race and stages of CKD

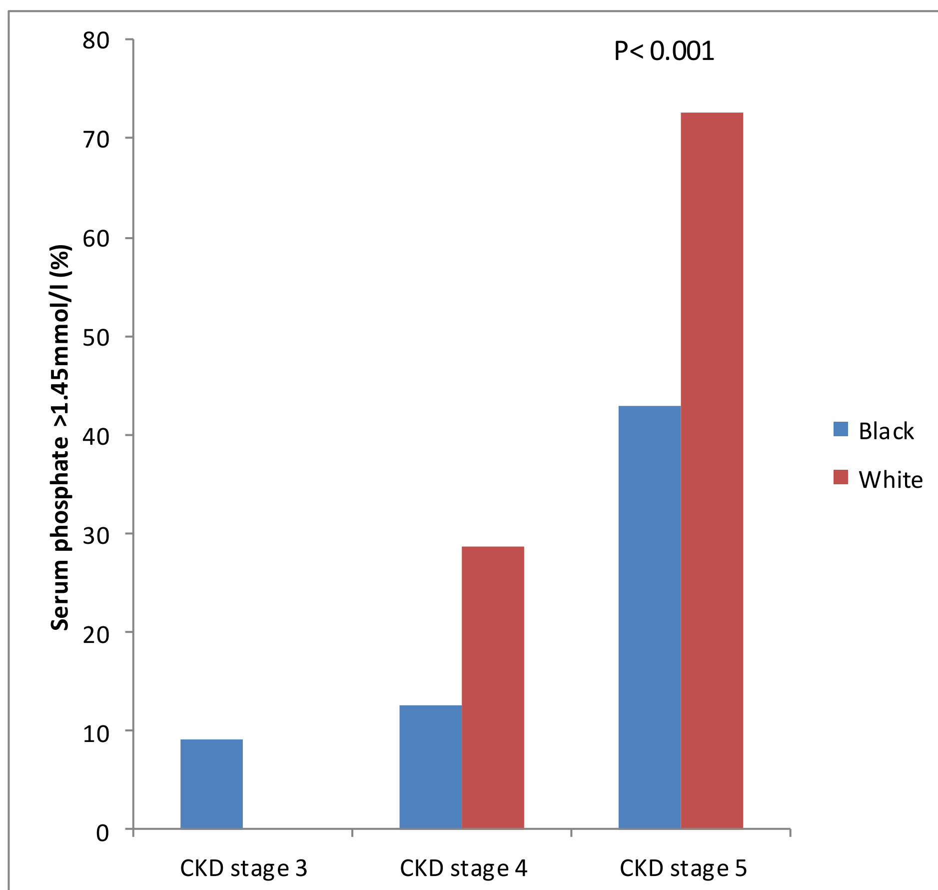

Figure 5. 2: Prevalence of hyperphosphataemia by race and stages of CKD.

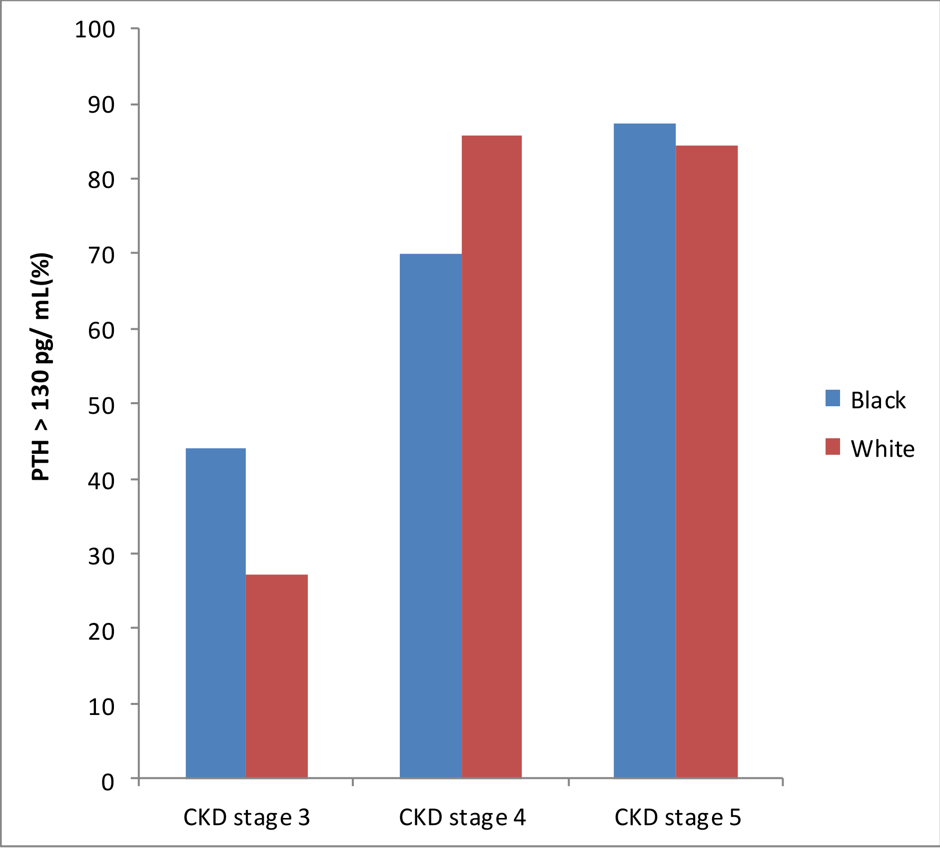

Figure 5. 3: Prevalence of hyperparathyroidism by race and stages of CKD

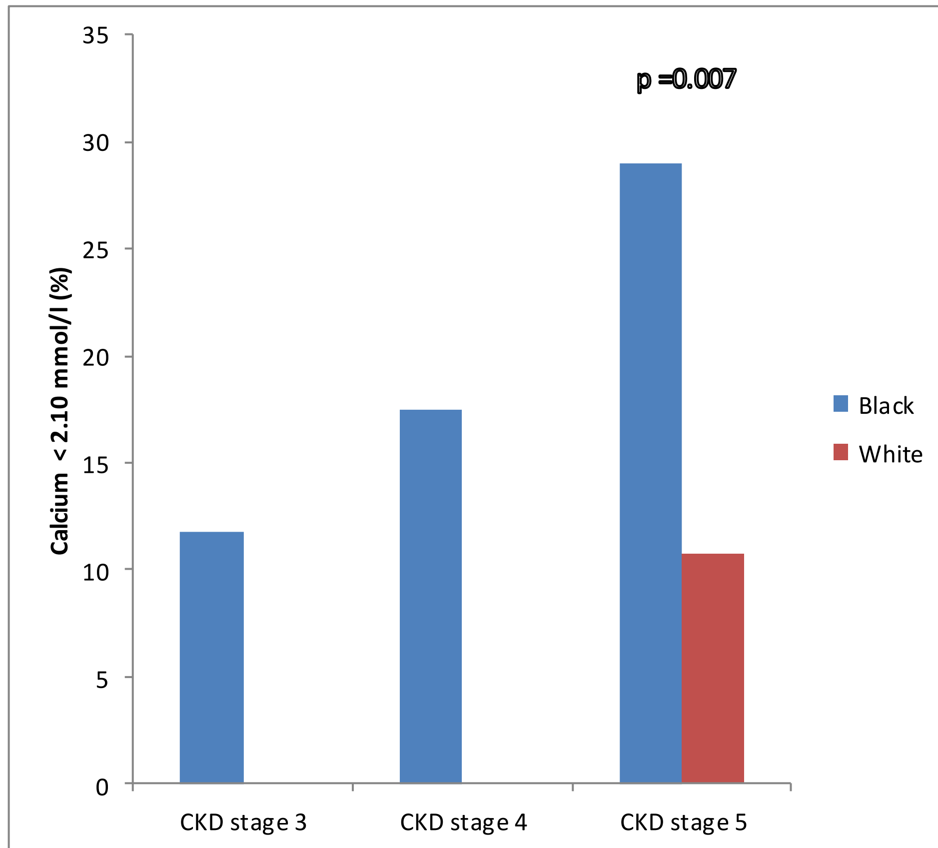

Figure 5. 4: Prevalence of hypocalcaemia by race and stages of CKD.

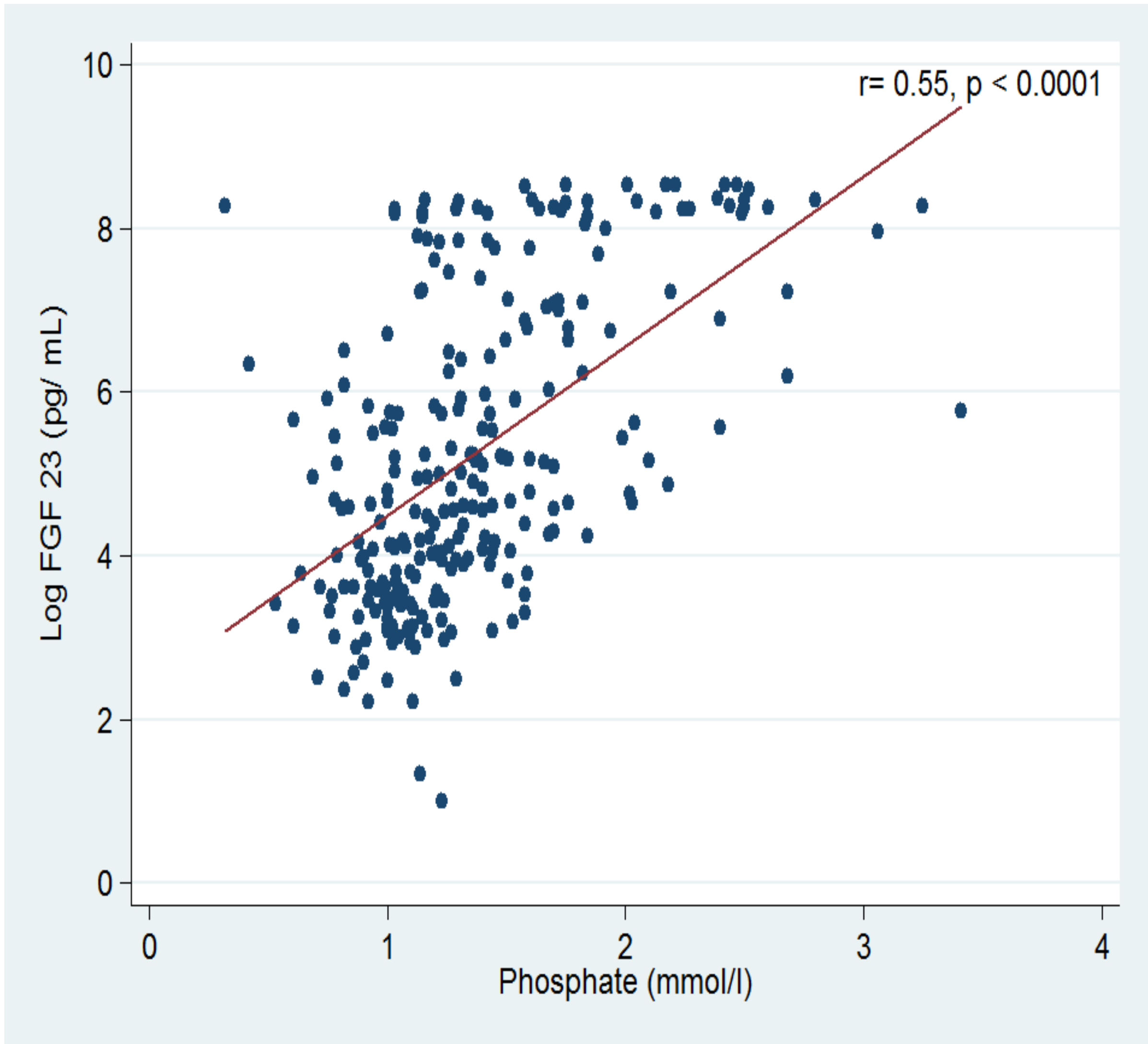

Figure 5. 5: Correlation between Log FGF23 and Phosphate

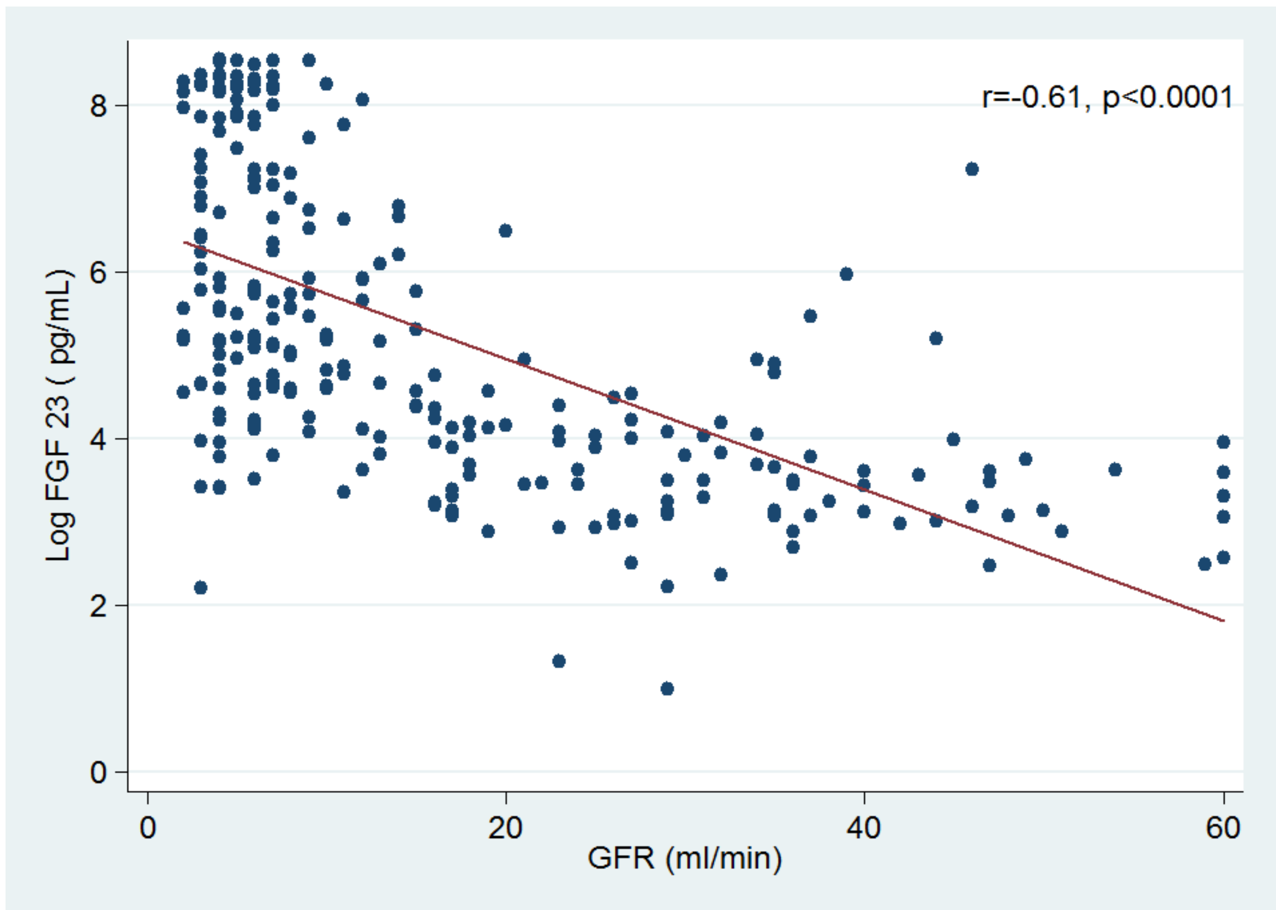

Figure 5. 6: Correlation between Log FGF23 and GFR

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CHAPTER 6: MANUSCRIPT 4

Influence of vitamin D receptor polymorphisms on biochemical markers of mineral bone disorders in South African patients with chronic kidney disease.

ABSTRACT

Background

It remains unclear whether genetic factors may explain the reported variation in the levels of biochemical markers of chronic kidney disease mineral and bone disorders (CKD-MBD) across ethnic groups. Therefore, the aim of this study was to examine the influence of VDR polymorphisms on secondary hyperparathyroidism and its association with vitamin D levels in black and white South African study participants.

Patients and Methods

This was a cross sectional study involving 272 CKD stage 3- 5D patients and 90 healthy controls. The four major VDR polymorphisms (*Bsm 1, Fok 1, Apa1, and Taq 1*) were genotyped using the polymerase chain reaction- restriction fragment length polymorphism (PCR –RFLP) method. In addition, the biochemical markers of CKD-MBD were measured to determine their associations with the four VDR polymorphisms.

Results

With the exception of *Taq I* polymorphism, the distribution of the VDR polymorphisms differed significantly between blacks and whites. In hemodialysis patients, the Bb genotype was significantly associated with moderate secondary hyperparathyroidism (OR, 3.88; 95 CI 1.13-13.25, p=0.03) and severe hyperparathyroidism (OR, 2.54; 95 CI 1.08-5.96, p=0.03). This was consistent with the observed higher levels of median PTH and mean phosphate in patients with Bb genotype. This candidate risk genotype (Bb) was over represented in blacks compared to whites (71.0 % versus 55.6 %, p <0.0001). In an unadjusted regression model, *FokI Ff* genotype was found to be significantly associated with the risk of developing severe vitamin D deficiency < 15ng/ml (OR, 1.89; 95 CI 1.17-3.07, p=0.01).

Conclusion.

The VDR Bb genotype is an independent predictor of developing secondary hyperparathyroidism in patients with end stage kidney disease. In addition, study participants with the *FokI Ff* genotype are at increased of developing severe 25(OH) D deficiency

6.1 Introduction

Vitamin D deficiency has been linked to various disease conditions and poor clinical outcomes (1-3). The widespread consequences of vitamin D deficiency have been partly attributed to the ubiquitous distribution of the vitamin D receptor (4). The vitamin D receptor (VDR) plays a vital role in mediating the effects of the biologically active form of vitamin D (1, 25, OH-D); therefore it is plausible that variations in these receptors will modulate the consequences associated with vitamin D deficiency (5). In 1994, Morrison et al. (6) were the first to report an association between VDR polymorphisms and bone metabolism, showing that the common allelic variants in the VDR encoding genes can predict differences in bone density in healthy individuals (6). Subsequently, several researchers have explored this relationship in CKD populations with emphasis on the calcium/ PTH/ calcitriol axis (7, 8). The *BsmI* polymorphism (BB genotype) has been associated with slower progression of secondary hyperparathyroidism and normal levels of calcitriol in pre dialysis CKD patients, and lower levels of parathyroid hormone (PTH) in hemodialysis, and a greater reduction in PTH levels in response to a single bolus of calcitriol therapy compared to patients with the bb genotype (8, 9). However, contrary to earlier studies, findings from subsequent studies on the associations between VDR polymorphisms and markers of mineral bone disease have been inconsistent. For instance, some studies reported no difference in PTH levels between the various *Bsm I* genotypes (10, 11), while Chudek et al. reported significantly lower levels of calcitriol in patients with BB genotype (12). Similarly, some studies have linked other VDR polymorphisms to mineral bone metabolism in hemodialysis patients. The VDR *Fok I* polymorphism (FF genotype) was reported to be associated with higher PTH levels (13).

Furthermore, the existence of racial disparities in abnormal markers of CKD-MBD and the better survival paradox in African Americans compared to white dialysis patients may be explained partly by the racial differences in the distribution of VDR polymorphisms and VDR receptor activation therapy. Most of these studies were conducted on European, Asian and American CKD populations, while studies from Africa were largely on non CKD

populations. Therefore, in line with ongoing efforts to greater understanding of the mechanisms behind racial disparities in markers of CKD-MBD, we aimed to explore the variations in the VDR polymorphisms between black and white African CKD patients and their relationship with markers of mineral bone disorders.

6.2 Patients and Methods

This was a cross- sectional study involving 272 CKD stage 3- 5D patients and 90 healthy controls. The study protocol was approved by the Health Research and Ethics committee (HREC) of the University of the Witwatersrand; clearance certificate number M141016. All participants gave written informed consent prior to enrolment. Exclusion criteria included active malignancies, aged < 18 years, and patients who withheld consent. Information on participants' demographic characteristics, duration on dialysis and use of medications related to CKD-MBD were obtained. Determination of race was based on self-reporting by the participants.

Laboratory measurements

Plasma intact PTH was measured by an electrochemiluminescence immunoassay (ECLIA) run on a Cobas 6000 auto analyzer (Roche Diagnostics, Mannheim, Germany).

FGF-23 was measured using an enzyme-linked immunosorbent assay kit from EMD Millipore Corporation (Billerica, MA, USA). Assay lower detect limit was 3.2 pg/ml. Plasma 25(OH) D was measured using the high performance liquid chromatography (HPLC) kit (Recipe, Munich, Germany). HPLC was used to selectively measure 25(OH) D2 and 25 (OH) D3 at a wave length of 264nm. The intra and inter assay coefficients of variation (CVs) were < 5%. Our institutional laboratory is a participating member in the vitamin D external quality assurance scheme (DEQAS). In this study, 25 (OH) D3 was used as a marker of vitamin D status to avoid confounding of our results by exogenous vitamin D supplementations. Serum calcium, phosphate and alkaline phosphatase were measured using the ADVIA 1800 centaur auto analyzer (Siemens Diagnostics, Tarrytown, USA).

Creatinine was measured by a modified Jaffe reaction and GFR was estimated using the four- variable Modified Diet Renal Disease (MDRD) equation (14): GFR (in mL/min per 1.73 m^2) = 175 × SCr (exp[−1.154]) × Age (exp[−0.203]) × (0.742 if female) × (1.21 if

Other biochemical parameters were determined using routine laboratory techniques.

Genotyping

DNA was extracted from whole blood using the Maxwell DNA purification kit (Promega AS1010, USA). Using appropriate primers and 50 ng of DNA polymerase chain reaction (PCR) products were amplified for *ApaI* (Forward: 5'CAGAGCATGGACAGGGAGCAAG 3' and Reverse: 5' GCAACTCCTCATGGCTGAGGTCTCA 3' with 65 ^{0}C as annealing temperature), *BsmI* (Forward : 5' CAACCAAGACTACAAGTACCGCGTCAGTGA 3' and Reverse: 5' AACCAGCGGGAAGAGGTCAAGGG 3 ' with 65 ^{0}C as annealing temperature), *FokI* (Forward: 5' AGCTGGCCCTGGCACTGACTCTTGCTCT 3' and Reverse: 5' ATGGAAACACCTTGCTTCTTCTCCCTC 3' with 67 ^{0}C annealing temperature), and *TaqI* (Forward:5'CAGAGCATGGACAGGGAGCAAG3' and Reverse :5'GCAACTCCTCATGGCTGAGGTCTCA 3' at an annealing temperature of 65 ^{0}C) VDR polymorphisms. The PCR products were then digested with enzymes *ApaI, BsmI, FokI,* and *TaqI* (New England Biolabs, Beverly, MA, USA) according to the supplier's protocol. Digestions for *BsmI and TaqI* were at 65 ^{0}C overnight, and 3hrs at 25 ^{0}C for *ApaI*, while *FokI* was incubated at 37 ^{0}C for 3 hrs. Restricted products were electrophoresed on either 10% polyacrylamide or 1.5% agarose gels and then visualized by the Gel Doc TM EZ imager (Bio-Rad systems, USA). Genotypes were scored based on the presence or absence of a restriction site for the enzymes *BsmI, ApaI, and TaqI* at the 3′ untranslated region and *FokI* at the N-terminal region of the gene.

Statistical analysis

The Fisher's exact test was utilized to compare differences in the frequency of genotypic distribution between groups. Based on the distribution of data, an independent t– test or Wilcoxon rank –sum test were used to compare continuous variables between two groups, while one- way ANOVA or Kruskal –Wallis tests were used for more than two groups. Both univariate and multivariable logistic regression models were used to determine the association between VDR genotypes, secondary hyperparathyroidism and vitamin D

deficiency. In the comparisons of the means and medians of the circulating markers of CKD-MBD across the genotypes, the P values for distribution between homozygous dominant and heterozygous genotypes were further determined separately due to the smaller numbers of the homozygous recessive. A backward selection procedure was used to fit the multiple regression model, which started with all potential predictor variables and subsequently removed the variables that had p values above the specified P=0.20.However, variables that are known to be biologically plausibly associated with secondary hyperparathyroidism were forced into the model despite not meeting inclusion criteria based on the stepwise approach. A post estimation test for Goodness of Fit of the models was carried out using Hosmer - Lemeshow goodness of fit test.

A p-value of less 0.05 was considered statistically significant at the 95% confidence interval. All analyses were performed using STATA version 12 (STATA Corp., TX, and USA).

6.3 Results

Description of the study population.

A total of 362 participants (272 CKD patients and 90 controls) were recruited for this study. The CKD group comprised of 156 CKD stage 5D and 116 CKD stages 3-5 patients. In the control group, 39 participants were self-identified as Whites, 60 as Blacks, and one Indian. The CKD group comprised of 73 Whites, 175 Blacks and 21 Indians. Fifteen patients were excluded from the genetic analysis due to failed genotyping (Figure1). Patients on haemodialysis were on three times weekly, 4 hr sessions of haemodialysis using polysulphone membranes and bicarbonate dialysate. Most of the patients were dialyzed with a dialysate calcium concentration of 1.50 mmol/L, which is usually modified based on serum levels of calcium. The blood and dialysis flow rates are generally 300–400 mls/min and 500 mls/min,

Table 6.1 shows the ethnic distribution of the VDR polymorphisms (*Bsm I, FokI, ApaI and Taq I*). In the VDR polymorphisms, blacks had significantly higher proportion of Bb genotype than whites (71.0 % versus 55.6 %), and lower frequency of BB genotype (24.1 % versus 44.4 %). Overall, the most common genotype was Bb (65.4%). Similarly, the distribution of *Fok I and Taq I* genotypes differed significantly between the groups; FF was

more frequent in blacks, while the Ff genotype was the most prevalent in whites. There was no significant ethnic variation in the distribution of the *ApaI* genotype.

Table 6.2 shows the distribution of the four VDR polymorphisms (*Bsm I, FokI, ApaI and Taq I*) between CKD patients and healthy controls, and the odds ratio of developing severe 25(OH) D severe deficiency (< 15ng/ml). The frequencies of these genotypes did not differ significantly between the CKD and control groups. Ff genotype showed a significant increase in odds of developing severe 25 (OH) D deficiency (OR, 1.89; 95 CI 1.17-3.07, P=0.01); a similar trend was found with combined Ff + ff genotypes (OR, 1.91; 95 CI 1.18-3.08, P=0.008). The remaining genotypes were not significantly associated with severe 25(OH) D deficiency.

The biochemical markers of CKD-MBD in the various genotypes are shown in Table 6.3. Median PTH and mean phosphate levels were significantly higher in patients with Bb genotype. In a restricted comparison between homozygous dominant genotype and heterozygous genotype due to smaller numbers of homozygous recessive genotype, the P values did not change significantly.

In a restricted analysis involving hemodialysis patients, the univariate and multivariate analyses for the odds of developing moderate and severe secondary hyperparathyroidism are shown in Table 6. 4 Moderate secondary hyperparathyroidism was defined as PTH >130 pg/ml (2 times the upper limit of normal) and severe secondary hyperparathyroidism as PTH > 585 pg/ml (9 times the upper limit of normal). After adjusting for serum calcium, phosphate, fibroblast growth factor 23, and use of alfacalcidol, the Bb genotype was a significant predictor of developing both moderate (OR,3.88; 95 CI 1.13-13.25, p=0.03) and severe hyperparathyroidism(OR, 2.54; 95 CI 1.08-5.96, p=0.03). The use of alfacalcidol was not eligible for inclusion into the final model, but was forced into the model due to a biologically plausible association between secondary hyperparathyroidism and the use of alfacalcidol. The post estimation test shows no lack of fit with the final models (p>0.05).

6.4 Discussion

In an attempt to unravel the complexity behind the pathophysiologic mechanisms of CKD - MBD, several investigators have looked at the relationship between VDR polymorphisms and the calcium/PTH/calcitriol axis with inconsistent findings (5, 12). In this present study,

consistent with some previous reports, we found a significant difference in PTH levels across *Bsm I* genotypes, patients with Bb genotype had a higher median PTH level compared to patients with BB and bb genotypes. In addition, the Bb genotype was independently associated with the risk of developing moderate and severe secondary hyperparathyroidism in patients with ESKD. The influence of *BsmI* on parathyroid function was also observed in pre dialysis CKD and transplant patients. Marco et al. (15) reported a slower progression of secondary hyperparathyroidism in pre dialysis CKD patients with BB genotype, while Messa et al. (16) reported lower PTH levels in transplant patients with BB genotypes. On the other hand, contrary to our findings, some studies have reported non-significant differences in PTH levels across *Bsm I* genotypes. However, it is noteworthy that the *Bsm I* genotype distribution varies greatly across ethnic groups, hampering comparisons of studies.

The molecular mechanisms by which *Bsm*I VDR polymorphisms influence hyperparathyroidism have been linked to presence of b alleles. Previous studies have reported a strong association between b alleles and decreased VDR gene transcription and/or m RNA stability, hence, affecting the regulatory actions of calcitriol on parathyroid glands (6, 16). For example, patients with the BB genotypes are less susceptible to having reduced 1α-hydroxylase levels compared to patients with bb genotypes. Therefore, patients with b alleles are less likely to have optimal levels of calcitriol required to inhibit PTH secretion and parathyroid cell proliferation.

A few studies have also investigated the associations between *FokI*, *ApaI* and secondary hyperparathyroidism in patients with CKD. Consistent with a prior study (13), although not statistically significant, patients with FF genotype had lower levels of PTH than patients with Ff in our study.

In addition to the complexity of CKD -MBD is the existence of the ethnic variability in the development and severity of secondary hyperparathyroidism among CKD patients. Several previous studies consistently showed that black patients have higher PTH levels and lower 25 (OH) D levels (17, 18). The mechanisms behind these dissimilarities may partly be explained by genetic factors. For example, some polymorphisms may be over represented in certain races and therefore alter their risk. In this present study, there was a statistically significant difference between black and white participants in the distribution of the VDR polymorphisms. The Bb genotype which is an independent predictor of hyperparathyroidism is over represented in black populations (71.0% versus 56.4 %, $p<0.0001$). In line with our

findings, previous studies have also reported ethnic variations in the distribution of VDR polymorphisms (19, 20). Uitterlinden et al. (20) reported that the frequency of the f allele of Fok1 was lower in Africans as compared to Caucasians (Caucasians 34% versus Africans 24%); similarly a significant difference was found in the frequency of the *Bsm1*, B allele was lower in the Asian population compared to other populations (Asians 7 %, Africans 36%, and 42 % in Caucasians). These observed ethnic variations in the frequency of the VDR polymorphisms may help in explaining the racial discrepancy in the markers of CKD-MBD.

Several studies have consistently associated vitamin D insufficiency to various skeletal and extra skeletal clinical end points, leading to a special interest in the determinants of vitamin D metabolites (20, 21). Thus far, well-established determinants of 25(OH) D levels include dietary sources and sun exposure (21). However, a genetic factor has been shown to play a vital role in the inter individual variation in circulating vitamin D levels. For example, in the classical twin study, Hunter et al. reported that the calcium/PTH/calcitriol axis is under strong genetic influence, accounting for 52% of calcium excretion, 74% of bone formation, 58% of bone resorption, 60% of PTH, and 65% of vitamin D variance (22). Similarly, a more recent large GWAS study has revealed a significant association with some genetic variants with 25(OH) D levels (21). These important findings were restricted to Caucasians, limiting their results to other ethnic groups. However, a few studies that explored these associations across races yielded similar results (23). In agreement with these studies, we found an increased risk of developing severe vitamin D deficiency with *FokI* Ff genotype and combined Ff+ff genotypes. In contrast, we did not find a significant difference in vitamin D levels across the various VDR genotypes.

The limitations of our study include the following: Firstly, the influence of some wild type genotype (homozygous minor) on the calcium/PTH/calcitriol axis could not be adequately determined due to their smaller numbers. Thus, a larger sample will be required to detect their associations with markers of CKD-MBD. Secondly, this was a cross-sectional study design; therefore we could not determine the longitudinal changes in markers of CKD-MBD, as well as seasonal variation in 25 (OH) D levels. Thirdly, information relating to UVB exposure and vitamin D dietary history are lacking.

The strength of this study lies in the heterogeneous nature of our study population (black and white patients) in an African setting which has allowed comparisons of data not only for Black Africans with Black Americans, but also between whites in Africa and USA/Europe.

6.5 Conclusion

We have demonstrated that both moderate and severe secondary hyperparathyroidism are predicted by *BsmI* Bb genotype, and the over expression of this genotype in black patients may partly explain the ethnic variations in the severity of secondary hyperparathyroidism in CKD population. In addition, the *Fok I* Ff genotype might be an important determinant of an individual's susceptibility to 25 (OH) D deficiency.

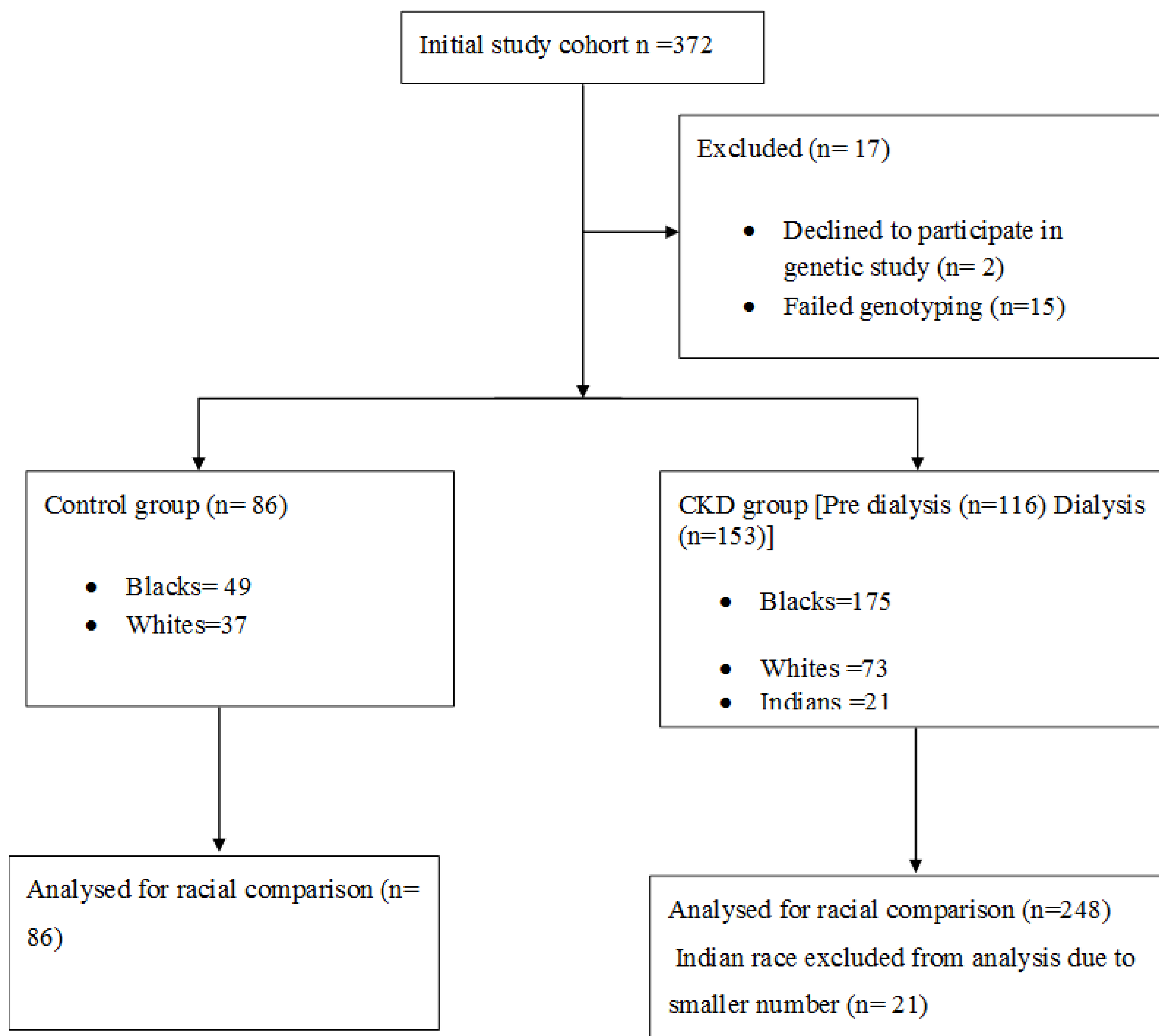

Figure 6. 1: Participant disposition and recruitment flow chart

Table 6. 1: Participants' characteristics and genotype frequencies by race

Parameters	Black(n=224)	White(n=110)	P
Age(years)	46.5±12.9	54.4±17.5	<0.0001
Gender n (%)			
Male	111(49.6%)	60(54.5%)	0.74
Female	113(50.4%)	50(45.5%)	
25(O H)D(ng/ml)	25.8±12.1	23.1±11.9	0.048
PTH(pg/ml)	214(61-872)	112(30-364)	0.001
Calcium (mmol/l)	2.22±0.25	2.29±0.18	0.06
TAP(U/L)	120(88-190)	110(74-145)	0.14
Phosphate (mmol/l)	1.29±0.47	1.48±0.49	0.003
FGF23(ng/ml)	59(23-307)	80(28-521)	0.20
VDR genotypes			
Bsm I			
BB	54(24.1%)	48(43.6%)	
Bb	159(71.0%)	62(56.4%)	P<0.0001
bb	11(4.9%)	0(0.00%)	
Fok I			
FF	151(67.4%)	38(34.6%)	
Ff	71(31.7%)	69(62.7%)	P<0.0001
ff	2(0.89%)	3(2.73%)	
Apa I			
AA	94(42.0%)	40(36.4%)	
Aa	127(56.7%)	69(62.7%)	0.61
aa	3(1.34%)	1(0.91%)	
Taq I			
TT	128(57.1%)	44(40.0%)	
Tt	80(35.7%)	52(47.3%)	0.01
tt	16(7.1%)	14(12.7%)	

Table6. 2: Distribution of VDR polymorphisms among CKD and control groups and the odds ratios for developing severe 25 -hydroxyvitamin D severe deficiency (< 15ng/ml)

VDR genotypes	Controls	CKD	p[a]	OR (95%CI)	P
Bsm I	N= 84	N=268			
BB	23(27.4%)	87(32.5%)	0.05	1.00(reference)	
Bb	55(65.5%)	176(65.7%)		0.85(0.51-1.42)	0.55
bb	6(7.14%)	5(1.87%)		0.28(0.03-2.32)	0.24
Dominant model					
BB	23(27.4%)	87(32.5%)			
Bb +bb	61(72.6%)	181(67.5%)	0.38	0.83(0.50-1.37)	0.46
Fok I	N=86	N=266			
FF	45(52.3%)	152(57.1%)	0.47	1.00(reference)	
Ff	39(45.4%)	111(41.7%)		1.89 (1.17-3.07)	0.01
ff	2 (2.3%)	3 (1.1%)		2.52 (0.41-15.59)	0.32
Dominant model					
FF	45(52.3%)	152(57.1%)	0.43	1.00 (reference)	
Ff+ ff	41(47.7%)	114(42.9%)		1.91(1.18-3.08)	0.008
Apa I	N=83	N=269			
AA	32(38.6%)	112(41.6%)	0.50	1.00(reference)	
Aa	51(61.4%)	152(56.5%)		1.44(0.88-2.37)	0.15
aa	0(0.0%)	5(1.86%)		2.33(0.37-14.57)	0.37
Dominant model					
AA	32(38.6%)	112(41.6%)	0.70	1.00(reference	
Aa +aa	51(61.4%)	157(59.0%)		1.46(0.89-2.40)	0.13
Taq I	N=84	N=268			
TT	37(44.1%)	146(54.5%)	0.05	1.00(reference)	
Tt	42(50.0%)	95(35.5%)		1.00(0.61-1.65)	0.99
tt	5(6.0%)	27(10.1%)		0.76(0.31-1.87)	0.60
Dominant model					
TT	37(44.1%)	146(54.5%)	0.06		
Tt +tt	47(56.0%)	122 (45.5%)		0.96(0.59-1.53)	0.85

OR= odds ratio, CI= confidence interval, CKD= chronic kidney disease, Pa value for comparison of genotype frequencies between control and CKD groups, VDR= vitamin D receptor.

Table 6. 3: Levels of markers of CKD-MBD across various VDR genotypes

Variable	Genotypes			p	P^c
Bsm I	BB(n=87)	Bb(n=176)	bb(n=5)		
25(OH) D(ng/ml)	21(14-33)	25(16-34)	27(19-31)	0.30	0.14
PTH(pg/ml)	231(111-593)	553(197-1230)	169(134-214)	<0.001	<0.001
Calcium (mmol/l)	2.20±0.75	2.21±0.64	2.20±0.10	0.99	0.98
Phosphate(mmol/l)	1.27±0.49	1.43±0.49	1.0±0.33	0.002	0.01
TAP(U/L)	121(83-153)	113(88-173)	80(57-141)	0.373	0.91
Medications n (%)					
Calcium carbonate	37(42.5)	89(50.6)	2(40.0)	0.19	0.07
Alfacalcidol	28(32.2)	80(45.5)	1(20.0)	0.09	0.047
Fok I	FF(n=152)	Ff(n=111)	ff(n=3)		
25(OH) D(ng/ml)	24(14-34)	22(14-33)	15(11-29)	0.31	0.30
PTH(pg/ml)	327(121-975)	360(166-735)	61(28-94)	0.12	0.86
Calcium (mmol/l)	2.21±0.61	2.19±0.76	2.17±0.27	0.97	0.83
Phosphate(mmol/l)	1.35±0.51	1.38±0.48	1.61±0.51	0.64	0.64
TAP(U/L)	123(91-160)	103(79-167)	258(69-312)	0.47	0.35
Medications n (%)					
Calcium carbonate	65(42.8)	59(44.1)	1(33.3)	0.23	0.11
Alfacalcidol	60(39.5)	45(40.5)	1(33.3)	0.91	0.74
Taq I	TT(n=146)	Tt (n=95)	tt (n=27)		
25(OH)D(ng/ml)	23(15-32)	25(16-36)	21(15-32)	0.31	0.23
PTH(pg/ml)	363(174-926)	327(109-913)	672(121-1314)	0.24	0.17
Calcium	2.21±0.61	2.17±0.82	2.36±0.31	0.47	0.71
Phosphate(ng/ml)	1.31±0.45	1.38±0.56	1.52±0.47	0.13	0.36
TAP(U/L)	110(82-154)	123(93-192)	121(75-167)	0.39	0.17
Medications n (%)					
Calcium carbonate	61(41.8)	52(54.7)	14(51.9)	0.41	0.18
Alfacalcidol	54(37.0)	43(45.3)	11(40.7)	0.50	0.24
Apa I	AA (n=112)	Aa(n=152)	aa (n=5)		
25 (OH)D(ng/ml)	24(17-37)	22(15-32)	19(15-28)	0.22	
PTH(pg/ml)	329(137-957)	383(99-814)	1889(1359-1889)	0.04	0.63
Phosphate(mmol/l)	1.34±0.52	1.37±0.48	1.63±0.47	0.46	0.52
Calcium(mmol/l)	2.22±0.66	2.19±0.68	2.41±0.16	0.79	0.76
TAP(U/L)	123(82-190)	115(88-149	160(91-440)	0.51	0.91
Medications n (%)					
Calcium carbonate	49(43.8)	74(48.7)	5(100.0)	0.04	0.23
Alfacalcidol	41(36.6)	64(42.1)	4(80.0)	0.29	0.33

Variables are presented as means± standard deviations or median(interquartile range), TAP= serum total alkaline phosphate, PTH= parathyroid hormone,25 (OH)D=25 hydroxyvitamin D 3, pc value: comparison between Homozygous dominant and heterozygous genotypes

Table 6. 4: Odds ratios for association between VDR polymorphisms and secondary hyperparathyroidism in haemodialysis patients

Polymorphisms	Crude OR 95%(CI)	P	Adjusted*OR 95%(CI)	P
Moderate secondary hyperparathyroidism (PTH>130 ng/ml)				
Bsm I				
BB	1.00(reference)		1.00(reference)	
Bb	3.12 (1.11-8.83)	0.03	3.88(1.13-13.25)	0.03
bb	N/A	N/A	N/A	
FokI				
FF	1.00(reference)		1.00(reference)	
Ff	0.87(0.31-2.39)	0.78	0.65(0.20-2.10)	0.47
ff	N/A	N/A	N/A	
Taq I				
TT	1.00(reference)		1.00(reference)	
Tt	0.27(0.09-0.84)	0.02	0.43(0.12-1.52)	0.19
tt	0.53(0.09-2.96)	0.47	0.76(0.11-5.19)	0.78
Apa I				
AA	1.00(reference)		1.00(reference)	
Aa	0.42(0.13-1.36)	0.15	0.25(0.06-1.01)	0.052
aa	0.28(0.3-3.14)	0.30	0.25(0.01-3.10)	0.25
Severe hyperparathyroidism (PTH>585 pg/ml)				
Bsm I				
BB	1.00(reference)		1.00(reference)	
Bb	2.55(1.19-5.47)	0.02	2.54(1.08-5.96)	0.032
bb	N/A	N/A	N/A	N/A
Fok I				
FF	1.00(reference)		1.00(reference)	
Ff	0.42(0.21-0.82)	0.01	0.37(0.17-0.81)	0.01
ff	N/A	N/A	N/A	
Taq I				
TT	1.00(reference)		1.00(reference)	
Tt	0.64(0.31-1.32)	0.23	0.71(0.32-1.59)	0.41
tt	1.37(0.44-4.24)	0.58	1.39(0.41-4.73)	0.39
Apa I				
AA	1.00(reference)		1.00(reference)	
Aa	0.85(0.43-1.69)	0.65	0.74(0.35-1.57)	0.43
aa	2.26(0.24-21.47)	0.48	2.84(0.27-30.22)	0.86

OR= Odds ratio, CI= confidence interval, N\A -= not applicable, *Adjusted Odd ratio= adjusted for Age, calcium, phosphate，25 hydroxyvitamin D 3，Fibroblast growth factor 23 and use of alfacalcidol,

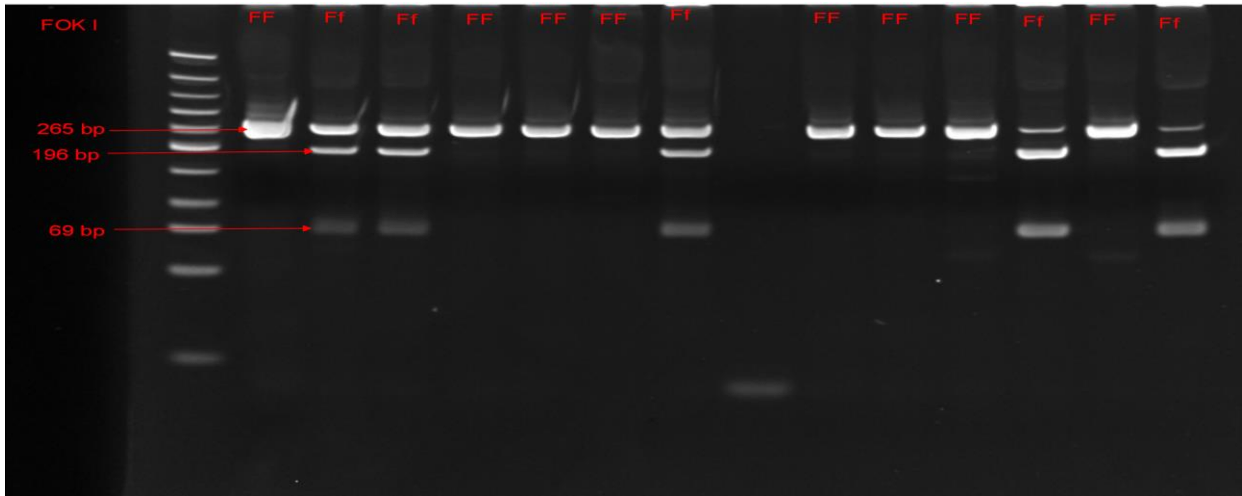

Figure 6. 2: Restriction Endonuclease digestion for *FokI* polymorphism

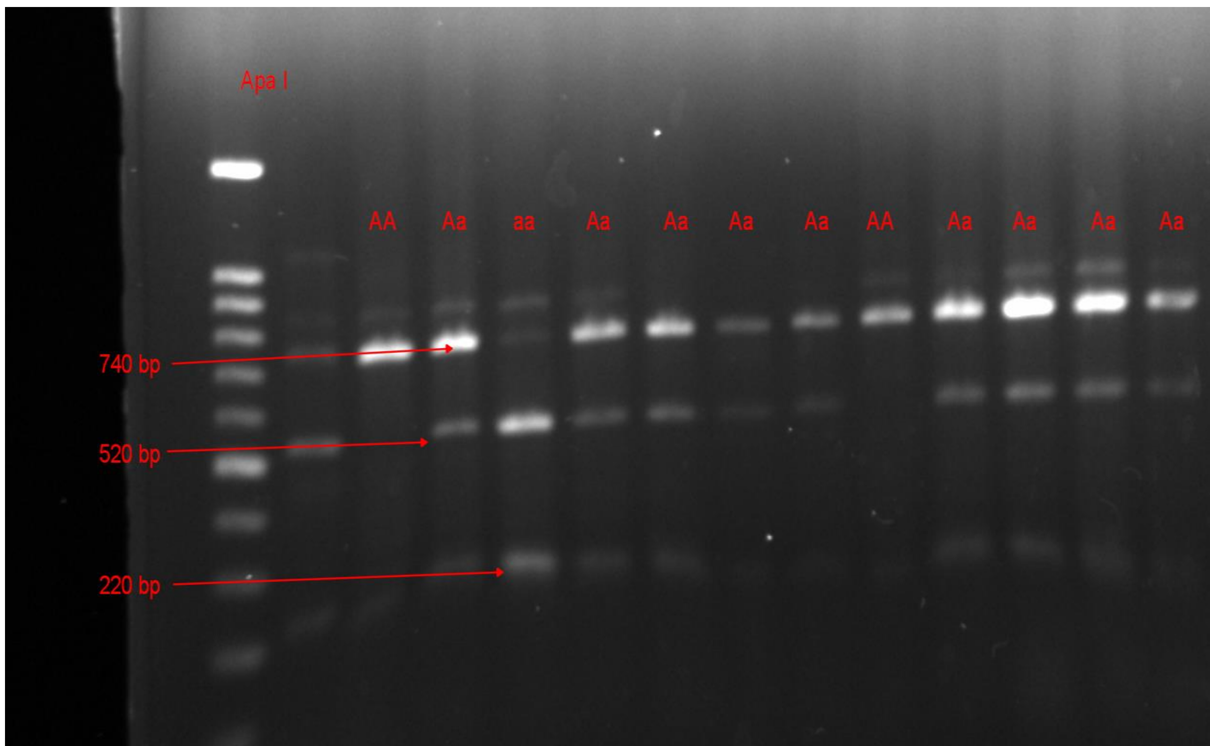

Figure 6. 3: Restriction Endonuclease digestion for *ApaI* polymorphism

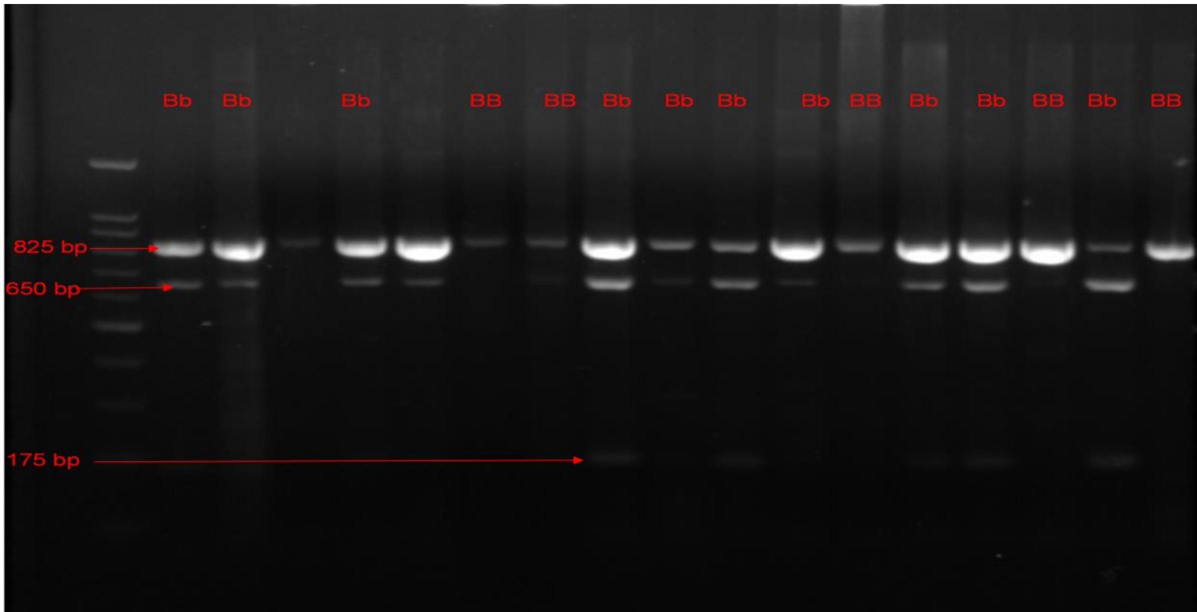

Figure 6. 4: Restriction Endonuclease digestion for *Bsm I* polymorphism

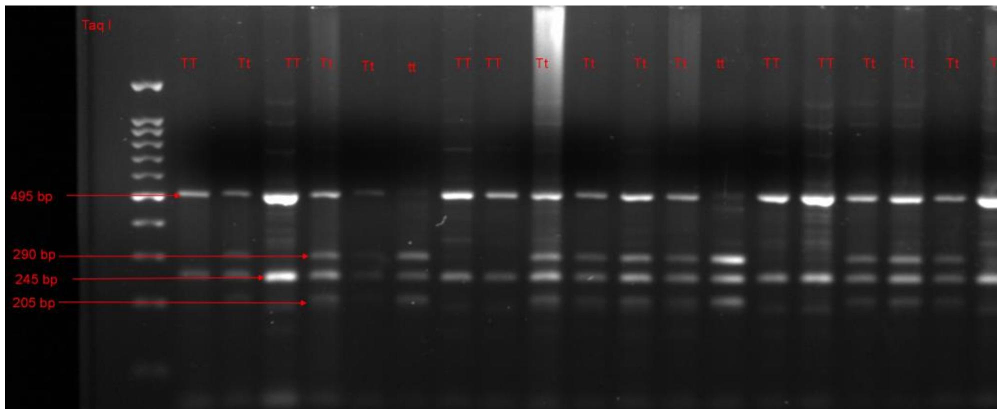

Figure 6. 5: Restriction Endonuclease digestion for *TaqI* polymorphism

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CHAPTER 7: DISCUSSION

7.1 Introduction

Although CKD-MBD has been extensively studied, surprisingly large gaps of knowledge still exist in this field of nephrology as highlighted by the KDIGO group in 2013(1).

In addition, the group agreed that further studies are needed to assist in updating some of the 2009 KDIGO clinical practice guidelines on the diagnosis and treatment of CKD-MBD. Therefore, in line with the KDIGO recommendations, we have conducted this study to assist in bridging the gaps in knowledge in the field of CKD-MBD.

This chapter highlights our relevant findings; followed by comparisons of our findings with the existing literature; recommendations and clinical implications; study limitations and conclusions.

7.2 The summary of our main findings are as follows:

7.2.1. Spectrum of CKD –MBD

The prevalence of hyperparathyroidism (iPTH>150 pg/mL), hyperphosphataemia, hypocalcaemia and 25-OH vitamin D deficiency (<30 ng/mL) was 73.4%, 57.0%, 20.3% and 80.7 % respectively in our MHD patients

7.2.2. Association between markers of CKD -MBD and mortality

Patients with high TAP had significantly higher risk of death compared to patients with TAP <112 U/L (hazard ratio, 2.50; 95% CI 1.24–5.01, P = 0.01). Similarly, serum calcium >2.75 mmol/L was associated with increased risk of death compared to patients within levels of 2.10–2.37 mmol/L (HR 6.34, 95% CI 1.40–28.76; P = 0.02). The HR for death in white patients compared to black patients was 6.88; 95% CI 1.82–25.88; P = 0.004.

7.2.3. Racial variations in markers of CKD-MBD

Our study highlighted the existence of racial differences in the circulating markers of mineral bone disorders in an African CKD population. Compared to whites, blacks had higher median intact PTH (498 [37-1084] versus 274[131-595] pg/ml; p=0.03), alkaline phosphatase (122[89-192] versus 103[74-144] U/L; p=0.03) and mean 25 OH vitamin D_3 (26.8±12.7 versus 22.7 ±12.2 ng/ml, p=0.01) levels, while their median FGF23 (100 [34-639] versus 233[80-1370] pg/ml; p=0.002) and mean serum phosphate (1.3±0.5 versus 1.5±0.5, p=0.001) levels were significantly lower

7.2.4. VDR polymorphisms and its association with markers of CKD –MBD

There was a significant difference in the distribution of VDR polymorphisms between black and white patients; blacks had significantly higher proportions of Bb genotype than whites (71.0 % versus 55.6 %), and lower frequency of BB genotype (24.1 % versus 44.4 %). The VDR Bb genotype was significantly associated with moderate secondary hyperparathyroidism (OR, 0.3.12; 95 CI 1.11-8.83, p=0.03) and severe hyperparathyroidism (OR, 2.55; 95 CI 1.19-5.47, P=0.02).

7.3 Comparisons with the existing literature

In Africa, data on the prevalence of secondary hyperparathyroidism and vitamin D status in the CKD population is sparse and limited by small sample size. In our study, the prevalence of secondary hyperparathyroidism (iPTH >150pg/mL) in maintenance haemodialysis patients was 73.4%. This high prevalence is consistent with previous studies from Africa (2, 3), despite utilizing different cut-off values. The strength of our study is the larger sample size compared to other previous studies from Africa, in addition to providing data on 25-(OH) vitamin D status in our patients.

In spite of the geographical location with high levels of vitamin D from sunlight, the majority of our MHD patients had 25 - (OH) vitamin D insufficiency. Inadequate vitamin D status defined as 25 - (OH) vitamin D level < 30 ng/ml was found in 80.7% of our study population. Similarly studies from countries with abundant sunlight have reported high prevalence of

vitamin D deficiency in healthy children and adults (4, 5). In fact, vitamin D deficiency has been termed a global health problem affecting over 1 billion of the world's population (6).

The consequences of deranged markers of CKD-MBD have been documented by studies mainly from American and Europe with few or no data from Africa. In our study we have highlighted that high levels of alkaline phosphatase, hypercalcaemia and white race are associated with increased risk of mortality. Our finding of hypercalcaemia and increased mortality is consistent with the three phases of the dialysis outcomes and practice patterns study (DOPPSI, II and III) with 25,588 HD patients, which showed that calcium levels greater than 10.0 mg/dL (>2.5 mmol/L) were significantly associated with greater risk of all cause and cardiovascular mortality in both baseline and time dependent models(7).

The reawakened interest in alkaline phosphatase, which is one of the pioneer markers of bone turnover, is due to emerging evidence that has consistently associated high levels of alkaline phosphatase with increased risk of mortality (8, 9). These studies have indicated that alkaline phosphatase is not just a marker of bone turnover but could also serve as a predictor of mortality. In line with previous studies, we have shown an association between high levels of alkaline phosphatase and mortality in South Africa MHD patients. The mechanisms for the association between high TAP and increased mortality have been linked to enhanced vascular calcification by high levels of serum TAP through hydrolysis of pyrophosphate or activation of apatite crystal formation (10). In addition to vascular calcification, elevated levels of TAP have been associated with high C reactive protein, insulin resistance, and 25-OH vitamin D deficiency

Despite racial disparity in access to health care and associated poor predictors of adverse outcome with black race in the general population, several studies from the USA have shown that black patients on haemodialysis have better survival than whites MHD patients (11, 12). This is the first study in Africa to highlight the existence of this association in African MHD patients. The mechanisms behind this survival paradox remain unclear; however, several reasons have been reported by some studies. For example, a large USA observational study involving 1,330,007 incident end-stage renal disease reported that the widely perceived survival advantage for black dialysis patients applies only to older adults, with a reversal of the higher risk of death in the younger age group (<50 years) (13). This is contrary to several studies including the current study, where the risk persisted after adjusting for the significantly higher mean age in the white patients (11, 12). Another reported explanation

was that racial variations in markers of nutritional status and response to inflammation may account for the survival paradox in MHD patients (11, 12). For example, Streja et al. (11) reported that markers of worse nutritional status and increased body fat in African American patients correlated less strongly with mortality than in whites. Additionally, psychosocial and coping mechanisms with disease conditions may vary across race.

In an attempt to reduce adverse clinical outcomes associated with CKD-MBD, guidelines were proposed by various global and regional societies to assist physicians in the management of CKD -MBD. However, biochemical markers of CKD-MBD have been shown to differ across different races and thus there is the need to establish race specific target values for these markers of CKD-MBD. For example, in this present study, with the exception of 25-(OH) vitamin D3 levels, our findings which are consistent with those of previous studies, showed that PTH and alkaline phosphatase are higher in black than white patients in CKD stage 5 and CKD stage 4 respectively. The mechanisms behind this discrepancy remain largely unclear. Therefore, in line with ongoing efforts to gain greater understanding of the mechanisms behind racial disparities in markers of CKD-MBD, we also explored the variations in VDR polymorphisms between black and white African CKD patients and its relationship with markers of mineral bone disorders. The influence of genetic factors on the inter-individual variation in circulating markers of CKD-MBD was first reported by Hunter et al.(12) who reported that the calcium/PTH/calcitriol axis is under strong genetic influence, accounting for 52% of calcium excretion, 74% of bone formation, 58% of bone resorption, 60% of PTH, and 65% of vitamin D variance. In our study, there was a statistically significant difference between black and white participants in the distribution of the VDR polymorphisms. The Bb genotype which is an independent predictor of hyperparathyroidism is overexpressed in the black populations (71.0% versus 56.4 %, $p<0.0001$). In line with our findings, some previous studies have also reported ethnic variations in the distribution of VDR polymorphisms. Uitterlinden et al. (14) reported that the frequency of the f allele of *Fok1* was lower in Africans as compared to Caucasians (Caucasians 34% versus Africans 24%); similarly, a significant difference was found in the frequency of the *Bsm1* B allele which was lower in the Asian population compared to other populations (Asians 7 %, Africans 36%, and 42 % in Caucasians). Therefore, these observed ethnic variations in the frequency of the VDR polymorphism may help in explaining the racial discrepancy in the markers of CKD -MBD.

7.4 Clinical implications of our findings and recommendations

Surprisingly, despite the strong association between abnormal markers of CKD-MBD, there is a paucity of data on the prevalence of secondary hyperparathyroidism in African MHD patients. Therefore, the findings from this study have provided us with important insights on the spectrum of CKD-MBD in African MHD patients. The highlighted high prevalence of secondary hyperparathyroidism and vitamin D deficiency in MHD patients has drawn our attention to the need to aggressively manage secondary hyperparathyroidism in our MHD patients. In addition, emphasis should be placed on mitigating the wider effects of vitamin D insufficiency through vitamin D supplementation.

The consistent linear association between alkaline phosphatase and mortality as demonstrated in this study and several other studies has further emphasized the role of total alkaline phosphatase in the management of CKD-MBD. This cheap and readily available test could be utilized in resource poor countries as a surrogate marker for monitoring CKD-MBD.

Although the association between hypercalcaemia and increased mortality has been established by previous studies, our study reaffirms the need to pay more attention to prevention and correction of hypercalcemia. The majority of our patients are on a calcium-based phosphate binder which is cheap and effective in controlling serum phosphate. However, use of calcium based phosphate binders has been associated with progression of vascular calcification (15, 16). Therefore, it is recommended that calcium-based phosphate binders should be avoided in patients with adynamic bone disease, hypercalcemia, and vascular calcification. On the other hand, cinacalcet which is one of the newer drugs that effectively lowers PTH without raising serum calcium levels recently became available in South Africa; however, it is quite expensive, thus limiting its use to a few of the patients who are able to access it.

The finding of the existence of racial variations in makers of CKD-MBD in our CKD patients further supports the notion that the present guidelines may not to be appropriate for all races and thus the need for race-specific target values.

The observed association between VDR Bb genotype with severity of secondary hyperparathyroidism may assist in identifying individuals at an increased risk of developing moderate to severe secondary hyperparathyroidism who may require more aggressive

management to prevent its development. In addition, genetic factors should be considered when designing intervention strategies for secondary hyperparathyroidism.

7.5 Study limitations

Our findings should be considered in the context of the following limitations. Firstly, due to the cross-sectional study design, we could not determine the longitudinal changes in markers of CKD-MBD, as well as seasonal variations in 25 (OH) D levels.

Secondly, information relating to UVB exposure and dietary phosphate are lacking. Thirdly, there is the lack of bone biopsies to definitively describe the pattern of MBD in our patients. However, studies have shown a good association between PTH and histological findings.

Finally, the influence of some wild type genotype (homozygous minor) on the calcium/PTH/calcitriol axis could not be adequately determined due to their small numbers. Thus, a larger sample will be required to detect their associations with markers of CKD-MBD.

However, despite the highlighted limitations, our study has assisted in bridging the knowledge gaps in the field of CKD-MBD by providing findings that has allowed comparisons of data not only for Black Africans with Black Americans, but also between whites in Africa and USA/Europe.

7.6 Conclusions

Our study showed that the abnormalities of biochemical markers of mineral bone disorder were common in our MHD patients and a moderately large proportion of the patients was outside the KDIGO recommended target levels. Our study also revealed a significant association between high levels of total alkaline phosphatase, hypercalcaemia, and white race with death in MHD patients, reaffirming the need to pay more attention to the two modifiable risk factors (calcium and TAP) in the management of CKD-MBD. Furthermore, we have also demonstrated the existence of racial variations in the markers of CKD- MBD. Finally, we have also demonstrated that both moderate and severe secondary hyperparathyroidism were predicted by the *Bsm I* Bb genotype, and the over expression of this genotype in black patients may partly explain the ethnic variations in the severity of secondary hyperparathyroidism in our CKD population.

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Appendix A: Ethics clearance certificate

R14/49 Dr Bala Waziri

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M141016

NAME: (Principal Investigator)	Dr Bala Waziri
DEPARTMENT:	Internal Medicine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Markers of mineral bone disease in Black South Africans with chronic kidney disease
DATE CONSIDERED:	31/10/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Saraladevi Naiker

APPROVED BY: Professor PE Cleaton-Jones Chairperson, HREC (Medical)

DATE OF APPROVAL: 05/12/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

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 ____________

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B : Consent form

UNIVERSITY OF THE WITWATERSRAND

Participant information and consent form (patient)

Section A

STUDY TITLE: Biochemical and Genetic Markers of Mineral Bone Disease in South African patients with Chronic Kidney Disease.

INVESTIGATOR: Dr Bala Waziri

SITE: Charlotte Maxeke Johannesburg Academic Hospital

TELEPHONE: Cell: 0848329071

Land line (Department): 011-488-3672

Section B

Introduction

Good day, my name is Bala Waziri. I am a PhD student doing research at the University of the Witwatersrand. Research is an act of investigation or study in order to establish facts and reach new conclusions. I am conducting a study on "Biochemical and Genetic Markers of Mineral Bone Disease in South Africans with Chronic Kidney Disease".

The information that I will collect from this research project will be kept confidential. You are hereby invited to take part in the study and your participation in this research is entirely voluntary. It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive at this hospital will continue and nothing will change. You may change your mind later and stop participating even if you agreed earlier.

If you have any questions you may ask them now or later, even after the study has started.

It is important that you read and fully understand information on this leaflet as it will help you in making the decision. If unable to read or understand English, an interpreter will be provided for you.

Purpose of the study

I am conducting a study to look at people with chronic kidney disease and why they develop bone disease which is one of the complications of chronic kidney disease. The human body has two kidneys, which are bean shaped and perform different functions; such as the removal of waste products and forming urine. One of the objectives of this study is to determine the frequency of bone disease in people with chronic kidney disease. I will also look for inheritable factors called genes that regulate the development of bone disease..

Genes are basically a collection of information or instructions that form the makeup of a human being and decide how he/she behaves.

I will assess fibroblast growth factor 23 and vitamin D levels and look for the relationship between their genes. Fibroblast growth factor 23 and vitamin D are responsible for controlling bone disease in people with chronic kidney disease.

Some people with genetic changes have high risk of developing bone disease compared to those with normal genes. One way to demonstrate this relationship is though DNA testing. DNA is the chemical compound which genes are made of and is often referred to as the building blocks of life. Genetic testing may help in the diagnosis of a disorder. Genetic testing can also be performed for research. When a gene has changes it is called a mutation.

DNA testing is optional and should you agree to participate, a separate DNA consent form will be given to read and sign.

Procedures of the study

If you agree to participate in the study, your medical records will be reviewed. You will be examined by a medical doctor and also interviewed to collect information regarding bone

disease. Blood and urine samples will be collected. A total of 10mls (2 teaspoons of blood) will be drawn to measure the markers of bone disease and analyze for the genetic changes.

The blood will be spun very quickly and the serum separated out. The serum which appears as a clear fluid will be kept frozen in a small test-tube. Your coded blood samples will be sent to Department of Internal Medicine research laboratory for the above mentioned tests. 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 you 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.

Benefits of the study

The benefits of participating in this study are that we will be able to identify the magnitude of bone disease in people with chronic kidney disease. Bone disease is associated with an increase in death and this study will assist in picking up bone disease early in people with kidney disease. Information that will be obtained from this study will guide our decision in prompt management of bone disease which will subsequently reduce the high rate of deaths in this group of people.

Possible risks

Possible side effects from drawing the blood sample include mild pain, bleeding, and bruising at the site of the needle insertion. Qualified personnel will collect the blood samples to prevent such complications.

Financial arrangements

You will not be paid for participating in the study. However, there will be no costs to you for any related study visits and procedures, as any costs incurred will be compensated by research funds.

Confidentiality

All information obtained during the course of this study will be kept strictly confidential. Your records will be given unique identification numbers and the initial identification details won't be used. All physical records will be kept in a locked locker with access limited to the research team. Electronic data will be password protected.

Source of information

If you have any questions, queries and clarifications, please contact the following; Dr Bala Waziri will be reachable 24 hours every day on the number, 0848329071.

Additional information can be obtained from the chairperson of Witwatersrand University Human Research Ethics Committee, Professor Cleaton Jones on 011-717-2301

Section C

Informed Consent Form: General (patient)

I confirm that I have been informed about the study by Dr Bala Waziri. I understand that my personal details will be kept strictly confidential and that I may at any stage withdraw my consent and participation in the study and continue to receive the appropriate treatment. 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.

PARTICIPANT (name)……………………………………………………………..

Signature or thumb print ……………………………… **Date** **………………………………..**

Witness (printed name)…………………………………………………………………………

Signature……………………………………………...Date…………………………………….

I, Dr Bala Waziri confirm that the participant has been fully informed about the nature of the above study.

STUDY INVESTIGATOR

…………………………………….. Signature……………Date………………..

Printed Name

Section D

Informed consent form: DNA Storage (Patient)

I hereby confirm that I have been informed about the study by Dr. Bala Waziri about the nature, benefits and risks of the genes study.

I understand that my blood sample will be stored for future testing.

I understand that my 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.

PARTICIPANT (name)…………………………………………………………………..

Signature or thumb print ……………………………… Date ………………………………..

Witness (printed name)…………………………………………………………………

Signature…………………………………………...Date……………………………………….

I, Dr Bala Waziri confirm that the participant has been fully informed about the nature of the above study.

STUDY INVESTIGATOR

Name ………………………….. Signature……………Date…………………

Section E

Informed consent form: DNA testing (patient)

I hereby confirm that I have been informed about the study by Dr. Bala Waziri about the nature, benefits and risks of the genes study. I understand that my 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.

PARTICIPANT (name)……………………………………………………………..

Signature or thumb print ……………………………… Date ………………………………..

Witness (printed name)……………………………………………………………………

Signature…………………………………………...Date……………………………………….

I, Dr Bala Waziri confirm that the participant has been fully informed about the nature of the above study.

STUDY INVESTIGATOR

Name ………………………….. Signature……………Date…………………

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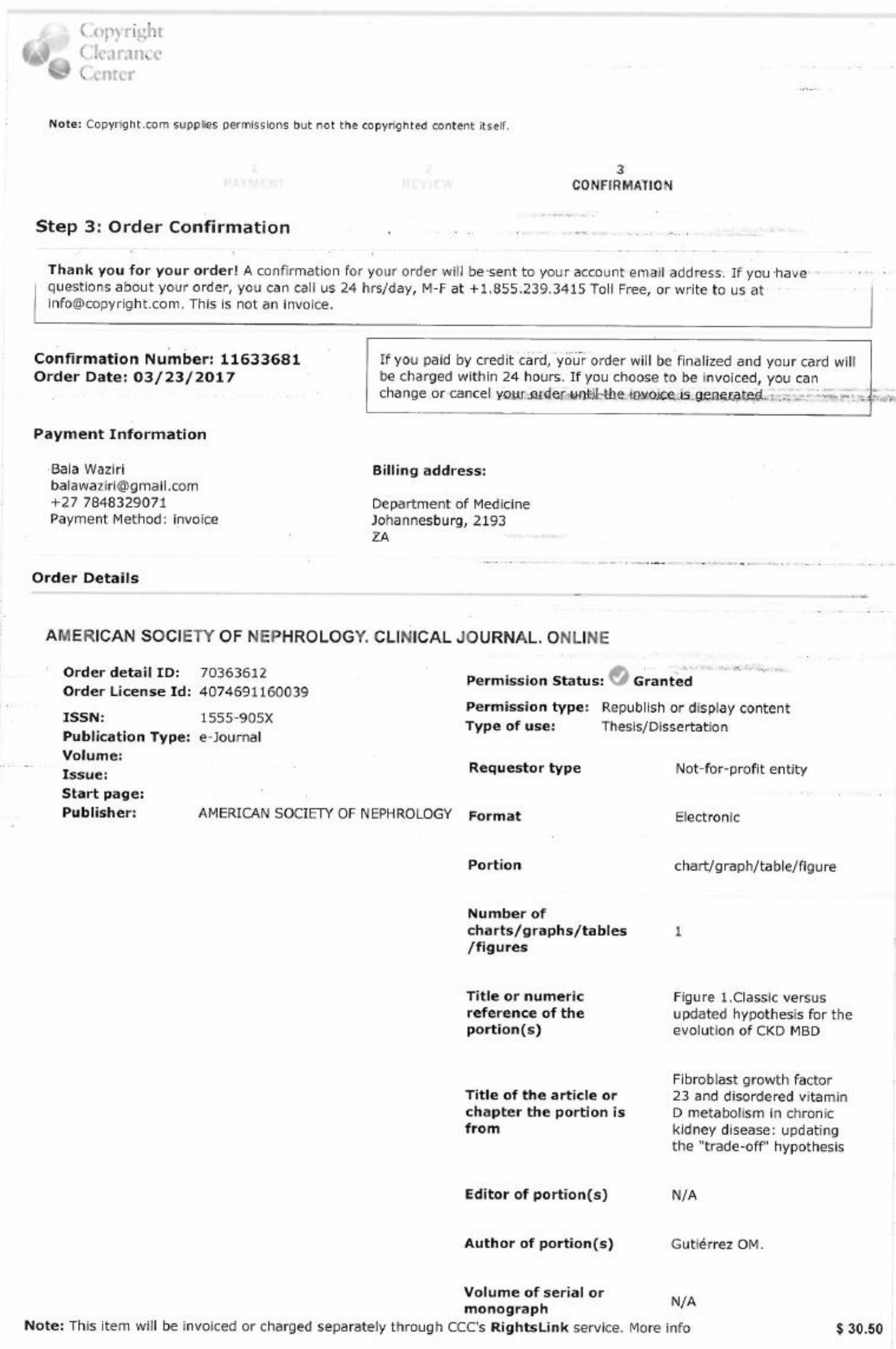

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Number of charts/graphs/tables /figures	1
Title or numeric reference of the portion(s)	Figure 1.Classic versus updated hypothesis for the evolution of CKD MBD
Title of the article or chapter the portion is from	Fibroblast growth factor 23 and disordered vitamin D metabolism in chronic kidney disease: updating the "trade-off" hypothesis
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Appendix D: Data sheet

Study: Biochemical and Genetic Markers of Mineral Bone Disease in Black South Africans with Chronic Kidney Disease.

Patients Data Sheet

Serial Number: ____ Hospital No: ________ Phone No.____________

1. Age at last Birthday (Years) ____________

2. Sex: Male ( ) Female ( )

3. Ethnicity ______________________ Black ( ) White ( )

4. Marital status: Single ( ) Married ( ) Widow/Widower ( ) Separated/Divorced ( )

5. Occupation ___________________

6. History of bone pain in the last one month (a) Yes (b) No

7. Presence of Hypertension: (a) Yes (b) No

8. Do you have diabetes mellitus? (a) Yes (b) No

9. Drug history : (a) Calcium supplement ________ i.Duration (months)._____Dose____

(b) Vitamin D supplement _______i.Duration (months) ._____Dose____

(c) Others____________

10. Aetiology of CKD_________________

11. Do you smoke cigarette? (a) Yes (b) No

12. Are you on dialysis (a) Yes (b) No

13. If answer to 12) is yes what type (a) Peritoneal (a.i) duration (months)____________

(b) Haemodialysis (b. i) duration (months) _______

Clinical Parameters

1) Weight (Kg) ______________

2) Height (m) _______________

3) BMI (Kg/m^2) _______________

4) Blood Pressure (mmHg) _____________

Investigations.

Parathyroid hormone (PTH)______________

Fibroblast growth factor 23 (FGF23) ______________

Albumin ______________ Calcium ______________

Phosphate ______________ Ca× PO_4 ______________

Total alkaline phosphate (TAP) _____________

25-OH vitaminD______________ Serum Creatinine (µmol/L) ______________

Urea (mmol/L) ______________ Total Cholesterol______________

LDL ______________ HDL_______________ TG _______________

Estimated GFR: ______________

Genetic analysis

VDR genotypes:

Appendix E: Pdf for manuscript 1

Hindawi
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Research Article

High Serum Alkaline Phosphatase, Hypercalcaemia, Race, and Mortality in South African Maintenance Haemodialysis Patients

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Objective. To determine the association between serum total alkaline phosphatase (TAP) and mortality in African maintenance haemodialysis patients (MHD). *Patients and Methods.* The study enrolled a total of 213 patients on MHD from two dialysis centers in Johannesburg between January 2009 and March 2016. Patients were categorized into a low TAP group (≤112 U/L) versus a high TAP group (>112 U/L) based on a median TAP of 112 U/L. *Results.* During the follow-up period of 7 years, there were 55 (25.8%) deaths. After adjusting for cofounders such as age, other markers of bone disorder, and comorbidity (diabetes mellitus), patients in the high TAP group had significantly higher risk of death compared to patients in the low TAP group (hazard ratio, 2.50; 95% CI 1.24–5.01, $P = 0.01$). Similarly, serum calcium >2.75 mmol/L was associated with increased risk of death compared to patients within levels of 2.10–2.37 mmol/L (HR 6.34, 95% CI 1.40–28.76; $P = 0.02$). The HR for death in white patients compared to black patients was 6.88; 95% CI 1.82–25.88; $P = 0.004$. *Conclusion.* High levels of serum alkaline phosphatase, hypercalcaemia, and white race are associated with increased risk of death in MHD patients.

1. Introduction

Prior to the availability of commercial intact parathyroid hormone (PTH) assays, serum total alkaline phosphatase (TAP) measurements were used as one of the surrogate markers of high bone turnover that was utilized in the management of chronic kidney disease mineral and bone disorder (CKD-MBD) [1]. Subsequently, in 2003 the Kidney Disease Outcome Quality Initiative (KDOQI) guidelines on CKD-MBD made no recommendations regarding the use of alkaline phosphatase and this has made it a less preferred marker to PTH. However, in 2009 the Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommended measurement of TAP every 12 months in CKD 4-5D [2] and more recently evidence continued to emerge on the importance of higher levels of alkaline phosphatase in the pathogenesis of vascular calcification via hydrolysis of pyrophosphate which is a potent inhibitor of vascular calcification [3–5]. This was further supported by a study that showed elevated levels of alkaline phosphatase, independent of PTH, calcium, or phosphorus as predictor of coronary artery calcification in haemodialysis patients [6]. Interestingly, in a recent secondary analysis of the handling erythropoietin resistance with oxypentifylline (HERO) trial, high levels of alkaline phosphatase were also associated with erythropoietin stimulating agent hyporesponsiveness [7]. These findings may likely explain the unclear pathophysiologic link between high serum alkaline phosphatase and mortality in haemodialysis patients [6].

Although the role of racial disparities in adverse clinical outcomes remains controversial and inconclusive, some studies have demonstrated survival benefits attributable to race in patients undergoing MHD [8, 9]. In addition, the impact of these biochemical abnormalities have been shown to differ across race and thus the need for race specific target values for these markers of mineral bone disorder [10, 11].

Therefore, the aim of this study was to determine if there is a link between high serum alkaline phosphatase and mortality in African MHD patients.

2. Patients and Methods

This study was a retrospective review of patients undergoing MHD from two dialysis centers in Johannesburg between

Appendix F: Pdf for manuscript 2

Biochemical markers of mineral bone disorder in South African patients on maintenance haemodialysis.

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Abstract

Background and objective: Despite the high mortality and morbidity associated with abnormalities in mineral and bone metabolism in haemodialysis patients, there is limited data on the pattern of mineral bone disorder in African CKD population. Therefore, the purpose of this study was to describe the pattern of mineral bone disease by evaluating biochemical parameters in patients on maintenance haemodialysis (MHD).

Methods: We evaluated the serum/plasma intact parathyroid hormone (iPTH), corrected calcium, phosphate, total alkaline phosphatase (TALP) and 25 –OH vitamin D levels of two hundred and seven patients undergoing MHD at two dialysis centers in Johannesburg.

Results: The MHD patients (133 men, 74 women) had a mean age of 54.5±15.6 years with a median dialysis vintage of 24 months (IQR, 12-48) and a mean kt/V of 1.45±0.28. The prevalence of hyperparathyroidism (iPTH >150 pg/ml), hyperphosphataemia, hypocalcaemia and 25-OH vitamin D deficiency (<30 ng/ml) was 73.4%, 57.0%, 20.3% and 80.7 % respectively. The combination of markers of bone turnover (iPTH >150pg/ml and TALP> 112 U/L) suggestive of high turnover bone disease, was present in 47.3 % of the study population. In multiple-logistic regression analysis, the odds ratio for developing hyperparathyroidism with hypocalcaemia and hyperphosphataemia were 5.32 (95% CI 1.10 - 25.9, P = 0.03) and 3.06(95 % CI 1.15 - 8.10, P=0.02) respectively. Ninety eight (47.3%) of the MHD patients had iPTH within the recommended kidney disease improving global outcome (KDIGO) guidelines.

Conclusion: Secondary hyperparathyroidism and 25–OH vitamin D deficiency were common in our haemodialysis patients. Hypocalcaemia and hyperphosphataemia were strong predictors for developing secondary hyperparathyroidism.

Keywords: Biochemical markers, guidelines, mineral bone disorder, haemodialysis.

DOI: https://dx.doi.org/10.4314/ahs.v17i2.19

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Introduction

Chronic kidney disease-mineral bone disorder (CKD MBD) is now defined 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, parathyroid hormone (PTH), or vitamin D metabolism; (ii) abnormalities in bone turnover, mineralization, volume, linear growth, or strength; or (iii) vascular or other soft tissue calcification and that the term renal osteodystrophy should exclusively be used to describe disorders in bone morphology associated with CKD[1,2]. Although bone biopsy is the gold standard for adequately describing the spectrum of CKD- MBD, it is less frequently utilized in clinical settings because of associated constraints. It is an invasive and cumbersome procedure that requires highly skilled personnel to interpret the obtained tissue samples. Therefore, clinicians largely depend on the biochemical parameters for monitoring and management of this important clinical entity that is associated with adverse clinical outcomes in CKD patients. In addition, the above aforementioned internationally acceptable definition has led to the ease of diagnosing CKD MBD and allows valid comparison of studies in this field.

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