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Association between fibroblast growth factor 23 and mortality in South African chronic kidney disease patients on chronic dialysis

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ASSOCIATION BETWEEN FIBROBLAST GROWTH FACTOR 23 AND MORTALITY IN SOUTH AFRICAN CHRONIC KIDNEY DISEASE PATIENTS ON CHRONIC DIALYSIS

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science in Epidemiology and Biostatistics

By

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October 2019

DECLARATION

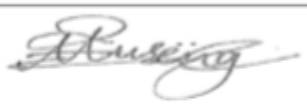
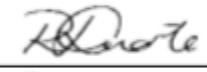
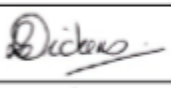
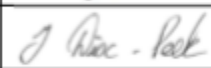
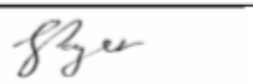
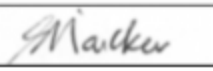
I declare that this research report is my own unaided work. It is being submitted for the degree of Master of Science in epidemiology and biostatistics at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

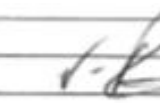


10th , October, 2019

RE-AGREEMENT BY CO-AUTHORS

Bala Waziri, student number 1111800, wrote the protocol, acquired ethical approval, cleaned the data, performed data analysis, wrote the first draft and received critical comment from all co-authors. By signing this declaration, the co-authors listed below agree to the use of the article by the student as part of his Research Report.

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DEDICATION

To patients with End Stage Kidney Disease

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS RESEARCH

Waziri B, Musenge E, Duarte R, Rekhviashvili V, Paget G, Naicker S. Plasma Fibroblast Growth Factor 23 and All- cause Mortality in South African Patients on Maintenance Haemodialysis**.** Oral presentation at the South African Renal Congress. 11-14 October 2018, Johannesburg.

Waziri B, Musenge E, Duarte R, Rekhviashvili V, Paget G, Naicker S. Associations of plasma fibroblast growth factor 23, calcium and parathyroid hormone with all-cause mortality in patients on maintenance dialysis. Oral Presentation at the 31st Annual Scientific Conference and General Meeting of Nephrology Association of Nigeria. 14-16 January 2019. Abakiliki, Nigeria.

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Waziri B, Musenge E, Duarte R, Dickens C, Dix Peek T, Rekhviashvili V, Paget G, Naicker S. Associations of plasma fibroblast growth factor 23, calcium and parathyroid hormone with all-cause mortality in patients on maintenance dialysis: A 3year Prospective Cohort Study. PLoS ONE 14(5): e0216656. https://doi.org/10.1371/journal.pone.0216656 Published in PloS ONE.

ABSTRACT

Background: Few studies have linked high levels of plasma C-terminal fibroblast growth factor 23 (FGF23) with poor clinical outcomes in patients on maintenance haemodialysis (MHD), while the association between intact FGF23 and mortality in this group of patients remains inconclusive.

Therefore, the aim of this study was to evaluate the association between plasma levels of intact FGF23 and mortality in dialysis patients.

Methods: A prospective multicenter study involving 165 patients undergoing dialysis at three dialysis centres in Johannesburg was undertaken between 1st October 2014 and 31st December 2017. The association between the quartiles of FGF23 and mortality was assessed using the Cox proportional hazard model.

Results: The study comprised 165 chronic dialysis patients (111 blacks, 54 whites) with a mean age of 46.6 ±14.2 years. During a three year follow up period, there were 46 deaths (1.03 per 100 person-years). The median plasma FGF23 level was 382 pg/ml (interquartile range [IQR], 145-2977). In adjusted multivariable analyses, there was a non-statistically significant increase in the risk of mortality with higher quartiles of FGF23 levels: the adjusted hazard ratios (HR) for the second, third and fourth quartiles were HR 3.20 (95% CI, 0.99-10.35; P=0.052), HR 2.43(95 % CI,0.65-9.09; P=0.19), and HR 2.09 (95% CI, 0.66-7.32; P= 0.25),respectively. Corrected serum calcium 2.38-2.5 mmol/l [HR 2.98 (95% CI, 1.07-8.29; P=0.04] and > 2.50 mmol/l [HR 5.50 (95% CI, 1.84-16.48; P=0.002] were independently associated with increased risk of death. Likewise, patients with intact parathyroid hormone > 600 pg/ml had a 3.46-fold higher risk of death (HR 3.46, 95% CI, 1.22-9.82 P=0.019). These findings persisted in time -dependent analyses.

Conclusion: Higher levels of intact FGF23 appear not to be independently associated with all-cause mortality in our dialysis patients, while hypercalcaemia and severe hyperparathyroidism were found to be independent predictors of mortality in this cohort of patients.

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TABLE OF CONTENTS

DECLARATION ii
DEDICATION iv
PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS RESEARCH v
ABSTRACT vi
ACKNOWLEDGEMENTS vii
LIST OF FIGURES xi
LIST OF TABLES xi
LIST OF ABBREVIATIONS xii
1. INTRODUCTION AND LITERATURE REVIEW 14
1.1 INTRODUCTION 14
1.2 LITERATURE REVIEW 15
1.2.1 Brief historical perspectives 15
1.2.2 Fibroblast growth factor 23 and Chronic Kidney Disease 15
1.2.3 Role of FGF23 in the pathogenesis of secondary hyperparathyroidism 16
1.2.4 Risk factors of mortality in patients on maintenance haemodialysis 17
1.2.5 Fibroblast growth factor 23 and mortality in maintenance haemodialysis 18
1.2.6 Association between traditional markers of CKD -MBD and mortality 19
1.2.7 Parathyroid hormone and mortality in chronic kidney disease 20
1.2.8 Phosphate and risk of mortality in CKD patients 21
1.2.9 Alkaline phosphatase and risk of mortality in patients with CKD 21
1.3 JUSTIFICATION FOR THE STUDY 22
1.4 RESEARCH QUESTION 22
1.5 AIM 22
1.5.1 Specific objectives 23
1.6 REFERENCES 23
2. SUBMISSIBLE MANUSCRIPT 30
2.1 ABSTRACT 31
2.2 INTRODUCTION 32

2.3 MATERIALS AND METHODS 34
2.3.1 Laboratory measurements 34
2.3.2 Primary outcome and exposure variables 35
2.3.3 Ethical consideration 36
2.3.4 Statistical analysis 36
2.4 RESULTS 38
2.4.1 FGF23 and mortality 40
2.4.2 Calcium and mortality 42
2.4.3 Intact PTH and mortality 43
2.4.4 Phosphate and risk of mortality 44
2.4.5 Bone specific alkaline phosphatase and mortality 44
2.4.6 Race, FGF23 and mortality 45
2.5 DISCUSSION 49
2.6 REFERENCES 55
3. EXTENDED METHODS AND RESULTS 62
3.1 STUDY DESIGN, POPULATION AND SITES 62
3.1.1 Study sample 62
3.1.2 Determination of sample size 62
3.1.3 Study sites 64
3.1.4 Inclusion Criteria 65
3.1.5 Exclusion criteria 65
3.2 ETHICAL CONSIDERATION 65
3.3 BLOOD COLLECTION AND LABORATORY MEASUREMENTS 65
3.4 TEST METHODOLOGIES 66
4. CONCLUSIONS AND RECOMMENDATIONS 73
4.1 RECOMMENDATIONS 75
APPENDICES 77
Plagiarism Form 77
Ethics clearance certificate 78
Patients Data sheet 79

Turnitin report 81

Journal guidelines 83

Do File 85

Plos One Reviewers' Comments and Point to Point responses 90

Informed Consent Form 98

LIST OF FIGURES

Figure 1. 1**:** Classic versus updated hypothesis for the evolution of CKD -MBD 17

Figure 2. 1: Kaplan Meier survival curves for different quartiles of plasma Fibroblast growth factor 23…… 45

Figure 2. 2: Kaplan Meier survival curves for different quartiles of serum calcium…… 46

Figure 2. 3: Kaplan Meier survival curves for categories of intact Parathyroid hormone…….47

Figure 2. 4: Hazard ratio of all- cause mortality for baseline serum calcium using fractional polynomial…… 48

Figure 2. 5: Hazard ratio of all-cause mortality for time dependent serum calcium using fractional polynomial…….49

LIST OF TABLES

Table 2. 1: Participants' characteristics by quartiles of plasma Fibroblast growth factor 23..... 38

Table 2. 2: Study participants characteristics by race..... 39

Table 2. 3: Fixed and time varying Hazard ratios for all-cause mortality 40

Table 2. 4: Hazard ratios for all-cause mortality factoring explanatory variables on a continuous scale..... 42

Table 3. 1: Baseline characteristics of patients by outcome status..... 67

Table 3. 2: Participants' characteristics by quartiles of intact Parathyroid hormone 68

Table 3. 3: Participants' characteristics by quartiles of serum calcium..... 70

LIST OF ABBREVIATIONS

b- ALP: Bone specific alkaline phosphatate

CKD: Chronic kidney disease

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

DEQAS: Vitamin D external quality assurance scheme

DM: Diabetes mellitus

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

iPTH: Intact Parathyroid hormone

KDIGO: Kidney Disease -Improving Global Outcomes

K/DOQI: Kidney Disease Outcomes Quality Initiative

MHD: Maintenance haemodialysis

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

ROD: Renal osteodystrophy

TAP: Total alkaline phosphatase

UK: United Kingdom

USA: United States of America

1. INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Chronic kidney disease, previously referred to as chronic kidney failure, is defined as the gradual loss of kidney function or structural abnormalities persisting for at least three months (1). The kidneys are responsible for elimination of wastes and excess fluids from the blood. Hence, impairment of the kidneys leads to fluid retention and electrolytes disturbances.

Over the past few decades, the developing countries have continued to undergo an epidemiological transition that is characterized by a rise in the burden of non-communicable diseases and a relative decline in the prevalence of infectious diseases because of improvement in immunization and sanitation programs. The dramatic global rise in the prevalence of diabetes mellitus and hypertension has made chronic kidney disease a global public health problem (2). Presently, CKD has been rated the 12^{th} highest cause of death and 17^{th} highest cause of disability worldwide (3). However, among the non-communicable diseases, CKD is a complex and intriguing condition that is caused by both non-communicable diseases (Diabetes mellitus and hypertension) and infection diseases such as HIV, hepatitis B, schistosomiasis and malaria (3). In Sub-Saharan Africa, it has been estimated that over half a million people develop end stage kidney disease (ESKD) annually (4), and mortality in this group of patients is significantly higher compared to patients in the developed countries (5). Some of the proposed reasons for this high mortality include increased prevalence of cardiovascular events and recurrent infection in haemodialysis patients (5). Furthermore, the disparity in mortality rate between the developed world and developing countries has also been attributed to inadequate resources allocated to renal replacement therapy in the developing countries. Kidney transplant, which is the mainstay of treatment for ESKD, remains largely scarce in Africa and thus the majority of these patients are dependent on chronic dialysis (6).

Therefore, identification of risk factors of mortality in patients on chronic dialysis is crucial in the care of these patients. Hence, information obtained from this study may assist us in reducing the high incidence of mortality in patients on maintenance dialysis by promptly addressing the identified modifiable risk factors.

1.2 LITERATURE REVIEW

1.2.1 Brief historical perspectives

When patients with CKD inevitably progressed to ESKD, they are left with two modalities of treatment namely haemodialysis and kidney transplant. In Africa, commencement of dialysis dates back to 1957 in Krugersdorp, South Africa (7). The dialysis machine was then used to manage acute renal failure by a physician in Krugersdorp.

A year later, the University of Cairo imported a dialyzer that was also used to treat a patient with acute renal failure who died after a few sessions of dialysis. In 1962, both countries reawakened their efforts by introducing peritoneal and haemodialysis which were used to manage patients with acute renal failure and poisoning in Johannesburg hospital and Cairo university hospital. Subsequently, three more countries Tunisia, Algeria and Kenya joined the league of countries offering dialysis (7). A rapid growth in number of countries have occurred over the years, a systematic review has shown the availability of maintenance haemodialysis in 32 African countries in 2007(8). However, sustainability of maintenance dialysis in most African countries has been hampered by the exorbitant cost of dialysis and limited man power. South Africa is one of the leading countries that is able to sustain its haemodialysis program through governmental support. Unfortunately, despite the availability of a kidney transplant program in South Africa, the majority of our patients remains dependent upon maintenance haemodialysis due scarcity of cadaveric kidneys and limited living donors (6). Therefore, it is paramount to identify potential modifiable risk factors of mortality in this population of patients as they await kidney transplant.

1.2.2 Fibroblast growth factor 23 and Chronic Kidney Disease

Since the discovery of fibroblast blast growth factor 23(FGF23) in 2000(9), this novel phosphaturic biomarker has gained global recognition and continues to be associated with several adverse clinical outcomes (10-12). Likewise, the discovery of FGF23 has led to further insights into the pathogenesis of chronic kidney disease – mineral bone disease (CKD-MBD), accounting for the paradigm shift from the classic trade off hypothesis to an updated trade off hypothesis (13).

Mortality remains unacceptably high in CKD patients and cardiovascular disease (CVD) is one of the leading causes of death in CKD populations, with CKD-MBD identified as an emerging entity responsible for the increased risk of CVD (14, 15). Hence, since FGF23 is one of the main regulators of the CKD-MBD axis, it became necessary to investigate its role as a risk factor for mortality in CKD populations.

1.2.3 Role of FGF23 in the pathogenesis of secondary hyperparathyroidism

The pathophysiology of secondary hyperparathyroidism has evolved with the discovery of FGF23. This has led to a paradigm shift from the classic trade off hypothesis to an updated trade off hypothesis as shown in figure 1.1 (13). Fibroblast growth factor 23 originates from osteocytes and has been considered to be a rate limiting factor in vitamin D and phosphate metabolism. It exerts its effect on target organs such as kidneys and parathyroid gland through Klotho which is a transmembrane protein (16). Fibroblast growth factor 23 stimulates excretion of phosphate via luminal sodium dependent phosphate channels in the proximal renal tubule. While in the intestine it inhibits phosphate absorption via NaPi cotransporter activity (17). Additionally, FGF23 also retards the production of 1, 25-dihydroxyvitamin D [1, 25(OH) $_{2D3}$] by inhibiting the activity of 1α-hydroxylase and conversely enhancing the activity of 24-hydroxylase (18). The ultimate effects of these activities by FGF23 lead to a triad of hypocalcaemia, low level of calcitriol and hyperphosphataemia that subsequently result to secondary hyperparathyroidism.

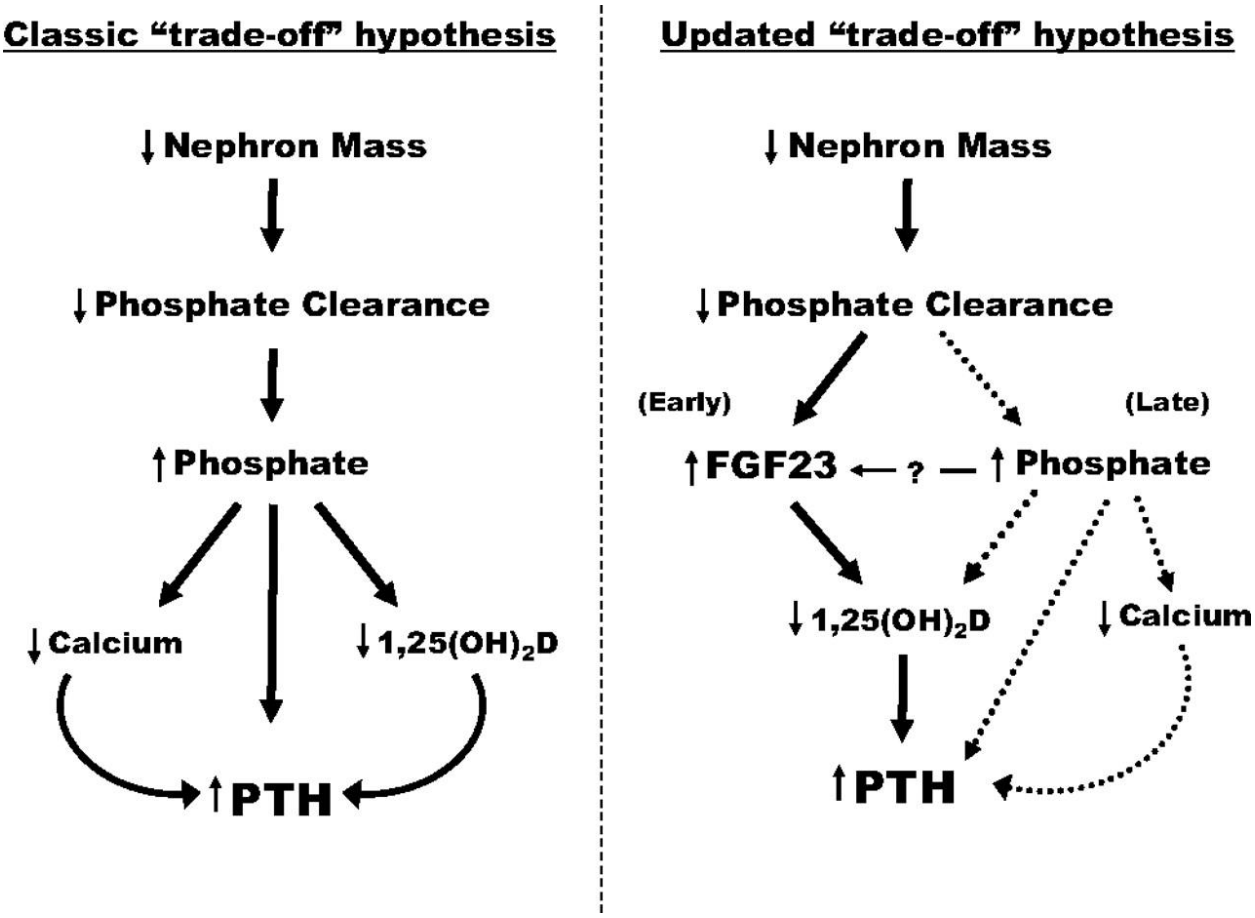


Figure 1. 1: Classic versus updated hypothesis for the evolution of CKD -MBD

1.2.4 Risk factors of mortality in patients on maintenance haemodialysis

Persons with stages 3-5 chronic kidney disease have three to thirteen times higher overall mortality than the general population (19). The factors responsible for this exceptionally high mortality in patients with ESKD on maintenance dialysis have been evaluated by several studies from the developed countries. Some studies have identified co morbid conditions (e.g. diabetes mellitus, cardiovascular conditions), nutritional factors and deranged markers of CKD- MBD as strong predictors of mortality in this group of patients (20). In a meta-analysis involving 86,915 patients from 23 studies, the findings revealed that age (per 1-year increment), diabetes mellitus, previous cardiovascular disease, had a significant harmful impact on the risk of both overall mortality and cardiac death in MHD patients (20). While some studies have revealed that worsening of

haematological indices is independently linked to high mortality in patients undergoing maintenance haemodailysis. For example, Ishii et al. showed that per 1 g/dL increment in the hemoglobin level there is a reduced risk of all-cause mortality (21).

Previous studies have also associated derangements of traditional markers of CKD-MBD with high incidence of mortality in patients on MHD (22). For example, elevated serum levels of phosphate, plasma parathyroid hormone and calcium have been strongly associated with cardiovascular mortality in patients with CKD. In a large, prospective, multicenter 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 maximum quartile of phosphate using both baseline and time-dependent values was reported (22). In another multicenter observational study in the USA, higher levels of alkaline phosphatase were associated with increased risk of death and hospitalization in haemodialysis patients (23).

However, despite attempts to modify some of these potential modifiable traditional risk factors, the mortality rate remains high and thus the need to further identify other non-traditional risk factors. Furthermore, most of these studies were largely conducted in developed countries and due to geographical variations and differences in practice pattern between the developed and developing countries it is unclear if the above aforementioned findings could be extrapolated to our patients. Thus, the need to embark on similar studies in Africa.

1.2.5 Fibroblast growth factor 23 and mortality in maintenance haemodialysis

Fibroblast growth factor 23 is a biochemical marker produced by the bony tissues and it is responsible for regulation of blood phosphate levels through the kidneys (17). It is measured in plasma or serum. A significant association between FGF23 and all-cause mortality was first reported by Gutierrez et al. In this study, involving incident haemodialysis patients from the US, a monotonic, dose–response relationship between FGF23 levels and mortality was reported; patients in the highest quartile of FGF23 had increased risk of death as compared to patients in the lowest quartile (10). Subsequent studies have revealed inconclusive findings, with some of the studies revealing a non-significant association between FGF23 and mortality (24, 25). The observed discrepancy in these studies was partly attributed to differences in assay methodology and whether intact or C terminal FGF23 was utilized. The intriguing complexity of assay

methodology is further compounded by racial variations in the levels of FGF23 as reported by prior studies (26, 27). For example, in a recent meta-analysis, a sub analysis based on race, FGF 23 assay type and dialysis modality showed variations in the association between elevated FGF-23 and mortality in haemodialysis patients (28).

Some of the studies that have reported a significant association between FGF23 and mortality include: the HOST (Homocysteine study) that showed that compared to patients with the minimum FGF23 levels, patients in the maximum quartile of baseline FGF23 had a hazard ratio of 2.58 for subsequent poor cardiovascular outcomes in univariable analysis. Elevated plasma levels of FGF23 remained an independent predictor of cardiovascular events, while vitamin D levels and plasma PTH were not. Regarding the composite cardiovascular clinical endpoint, elevated FGF23 was an independent predictor of myocardial infarction and amputation of lower extremity (29). These studies have also shown a link between markedly elevated levels of FGF23 in CKD, vascular calcification and mortality (29, 30). Similarly, in a Brazilian prospective study comprising type 2 DM patients with macroalbuminuric nephropathy, FGF23 was an independent predictor of the composite primary outcome defined as "death, doubling of baseline serum creatinine and/or need for dialysis, after adjustment for PTH and renal function (31).

On the other hand, a few studies have also reported a non-significant association between FGF23 and all-cause mortality. For example, Olauson et al, reported a lack of significant association between higher levels of FGF23 and increased mortality risk, when FGF23 was analyzed in quartiles and on a continuous scale (24). A similar trend was seen in a large cohort of patients with stage 3 CKD, where higher levels of FGF23 failed to be associated with all-cause mortality and progression to CKD (25).

1.2.6 Association between traditional markers of CKD -MBD and mortality

Abnormalities of markers of CKD-MBD have been consistently linked to poor clinical outcomes in patients with chronic kidney disease. This has led to emergence of several guidelines proposed by regional and global organization to assist clinicians in the management of CKD-MBD.

1.2.7 Parathyroid hormone and mortality in chronic kidney disease

Studies relating to intact PTH and mortality remain contradictory, while some studies have shown increased risk of death with elevated PTH, some have shown a non-significant association. The discrepancies between these studies may be attributed to different methodological issues, different cut off values used as reference points, study populations and practice patterns. For example, in a large European study comprising 8377 chronic haemodialysis participants, only intact PTH below the lower limit of KDIGO (<130pg/ml) target value was associated with lower survival, while PTH above the recommended upper limit (>585 pg/ml) was not predictive of mortality (32). In contrast to this finding, several studies have shown an increased risk of death with PTH above the upper limit. In the DOPPS study Tentori et al. found that iPTH level >600 pg/mL was an independent predictor of mortality (33). Similarly, Floege et al reported a 2-fold increase in risk of death with PTH > 600 pg/ml (34). The biological explanation for the strong association between PTH and mortality risk may likely be through a synergetic relationship with calcium. High levels of PTH will cause mobilization of excessive calcium from the bone which will in turn accelerate arterial calcification (14).

Similarly, findings relating to low PTH and patient's survival have been conflicting. For example, Floege et al (34) and Avram et al(35) reported increased risk of death with PTH levels below 75 pg/mL and 65 pg/mL, respectively. While on the other hand, some studies have shown increased risk of mortality with higher levels of PTH but not with lower levels.

Calcium and risk of mortality in patients with CKD

Several studies have consistently associated hypercalcaemia with increased risk for mortality in patients undergoing maintenance haemodialysis despite utilizing different cut off values of serum calcium (33, 36) . In a large multicenter study including the three phases of the dialysis outcomes and practice patterns study (DOPPSI, II and III) with 25,588 HD patients, calcium levels greater than10.0mg/dL (>2.5mmol/L) were significantly associated with higher risks for both cardiovascular and all-cause mortality (33). This consistent association is likely due to the fact that high levels of serum calcium play a vital role in arterial calcification, hence predisposing individuals with hypercalcaeamia to cardiovascular events which are the leading causes of death in patients with CKD (37).

Reports on the effect of low level of calcium on mortality has not been consistent. For instance, Block et al. (36) and Young et al. (38) reported survival advantage in patients with hypocalacaemia while Kalantar-Zadeh et al.(39) , Tentori et al.(33) and Floege et al.(34) reported increased risk of deaths with hypocalcaemia. The discrepant findings in these studies may be attributed to utilization of different cut off values for defining hypocalcaemia

1.2.8 Phosphate and risk of mortality in CKD patients

Several studies have reported increased risk of death with high and low levels of serum phosphate. In the COSMOS (Current management of Secondary hyperparathyroidism: a Multicentre Observational Study) involving 6797 haemodialysis patients from 20 European countries, reported a U- shaped relationship between serum phosphate and mortality (40),that is both low and high levels of phosphate were associated with higher risk of all -cause mortality. This finding was also consistent with the report from CORES study that showed increased risk of death with both high phosphorus level above >5.5 mg/dL and low phosphorus level below <3.0 mg/dL (41). These aforementioned large studies accounted for fluctuations in the levels of phosphate by using time dependent Cox models to assess the association between phosphate levels and all-cause mortality. Most of these studies have also served as a cornerstone for restriction phosphate and use of phosphate binders in patients with chronic kidney disease.
However, it is worth noting that most of these studies were largely from America, Asia and Europe. Hence, this current study will improve on the lack of data from sub -Saharan Africa relating to the effects of these biochemical markers on mortality in our CKD patients.

1.2.9 Alkaline phosphatase and risk of mortality in patients with CKD

A linear association between serum total alkaline phosphatase and mortality in healthy population and patients with chronic kidney disease has been reported by previous studies. The strong association between higher levels of total alkaline phosphatase and increased risk of death has been attributed to accelerated vascular calcification induced by elevated levels of total alkaline phosphatase via hydrolysis of pyrophosphate or activation of apatite crystal formation(42).

In Africa, the risk factors of mortality in patients undergoing haemodialysis are yet to be fully explored, with the majority of these studies focusing on traditional risk factors. Few if any studies have evaluated the association between FGF23 and mortality in Africa haemodialysis patients. Therefore, one of the objectives of this study is to determine the relationship between FGF23 and mortality in maintenance haemodialysis patients.

1.3 JUSTIFICATION FOR THE STUDY

More recently, studies from the developed countries have shown a strong association between elevated levels of novel marker of chronic kidney mineral bone disease known as fibroblast growth factor 23 (FGF23) and mortality in patients undergoing maintenance haemodialysis (MHD) (10, 30). However, In sub- Saharan Africa, no studies have investigated the existence of this association in MHD patients. Hence, one of the objectives of this study is to determine the association between FGF23 and mortality in South African patients on chronic dialysis. In addition, modification of traditional risk factors in patients with CKD has been shown to be less effective compared to the general population (43). Thus, identification of non-traditional risk factors and biomarkers such as FGF23 is crucial for achieving effective risk stratification strategy. Furthermore, the non-affordability of kidney transplant by most of the African chronic dialysis patients has made them dependent on chronic dialysis. Therefore, it is necessary to identify risk factors of mortality in this growing population of dialysis patients so as to improve their survival period.

1.4 RESEARCH QUESTION

Are higher plasma levels of intact fibroblast growth factor 23 associated with increased mortality in South African patients undergoing dialysis between October 2014 to December 2017.

1.5 AIM

To determine the association between fibroblast growth factor 23 and other risk factors of mortality in South African patients on maintenance dialysis.

1.5.1 Specific objectives

1. To determine a three year all-cause mortality in patients on maintenance dialysis
2. To determine the association between FGF23 and mortality in patients on maintenance dialysis.

3. To determine the association between traditional biochemical markers of chronic kidney disease mineral bone disease (serum phosphate, PTH, calcium) and mortality in patients on maintenance dialysis.

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2. SUBMISSIBLE MANUSCRIPT

Associations of Plasma Fibroblast Growth Factor 23, Calcium and Parathyroid hormone with All-Cause Mortality in South African Patients on Maintenance Dialysis: A 3-year Prospective Cohort Study.

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2.1 ABSTRACT

Introduction

Few studies have linked high levels of plasma C-terminal fibroblast growth factor 23 (FGF23) with poor clinical outcomes in patients on maintenance haemodialysis (MHD), while the association between intact FGF23 and mortality in this group of patients remains inconclusive.

Therefore, the aim of this study was to evaluate the association between plasma levels of intact FGF23 and mortality in dialysis patients.

Methods: A prospective multicenter study involving patients undergoing dialysis at three dialysis centres in Johannesburg was undertaken between 1st October 2014 and 31st December 2017.

Results

The study comprised 165 chronic dialysis patients (111 blacks, 54 whites) with a mean age of 46.6 ±14.2 years. During a three year follow up period, there were 46 deaths (1.03 per 100 person-years). The median plasma FGF 23 level was 382 pg/ml (interquartile range [IQR], 145-2977). In adjusted multivariable analyses, there was a non-statistically significant increase in the risk of mortality with higher quartiles of FGF 23 levels: the adjusted hazard ratios (HR) for the second, third and fourth quartiles were HR 3.20 (95% CI, 0.99-10.35; P=0.052), HR 2.43(95 % CI,0.65-9.09; P=0.19), and HR 2.09 (95% CI, 0.66-7.32; P= 0.25),respectively. Corrected serum calcium 2.38-2.5 mmol/l [HR 2.98 (95% CI, 1.07-8.29; P=0.04] and > 2.50 mmol/l [HR 5.50 (95% CI, 1.84-16.48; P=0.002] were independently associated with increased risk of death. Likewise, patients with intact parathyroid hormone > 600 pg/ml had a 3.46-fold higher risk of death (HR 3.46, 95% CI, 1.22-9.82 P=0.019). These findings persisted in time -dependent analyses.

Conclusion

Higher levels of intact FGF23 appear not to be independently associated with all-cause mortality in our dialysis patients, while hypercalcaemia and severe hyperparathyroidism were found to be independent predictors of mortality in this cohort of patients.

2.2 INTRODUCTION

Since the discovery of fibroblast blast growth factor 23(FGF23) in 2000[1], this novel phosphaturic biomarker has gained global recognition and continues to be associated with several adverse clinical outcomes[2-4]. Likewise, the discovery of FGF23 has led to further insights into the pathogenesis of chronic kidney disease – mineral bone disease (CKD-MBD), accounting for the paradigm shift from the classic trade off hypothesis to an updated trade off hypothesis[5].

The incidence of mortality remains unacceptably high in CKD patients, despite interventions on traditional risk factors and thus the need to identify other non-traditional risk factors for death in CKD patients, such as FGF23. Additionally, cardiovascular disease (CVD) is one of the leading causes of death in CKD populations, with CKD-MBD identified as an emerging entity responsible for the increased risk of CVD[6, 7]. Hence, since FGF23 is one of the main regulators of the CKD-MBD axis, it became necessary to investigate its role as a risk factor for mortality in CKD populations.

In 2008, Gutierrez et al. were the first to report a significant association between higher levels of FGF23 and mortality in a haemodialysis population. In this large US study, involving incident haemodialysis patients, a monotonic, dose–response relationship between FGF23 levels and mortality was reported; patients in the highest quartile of FGF23 had increased risk of death compared to patients in the lowest quartile[2]. However, these findings were not consistent nor replicated in all the subsequent studies, with some of the studies revealing a non-significant

association between FGF23 and mortality [8, 9]. The observed discrepancy in these studies was partly attributed to differences in assay methodology and whether intact or C terminal FGF 23 was utilized. The C- terminal enzyme-linked immunosorbent assay (ELISA) can recognize both the full length FGF23 and the C-terminal fragments obtained from FGF23 proteolysis. While the intact FGF23 assay detects the full length FGF23 molecule [10]. The results obtained by both assays methodology varied and thus lack of harmonization of these assays make it difficult to make comparisons across different studies. The intriguing complexity of assay methodology is further compounded by racial variations in the levels of FGF 23 as reported in prior studies [11, 12]. For example, in a recent meta-analysis, a sub analysis based on race, FGF 23 assay type and dialysis modality showed variations in the association between elevated FGF-23 and mortality in haemodialysis patients [13]. The pathophysiological explanation behind the reported association between FGF23 and mortality remains unclear. Some of the proposed mechanisms include a possible direct cytotoxic effect of excess FGF23 on the myocardium leading to left ventricular hypertrophy and arterial calcification through phosphorus and vitamin D regulation [3, 14, 15]. Similarly, since the regulation of CKD-MBD by FGF23 is through an interplay with other known traditional markers of CKD-MBD such as calcium, phosphate and PTH, this current study will further explore the relationships between these markers of CKD-MBD and mortality.

Furthermore, studies largely from Europe, America and Asia have also linked calcium, parathyroid hormone, phosphate to increased risk of mortality in patients undergoing dialysis [16, 17]. While in Africa, the risk factors of mortality in patients undergoing haemodialysis are yet to be fully explored, with most studies focusing on traditional risk factors such as diabetes mellitus, hypertension, dyslipidaemia and anaemia[18, 19]. Few if any studies have evaluated the association between FGF23 and mortality in African patients on dialysis. Therefore, this study

aimed to determine the relationship between FGF23, traditional markers of CKD-MBD and mortality in patients on dialysis; and tested the hypothesis that high level of plasma FGF23 is expected to be an independent predictor of all -cause mortality in South African patients on maintenance haemodialysis.

2.3 MATERIALS AND METHODS

This was a prospective multicenter study involving patients undergoing chronic haemodialysis and peritoneal dialysis at three dialysis centers in Johannesburg between October 2014 and December, 2017. Eligible patients were aged 18 years and above with no active malignancy or a history of parathyroidectomy.

Information obtained included participants‘ socio-demographic characteristics, history of comorbid conditions as diabetes mellitus, usage of CKD-MBD medications, aetiology of end stage kidney disease (ESKD) and blood pressure measurements. Determination of race was based on self-report by the participants. Although patients with active illness such as heart failure were excluded at baseline, development of other co morbid conditions such as coronary artery disease, stroke or myocardial infarction could not be ascertained during the follow up period.

2.3.1 Laboratory measurements

Detailed blood sample collections and test methodologies as previously published [12], are briefly described below:

Blood samples were collected at baseline for measurements of FGF23 and other biochemical markers of CKD-MBD. Plasma PTH, serum calcium and phosphate were repeatedly measured at intervals of three months. Results of six months post enrollment into the study were used for time varying analyses.

“Plasma FGF23 was measured using a sandwich-based enzyme-linked immunosorbent assay kit from EMD Millipore Corporation (Billerica, MA, USA); lower limit of detection was 3.2 pg/ml. The intra and inter assay coefficients of variation (CVs) were < 11%.

Plasma intact PTH was measured by an electrochemiluminescence immunoassay (ECLIA) run on a Cobas 6000 auto analyzer (Roche Diagnostics, Mannheim, Germany). The intra and inter assay coefficients of variation (CVs) were <2% and <3.4%, respectively.

Plasma 25 hydroxyvitamin D (25-OHD) was measured using the high-performance liquid chromatography (HPLC) kit (Recipe, Munich, Germany). 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).

Serum calcium, phosphate and alkaline phosphatase were measured using the ADVIA 1800 centaur auto analyzer; Siemens Diagnostics, Tarrytown, USA”.

2.3.2 Primary outcome and exposure variables

The primary endpoint was all-cause mortality, events other than this endpoint such as kidney transplantation, loss to follow up and still on dialysis at end of the study were censored. The primary exposure variable of interest was intact FGF23. Secondary exposure variables were traditional markers of CKD-MBD (phosphate, calcium, PTH, bone specific alkaline phosphatase, and 25-OHD).

In line with previous studies [2, 20], plasma FGF23 was categorized into quartiles based on 25, 50 and 75 percentile distribution and using levels lower than the 25 percentile as the reference value. Similarly, due to the lack of recommendations for clinical cut-off values for bone specific alkaline phosphatase (BSALP) and in line with a previous study [21], participants were categorized based

on 25, 50 and 75 percentile distribution and the interquartile range was taken as the reference value. The use of the interquartile range as the reference point, was due to the fact that both low and high levels of BSALP have been associated with low and high bone turnover [22].

Plasma Intact PTH, serum calcium and phosphate were categorized based on the Kidney Disease Improving Global Outcomes (KDIGO) recommended target values and in line with a previous study [16, 23]. Briefly, the KDIGO guidelines recommend maintaining intact PTH in the range of two to nine times the upper normal limit for the assay, while serum calcium and phosphate should be maintained within the normal laboratory reference values.

The exposure variables of interest were further modelled on a continuous scale.

Model 1 adjusted for age, calcium, phosphate, Bone Specific alkaline phosphate, intact PTH and 25-hydroxyvitamin D. Model 2 in addition to variables in model 1, further adjusted for race, gender, diabetes status, use of calcium carbonate, alfacalcidol, dialysis modality and vintage.

2.3.3 Ethical consideration

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

2.3.4 Statistical analysis

Descriptive statistics were used to characterize study participants. Comparison of baseline parameters between patients that died and survived was done using Chi square, unpaired t tests and Mann- Whitney tests for categorical outcomes, and normally distributed and non-normally distributed continuous variables respectively. We also used one way ANOVA and Kruskal -Wallis tests to compare participants' parameters across quartiles of FGF23, PTH and calcium. A Cox

regression model was initially fitted with baseline fixed variables (calcium, phosphate and PTH) and then repeated as time varying covariates. Eligibility of variables into the Cox multivariable regression model was determined using a stepwise regression strategy; a backward elimination method was used to include variables with specified P values less than 0.20 into the model. Variables that are known to be plausibly associated with death in CKD were assessed for eligibility and forced into the model even if their P values were >0.2. Plasma FGF23 and PTH were log transformed and then introduced along with other exposure variables into the Cox model on a continuous scale. The nonlinear association between the explanatory variables and all-cause mortality was explored using a fractional polynomial. The proportional hazards assumption was checked using Schoenfeld residuals test. The Schoenfeld global test Chi square was 18.96 with a p value of 0.65 and the individual p values for the fitted Cox models were >0.05. Hence, we failed to reject the null hypothesis that states no violation of proportional-hazards assumption.

We further explored whether race modified the effects of our primary exposure variable on all-cause mortality by adding interaction terms of race with categories of FGF23 into the model. This model was compared to model 2 (fully adjusted model) using the likelihood-ratio test.

Model 1 adjusted for age, calcium, phosphate, Bone Specific alkaline phosphate, intact PTH and 25-hydroxyvitamin D.

Model 2 in addition to variables in model 1, further adjusted for race, gender, diabetes status, use of calcium carbonate, alfacalcidol, dialysis modality and vintage.

All analyses were performed using STATA version 12 (STATA Corp., TX, USA).

2.4 RESULTS

The study comprised 165 dialysis patients (111 blacks, 54 whites) with a mean age of 46.5 ±14.2 years. Most of the study participants were on CKD –MBD related medications: calcium carbonate (66. 7 %) and Alfacalcidol (63.0 %) (Table 2.1).

The mean serum phosphate was higher in patients within the highest quartile of FGF23 and this increased in ascending order across the quartiles. Other baseline parameters were comparable across the quartiles of FGF23 (Table 2.1).

As shown in the supplementary S1 Table, Patients with PTH within KDIGO recommended target values tend to be older.

Patients with serum calcium within the range of 2.38-2.51 mmol/l had significantly higher levels of plasma PTH. Other characteristics were comparable across the quartiles of serum calcium (S2 Table).

Table 2. 1: Participants' characteristics by quartiles of plasma Fibroblast growth factor 23

Variables	All(N=165)	Intact FGF23 Quartiles (pg/ml)				P Value
		<148 (n=42)	148-382 (n=40)	383-2977 (n=41)	>2977 (n=42)	
Age (years)	46.6±14.2	46.2±13.9	49.6±15.7	47.7±14.2	42.7±11.6	0.16
Gender n(%)						
Male	90 (54.5)	22(52.4)	19(47.5)	23 (56.1)	26 (61.9)	0.39
Female	75(45.5)	20(47.6)	21(52.5)	18(43.9)	16 (38.1)	
Race n(%)						
Black	111(67.3)	32(76.2)	27 (67.5)	28 (68.3)	24 (57.1)	0.18
White	54(32.7)	10(23.8)	13 (32.5)	13 (31.7)	18 (42.9)	
Dialysis Vintage(months)	61(43-96)	59(42-91)	59(39-108)	68(42-102)	62(48-85)	0.91
DM n(%)	13 (7.9)	3 (7.1)	2 (5.0)	3 (7.3)	4 (9.5)	0.83
Systolic BP(mmHg)	143±26	144±27	141±22	145±28	143±26	0.91
Diastolic BP (mmHg)	86±23	93±30	83±19	83±21	86±16	0.25
Hb (g/dl)	10.8±2.1	10.8±2.4	11.2±2.0	10.5±1.8	10.5±2.4	0.39
T.cholesterol (mmol/l)	4.30±1.43	4.26±0.95	4.20±1.35	4.20±1.41	4.53±1.96	0.80
Calcium (mmol/l)	2.21±0.28	2.14±0.27	2.14±0.29	2.27±0.15	2.27±0.33	0.03
IPTH(pg/ml)	750(268-1359)	534(226-1193)	694(213-1034)	784(312-1736)	888 (406-1560)	0.11

Phosphate (mmol/l)	1.52±0.55	1.23±0.34	1.39±0.51	1.58±0.52	1.89±0.60	<0.001
BSALP (IU/L)	15.8±5.5	16.4±5.4	15.6±5.0	15.3±4.7	15.6±6.2	0.87
Albumin (g/dl)		36.3±6.9	37.2±4.7	35.7±5.3	35.9±7.1	0.74
25-OHD (ng/ml)	27.7±13.6	27.6±15.1	24.5±11.9	31.9±14.2	26.0±13.5	0.09
Alfacalcidol n(%)	104 (63.0)	24(57.1)	25(62.5)	28(68.3)	27(64.3)	0.30
$CaCo_3$ n(%)	110(66.7)	26(61.9)	28(70.0)	26(63.4)	30(71.4)	0.47

T=Total; BP= Blood pressure; FGF23= Fibroblast growth factor; BSALP=Bone specific alkaline phosphatase; PTH=Parathyroid hormone, 25-OHD= 25 - hydroxyvitamin D; CKD-MBD-Chronic kidney disease- mineral bone disease; Caco3 =Calcium carbonate, continuous variables are reported as means± standard deviations or medians (interquartile ranges)

During a follow up period of 3 years with a mean time of 27±9 months, 46 deaths (1.03 per 100 person- years) occurred, 11 (6.7%) patients had a successful kidney transplant and only 2 (1.2 %) patients were lost to follow-up.

The baseline clinical characteristics of the study participants by race are shown in Table 2.2.

In comparison to Whites, Blacks were significantly younger with higher median intact PTH, mean 25-OHD and lower median plasma FGF23 levels .

Table 2. 2: Study participants characteristics by race

Variable	All(N=165)	Black(n=111)	White(n=54)	P Value
Age (years)	46.6±14.2	42.8±11.5	54.4±16.0	<0.001
Outcome n (%)				
Dead	46 (27.9)	27 (24.3)	19 (35.2)	0.14
Alive	119 (72.1)	84 (75.7)	35(64.8)	
SBP (mmHg)	143±26	142±24	146±29	0.47
Hb (g/dl)	10.8±2.1	10.7±2.2	11.0±2.0	0.32
T. Cholesterol (mmol/l)	4.30±1.43	4.28±1.44	4.36±1.43	0.79
Calcium (mmol/l)	2.21±0.28	2.18±0.31	2.27±0.21	0.06
Albumin (g/L)	36.1±6.7	36.6±6.8	35.8±4.1	0.51
Phosphate (mmol/l)	1.52±0.55	1.44±0.57	1.72±0.45	0.003
BSALP (U/L)	15.8±5.3	16.1±5.7	15.0±4.2	0.19
PTH (Pg/ml)	750 (268-1359)	856(314-1636)	440 (161-890)	0.003
25-OH vit D (ng/ml)	27.7±13.6	29.9±13.7	22.3±12.6	<0.001
FGF 23 (pg/mL)	382 (145-2977)	322 (105-2543)	970 (187-3777)	0.01
CKD-MBD med n(%)				

Alfacalcidol	104(63.0)	74(66.7)	30(55.5)	0.41
Calcium carbonate	110(66.7)	77(69.3)	33 (61.1)	0.13

T=Total; SBP= Systolic Blood pressure; Hb= Haemoglobin; FGF23= Fibroblast growth factor; BSALP=Bone specific alkaline phosphatase;

PTH=Parathyroid hormone, 25-OHD= 25 -hydroxyvitamin D; CKD-MBD-Chronic kidney disease- mineral bone disease.

2.4.1 FGF23 and mortality

In comparison to patients in the lowest quartile of FGF23, patients in the second, third and fourth quartiles had non-statistically significant increased unadjusted hazard ratios (HR) in the univariable regression analysis. Although the HR became accentuated in the multivariable Cox analysis, it still failed to reach statistical significance. The fully adjusted hazard ratios (HR) for the second, third and fourth quartiles were HR 3.20 (95% CI, 0.99-10.35; P=0.052), 2.43 (95 % CI,(0.65-9.09; P=0.19, and HR 2.09 (95% CI, 0.66-7.32; P= 0.25), respectively (Table 2.3, model 2).

Table 2. 3: Fixed and time varying Hazard ratios for all-cause mortality

Fixed Hazard ratios for all- cause mortality						
Variable	Unadjusted HR(95 CI)	P-value	Adjusted HR (95%CI) Model 1	P-value	Adjusted HR(95 CI) Model 2	P-value
FGF 23						
<148	1.00 (reference)		1.00(reference)		1.00(reference)	
148-382	1.80 (0.76-4.23)	0.18	2.31(0.87-6.08)	0.09	3.20(0.99-10.35)	0.052
383-2977	1.49 (0.63-3.48)	0.36	1.11(0.41-3.02)	0.83	2.43(0.65-9.09)	0.19
>2977	1.28 (0.52-3.15)	0.60	1.11(0.39-3.12)	0.85	2.09(0.66-7.32)	0.25
Calcium						
<1.80	1.45 (0.49-4.27)	0.50	1.68(0.51-5.50)	0.39	1.08(0.20-5.35)	0.93
1.80-2.10	0.50 (0.15-1.69)	0.27	0.75(0.21-2.71)	0.66	0.98(0.25-3.77)	0.98
2.11-2.37	1.00 (reference)		1.00(reference)		1.00(reference)	
2.38-2.50	2.21(1.05-4.66)	0.04	3.32(1.35-8.15)	0.009	2.98(1.07-8.29)	0.04
>2.50	2.94(1.17-7.40)	0.02	5.14(1.88-14.03)	0.001	5.50(1.84-16.48)	0.002
PTH						
<130	1.03(0.22-4.87)	0.97	1.05(0.30-3.73)	0.94	1.15(0.29-4.63)	0.96
≥130-≤600	1.00		1.00(reference)		1.00(reference)	
>600	2.23(1.03-4.85)	0.04	2.77(1.09-7.08)	0.03	3.46(1.22-9.82)	0.019
Phosphate						
<1.15	0.80(0.35-1.81)	0.59	1.50(0.57-3.91)	0.42	2.76(0.93-8.19)	0.07
1.14-1.75	1.00		1.00(reference)		1.00(reference)	

>1.75	1.41(0.72-2.78)	0.32	2.35(1.09-5.06)	0.03	1.64(0.60-4.51)	0.33
BSALP						
<12	1.87(0.68-5.14)	0.23	3.20(0.99-10.28)	0.051	1.64(0.44-6.12)	0.46
12-15	1.00(reference)		1.00(reference)		1.00(reference)	
>15	2.37(0.98-5.71)	0.055	2.91(1.12-7.54)	0.028	2.12(0.77-5.87)	0.15
Time Dependent Hazard ratios						
FGF 23						
<148	1.00(reference)		1.00(reference)		1.00(reference)	
148-382	1.80(0.76-4.23)	0.18	2.49(0.99-6.21)	0.051	2.61 (0.68-9.91)	0.16
383-2977	1.49(0.63-3.48)	0.36	1.54(0.61-3.89)	0.36	1.89(0.48-9.91)	0.36
>2977	1.28(0.52-3.15)	0.60	1.48(0.57-3.84)	0.42	2.06(0.64-6.57)	0.69
Calcium						
<1.80	2.02(0.69-5.92)	0.20	2.34(0.72-7.64)	0.16	2.56 (0.63-10.25)	0.18
1.80-2.10	0.80(0.33-2.09)	0.71	1.19(0.45-3.88)	0.72	0.96 (0.31-2.94)	0.94
2.11-2.37	1.00(reference)		1.00(reference)		1.00 (reference)	
2.38-2.50	1.70(0.72-4.02)	0.23	2.03(0.72-5.74)	0.18	1.62 (0.51-5.22)	0.46
>2.50	4.54(2.05-10.05)	<0.001	4.59(1.82-11.62)	0.001	6.44 (2.24-18.49)	0.001
PTH						
<130	1.02(0.12-8.29)	0.99	1.73(0.41-7.25)	0.45	1.15(0.23-5.69)	0.87
≥130-≤600	1.00(reference)		1.00(reference)		1.00(reference)	
>600	3.02(1.34-6.82)	0.008	3.51(1.36-9.07)	0.009	3.75(1.39-10.07)	0.009
Phosphate						
<1.15	0.81(0.38-1.72)	0.58	1.54(0.64-3.68)	0.33	2.13 (0.56-8.16)	0.26
1.15-1.75	1.00(reference)		1.00(reference)		1.00 (reference)	
>1.75	1.15(0.59-2.22)	0.68	1.35(0.65-2.83)	0.43	1.30 (0.42-3.99)	0.65
BSALP						
<12	1.87(0.68-5.14)	0.23	2.47(0.81-7.53)	0.11	1.23(0.35-4.43)	0.72
12-15	1.00(reference)		1.00(reference)		1.00(reference)	
>15	2.37(0.98-5.71)	0.055	2.61(1.03-6.34)	0.04	1.92(0.69-5.23)	0.20

HR= Hazard ratio, PTH=Parathyroid hormone, BSALP= Bone Specific Alkaline Phosphate, FGF23=Fibroblast growth factor 23

Model 1 adjusted for age, calcium, phosphate, Bone Specific alkaline phosphate, intact PTH and 25-hydroxyvitamin D.

Model 2 in addition to variables in model 1, further adjusted for race, gender, diabetes status, use of calcium carbonate, alfacalcidol ,dialysis modality and vintage.

Since categorizing explanatory variables may be associated with loss of information, further examination of FGF23 on a continuous scale did not reveal a statistical significant association at both univariable (HR per unit increase in natural logFGF23, 1.04; 95 % CI , 0.87-1.25, P=0.86) and multivariable Cox regression analyses (HR per unit increase in natural logFGF23, 1.02; 95 % CI , 0.81-1.29, P=0.54)(Table 2.4).

Table 2. 4: Hazard ratios for all-cause mortality factoring explanatory variables on a continuous scale

Variable	Univariable	P Value	Adjusted HR (95%CI) model 1	P value	Adjusted HR (95%CI) Model 2	P Value
Log FGF23	1.04(0.87-1.25)	0.86	0.90 (0.71-1.14)	0.38	1.02 (0.81-1.29)	0.86
Log PTH	2.26(1.26-4.04)	0.006	3.13 (1.54-6.37)	0.002	2.21 (1.07-4.55)	0.03
Calcium	3.01(1.005-9.61)	0.049	5.13 (1.24-21.09)	0.02	5.34 (1.11-25.73)	0.04
Phosphate	1.06(0.96-1.18)	0.24	2.27 (1.11-4.64)	0.03	2.12 (1.02-4.42)	0.045
BSALP	1.01(0.96-1.07)	0.62	1.02 (0.97-1.09)	0.38	1.03 (0.96-1.10)	0.41

FGF23=Fibroblast growth factor 23, BSALP= Bone Specific Alkaline Phosphatase, CI= Confidence interval, HR= Hazard ratios

Model 1 adjusted for age, calcium, phosphate, Bone Specific alkaline phosphate, intact PTH and 25-hydroxyvitamin D.

Model 2 in addition to variables in model 1, further adjusted for race, gender, diabetes status, use of calcium carbonate, alfacalcidol and dialysis modality

Fig 2.1 represents the Kaplan Meir survival curves by different quartiles of FGF23; there is no statistically significant difference in the survival functions across the quartiles (log rank P >0.05).

Fig2.1. Kaplan Meier survival curves for different quartiles of plasma Fibroblast growth factor 23

2.4.2 Calcium and mortality

Participants whose calcium levels were outside the laboratory normal range had increased risk of death. The HRs for baseline serum calcium values 2.38-2.5 mmol/l and > 2.5 mmol/l remained persistently high and significant throughout the models(1 and 2): the final model 2 adjusted HR for serum calcium 2.38-2.5 mmol and > 2.50 mmol were HR 2.98 (95% CI, 1.07-8.29; P=0.04) and HR 5.50 (95% CI, 1.84-16.48; P=0.002), respectively (Table 2.3). Fig2.2 represents the

Kaplan Meier survival curves by different quartiles of serum calcium; patients with serum calcium ≥2.38 mmol has worst survival function.

Fig2.2. Kaplan Meier survival curves for different quartiles of serum calcium

Although the association between mortality and hypocalcaemia is not significant, the overall relationship seems to take a U- shaped pattern (Figs 2.4 and 2.5).

Fig2.4. Hazard ratio of all- cause mortality for baseline serum calcium using fractional polynomial

Fig2.5. Hazard ratio of all-cause mortality for time dependent serum calcium using fractional polynomial.

Similarly, when calcium is being considered as a time varying covariate, the increased risk of death with calcium remains unchanged (Table 2.3).

The significant association between serum calcium and all-cause mortality remains sustained even when serum calcium was treated as a continuous exposure variable [HR per unit increase in calcium; 5.34 (95 % CI, 1.11-25.73), P= 0.04] (Table 2.4).

The fractional polynomial analysis furthers confirms this association and the U-shaped relationship (Figs2. 4 and 2.5)

2.4.3 Intact PTH and mortality

In the fully adjusted Cox regression model 2, participants with intact PTH above the upper limit of the KDIGO recommended target value versus those within the target values had a 3.46 fold higher risk of death (HR 3.46, 95% CI, 1.22-9.82 P=0.019).This relationship persisted when

PTH was considered as a time varying co-variate (HR 3.75, 95% CI, 1.39-10.07, P=0.009 (Table 2.3).

The increased risk of death with higher levels of PTH remained unaltered when PTH was considered as a continuous exposure variable, HR per unit increased in log transformed PTH, 2.21; 95% CI, 1.07-4.55, P=0.03 (Table 4). Fig2.3 represents the Kaplan Meier survival curves by categories of intact parathyroid hormone.

Fig2.3. Kaplan Meier survival curves for categories of intact Parathyroid hormone

2.4.4 Phosphate and risk of mortality

In the baseline fixed model 1 that adjusted for markers of CKD –MBD, patients with high phosphate levels had increased risk of death compared to those with phosphate within the normal range, HR 2.35 (95% CI, 1.09-5.06; P=0.03). This finding became attenuated and non-significant in the fully adjusted model 2, HR 1.64 (95% CI, 0.60-4.51; P=0.33). This finding is also not consistent with time dependent analysis, which showed a non- significant association between higher levels of phosphate in both model 1 and model 2. However, when phosphate was analyzed as a continuous variable, increasing levels of phosphate were significantly associated with an increased risk of death in both model 1 [HR per unit increase in phosphate; 2.27 (95 % CI, 1.11-4.64), P= 0.03] and model 2 [HR per unit increase in phosphate; 2.12 (95 % CI, 1.02-4.42), P= 0.045] (Table 2.4).

2.4.5 Bone specific alkaline phosphatase and mortality

In model 1 that adjusted for age and other biochemical markers of CKD MBD, patients with BSALP > 15 IU/L versus 12-15 IU/L had a 2.91fold significantly greater risk of death (HR 2.91, 95% CI, 1.12-7.54; P=0.028). This significant association did not persist in the fully adjusted model (HR 2.12, 95 % CI,0.77-5.87; P=0.15).

2.4.6 Race, FGF23 and mortality

In the fully adjusted model including an interaction between FGF 23 and race, using black patients with FGF 23 lower than the overall median value as the reference, white participants with FGF 23 above the median value had similar HR with black patients with FGF 23 above the median value (HR 3.24, 95% CI, 070-15.05; p interaction=0.13 versus HR 3.28, 95% CI, 0.65-16.52; p interaction=0.15).

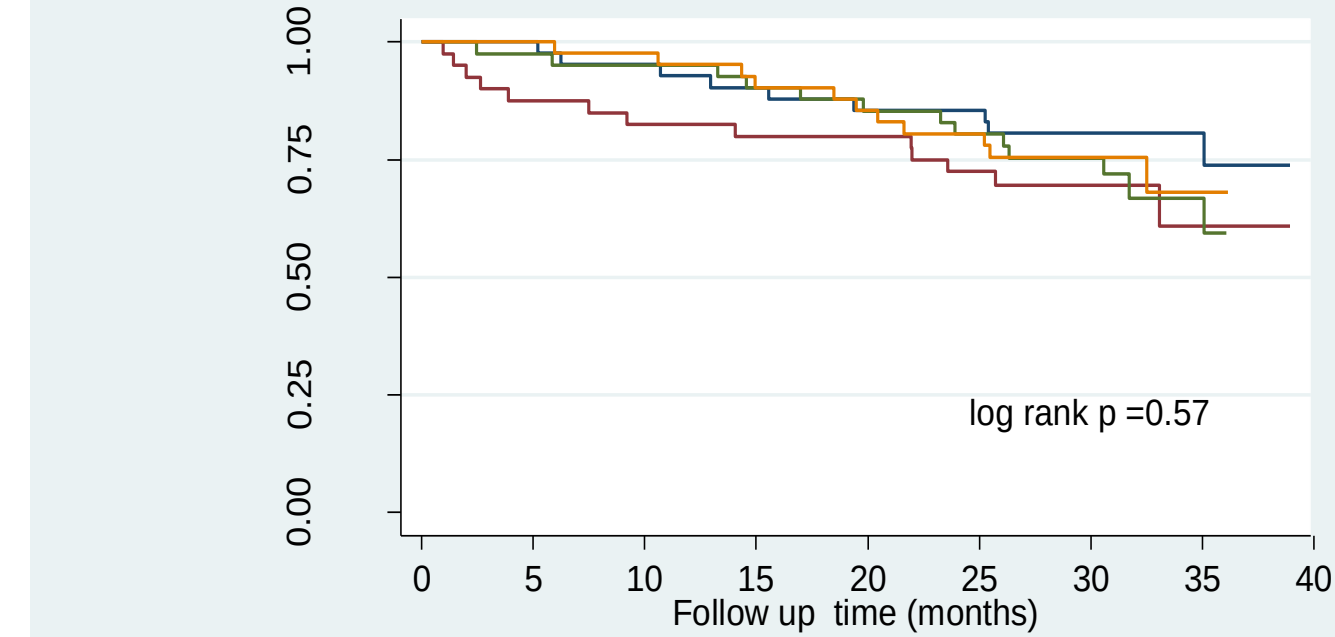


Figure 2. 1: Kaplan Meier survival curves for different quartiles of plasma Fibroblast growth factor 23

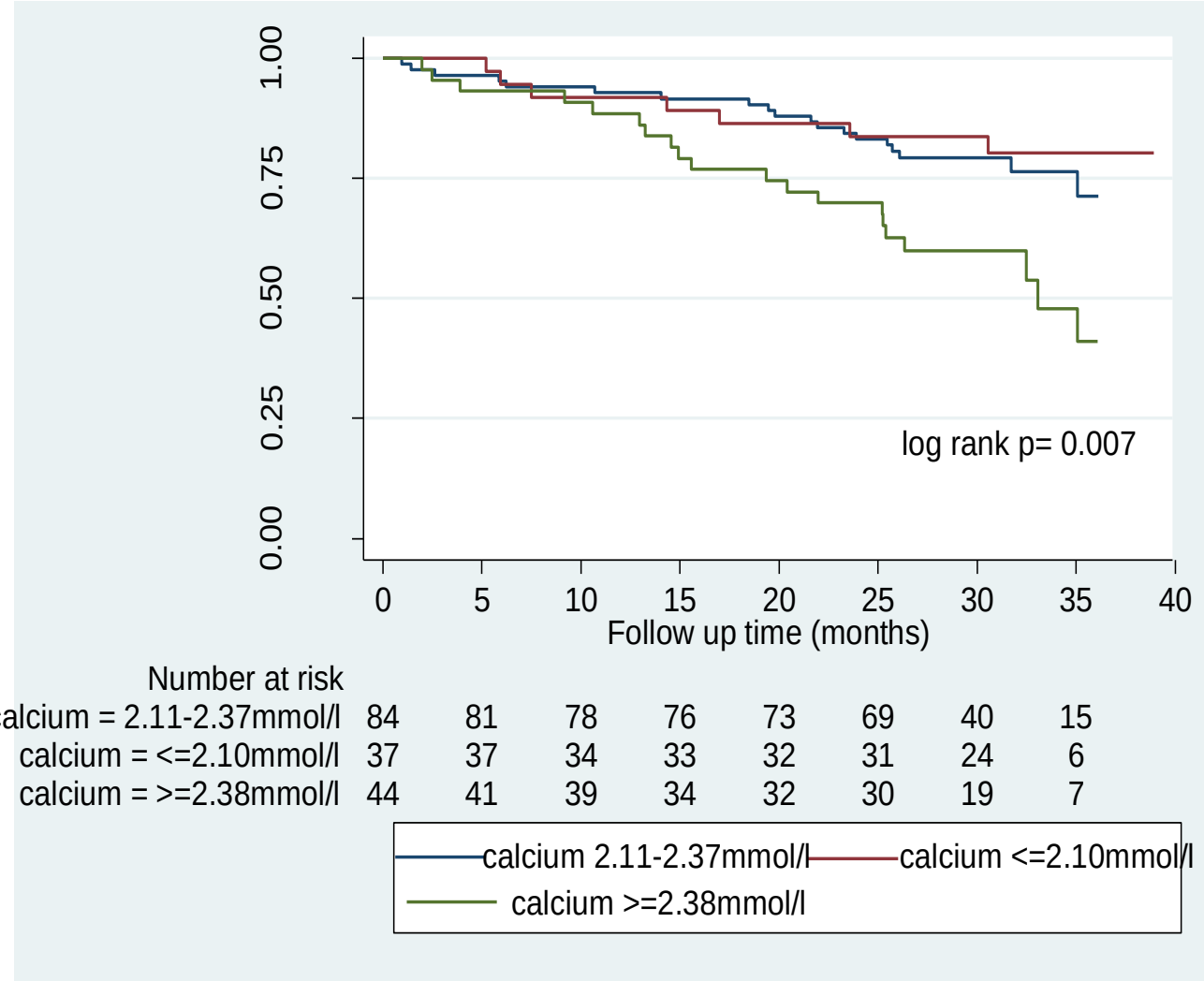


Figure 2. 2: Kaplan Meier survival curves for different quartiles of serum calcium

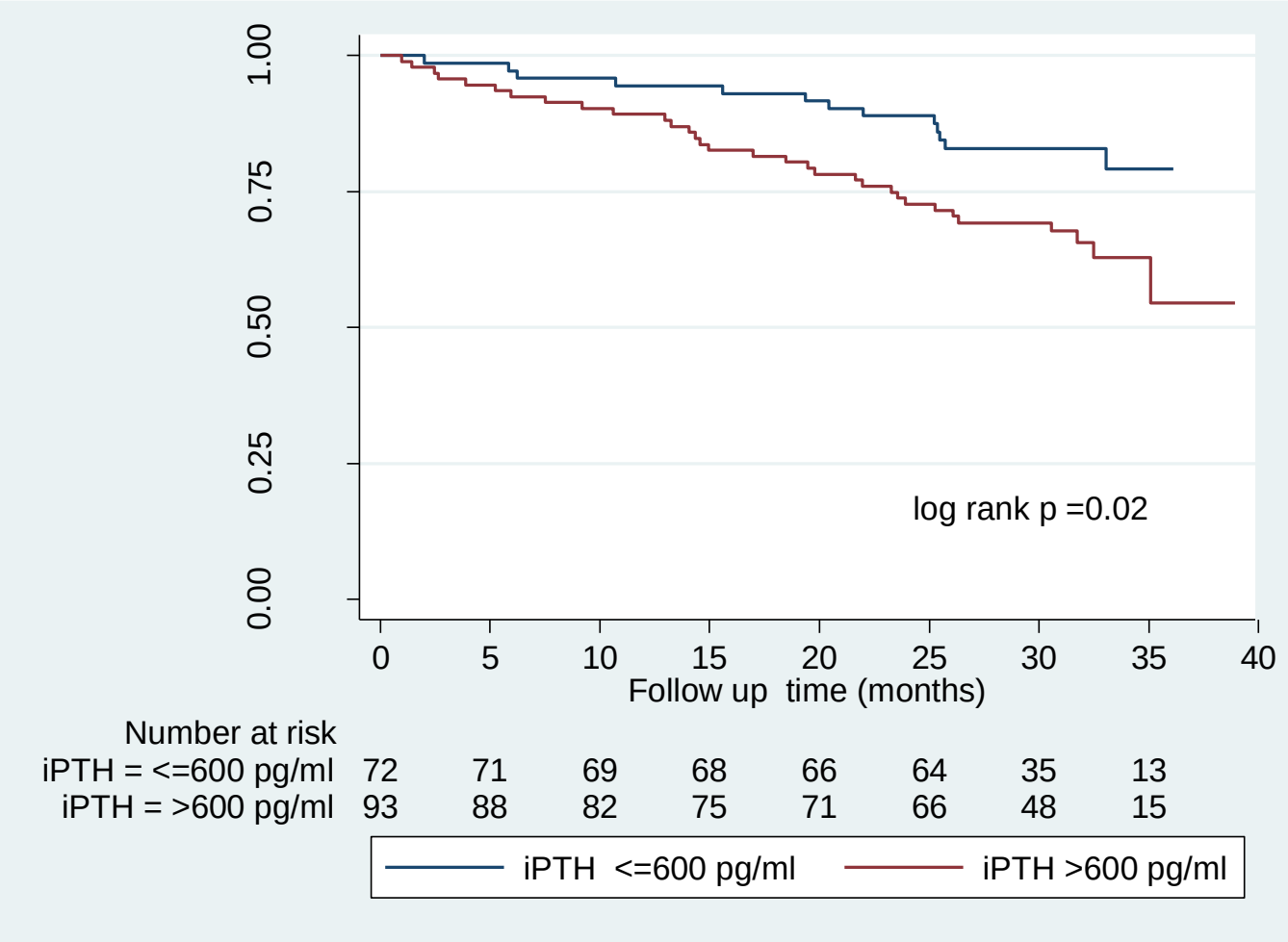


Figure 2. 3: Kaplan Meier survival curves for categories of intact Parathyroid hormone

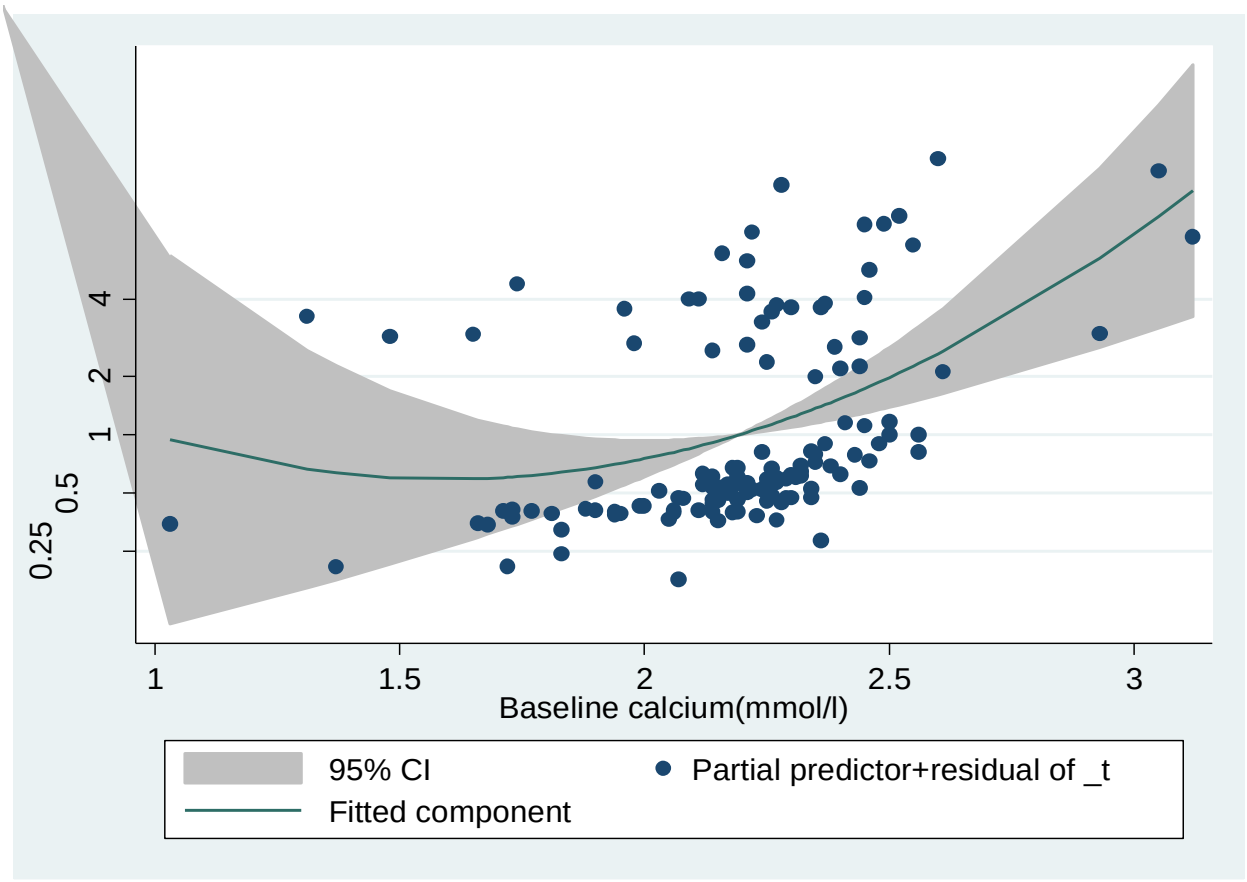


Figure 2. 4: Hazard ratio of all- cause mortality for baseline serum calcium using fractional polynomial

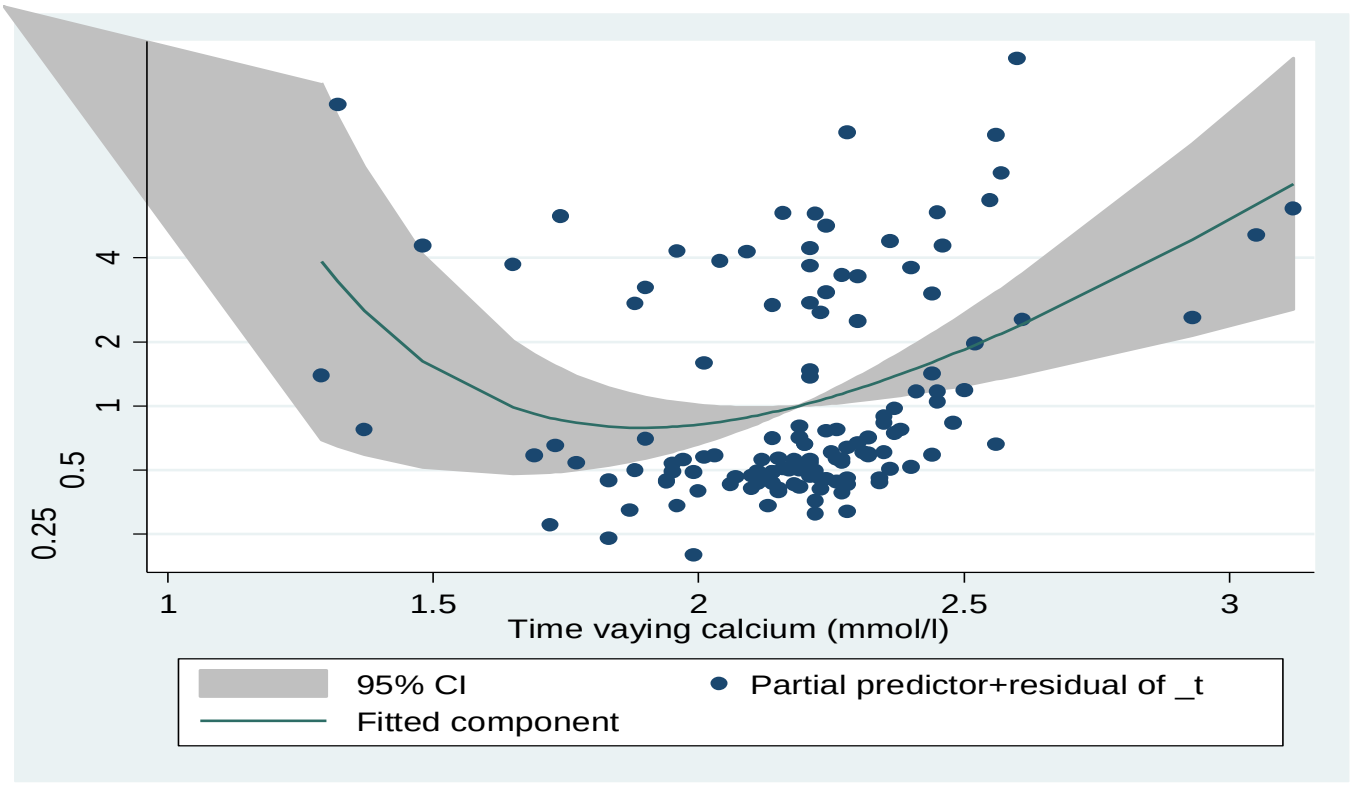


Figure 2. 5: Hazard ratio of all-cause mortality for time dependent serum calcium using fractional polynomial

2.5 DISCUSSION

In this prospective African study with diverse ethnicity (Blacks and Whites) on maintenance dialysis, higher levels of intact FGF 23 levels did not seem to predict all-cause mortality. Conversely, higher levels of calcium and severe hyperparathyroidism were independently associated with all-cause mortality.

Previous studies relating to the association between FGF23 and clinical adverse outcomes have yielded conflicting results. Our findings of a lack of significant association between intact FGF23 levels was contrary to some previous studies [2, 14]. The discrepancy may partly be attributed to the use of different FGF23 assay methodologies and study populations. Although some of these studies similarly utilized intact FGF 23, it was reported that cardiovascular risk factors may be

more specific with C- terminal FGF23[24]. For example, in a recent meta-analysis cumulative HR for c- terminal revealed a 2.3-fold increased risk of all-cause mortality, while higher levels of intact FGF 23 were not significantly associated with mortality [13].

Although this meta-analysis excluded Gutierrez's study in the pool analysis because odds ratios was reported as the measure of association with the outcome, the study utilized both intact FGF23 and C-terminal FGF23 assays revealing a strong linear correlation and similar findings with the two assays[2]. While some studies have shown a significant disagreement between the two assays [25, 26].

In addition to the use of different assay methodology, the impact of high levels of FGF23 also varied across races. For instance, in the same meta-analysis, no association was found between level of FGF23 and mortality in the Asian populations (HR: 0.95; 95% CI: 0.37–2.43), while in non- Asian population elevated FGF23 was significantly associated to mortality (HR: 1.69; 95% CI: 1.11–2.57) [13].

A similar trend in the variation of consequences of disordered markers of CKD -MBD across races was reported by the Multi-Ethnic Study of Atherosclerosis (MESA); in which 25-OHD deficiency was associated with an increased risk of coronary heart diseases in whites but not in blacks[27]. These contrasting findings may partly be explained by racial variations in the levels of FGF23 and some markers of CKD-MBD such as PTH, 25-OHD, and phosphate. For example, similar to this current study, other previous studies have shown that blacks have lower levels of FGF23 and higher PTH levels compared with whites [2, 11, 28].

The overall mechanisms proposed for the excess risk in mortality with elevated levels of C-terminal FGF 23 are unclear. Some of the repeatedly documented mechanisms, though remaining

speculative, include the direct cytotoxic effects of excess FGF23 on the myocardium leading to left ventricular hypertrophy, endothelial dysfunction, and arterial calcification [14, 15]. The controversy relating to these postulated modes of action was compounded by the absence of a coreceptor (Klotho) in the cardiovascular system which is needed by FGF23 to exert its effects on these tissues [29]. For example, in Chronic Renal Insufficiency study, Scialla et al reported that baseline FGF23 was not associated with arterial calcification, and also noted the absence of mRNA expression for FGF23 and Klotho in both human and mouse vascular tissue [29].

Although some researchers have proposed that the effect of FGF23 on the cardiovascular system is mediated through Klotho independent pathways [4, 30], the controversies relating to its mechanism prevail.

In agreement with our findings, previous studies have also reported that higher levels of FGF23 do not predict mortality in haemodialysis patients. Olauson et al, whose study population was similar to the present study comprising both haemodialysis and peritoneal dialysis patients, reported the lack of a significant association between higher levels of FGF23 and increased mortality risk, when FGF23 was analyzed in quartiles and on a continuous scale [8]. A similar trend was seen in a large cohort of patients with stage 3 CKD, where higher levels of FGF 23 failed to be associated with all-cause mortality and progression to CKD [9].

As we have previously published a survival advantage and a significant lower level of FGF 23 in our black CKD patients compared to white patients [12, 31], we further explored if there is an interaction between FGF23 levels, race and mortality risk. Similar to previous studies [14, 32], in this current study, black race did not modify the effect of FGF23 on mortality.

Our finding of a significant association between high levels of serum calcium and increased mortality risk is consistent with the findings of previous studies [16, 33]. Floege et al have also shown that hypercalcemia is a potent predictor of mortality in an European dialysis population and this finding was consistent when calcium was analyzed as fixed baseline and time varying covariates[16]. In line with our study, their findings were reinforced by modelling serum calcium as a continuous exposure variable using the fractional polynomial method. This limits loss of information associated with categorizing explanatory variables. Although cause and effect associations are difficult to be established with observational studies, the biological plausible explanation for the poor survival rate with high levels of calcium may be linked to the acceleration of arterial calcification by excess calcium.

Of note, we adjusted for the use of CKD-MBD medications (alfacalcidol and calcium carbonate) which have been associated with levels of serum calcium and phosphate, in addition to an inconclusive evidence that these medications may modify mortality in patients on haemodialysis [34].

Reports relating to PTH and mortality remain contradictory, while some studies have shown increased risk of death with elevated PTH, some have reported mixed results. The discrepancies between these studies may be related to different methodological issues, different cut off values used as reference points, study populations and practice patterns. For example, in a large European study comprising 8377 chronic haemodialysis participants, only intact PTH below the lower limit of KDIGO (<130pg/ml) target value was associated with lower survival, while PTH above the recommended upper limit (>585 pg/ml) was not predictive of mortality [35]. In contrast to this finding, several studies including this current study have shown an increased risk of death with PTH above the upper limit. In the DOPPS study Tentori et al. found that iPTH level >600 pg/mL

was an independent predictor of mortality [36]. Similarly, Floege et al reported 2-fold increase in risk of death with PTH > 600 pg/ml [16]. The biological explanation for the strong association between PTH and mortality risk may likely be through a synergetic relationship with calcium. High levels of PTH will cause mobilization of excessive calcium from the bone which will in turn accelerate arterial calcification [6]. Moreover, the leading cause of mortality in dialysis patients has been repeatedly attributed to cardiovascular disease, with emerging evidence that arterial calcification may largely be responsible [6]; hence fulfilling some of the Brad Hill criteria of causation of an event.

However, despite the reported increased mortality risk with high levels of PTH, an available randomized controlled trial did not show a significant improvement in mortality with lowering PTH level. The trial titled "Evaluation of Cinacalcet Hydrochloride Therapy to Lower Cardiovascular Events (EVOLVE)" showed a non-significant 7 % reduction in the risk of death with the use of cinacalcet to lower PTH level in patients with moderate to severe secondary hyperparathyroidism [37]. Thus, more randomized controlled trials are needed to ascertain the benefits of lowering PTH to recommended target values.

Another notable finding in this current study was the association between phosphate and increased risk of death when phosphate was factored into the model as a continuous variable, but not on categories. This further reiterates the fact that when exposure variables are analyzed only based on categories may erroneously lead to wrong conclusions. Hence, the need to further explore the relationship on a continuous scale as shown in this current study. This association is not surprising in the light of several studies that have consistently associated higher levels of phosphate with increased cardiovascular disease and all- cause mortality [33, 38]. This was reinforced by studies that reported improvement in survival with the use of phosphate binders to lower phosphate in

patients with advanced CKD [39, 40]. Similarly, Sciella et al in an experimental study reported that elevated phosphate induced arterial calcification in vitro and that FGF23 played no role in the phosphate uptake during the process of the calcification [29].

Limitations of our study include relatively small sample size probably precluding detection of a significant association between higher levels of FGF23 and mortality risk. However, as highlighted earlier, some studies with larger sample size with more events of interest also did not detect a significant association. FGF23 was measured at a single point in time, and since some exposures change over time, we were unable to model FGF23 as a time varying covariate. However, a recent study that assessed longitudinal FGF 23 trajectories and risk of mortality in CKD patients showed that plasma FGF23 levels were stable over time in the majority of their study cohort [41]. Additionally, despite attempt to adjust for potential confounders, we still could not have accounted for other residual confounding variables. Finally, we could not ascertain specific causes of death. Meanwhile, the strengths our study include the diverse study population involving both black and white South African populations, and patients on both peritoneal and haemodialysis. Hence, allowing comparison of data not only between Black Africans and Black Americans but also between Whites in Africa and USA/Europe. In addition, previous studies relating to FGF23 were largely from US, Europe and Asia and thus this current study has given insights on the burden of FGF23 and traditional markers of CKD-MBD in an African dialysis population.

Repeated measurements of the traditional markers of CKD- MBD have allowed us to account for variations of these markers on the impact of our primary endpoint over a period of time. Furthermore, modelling our explanatory variables on a continuous scale has further reinforced our derived findings based on categorical variables.

In conclusion, high levels of plasma PTH and serum calcium independently predicted all- cause mortality in diverse South African ESKD patients on dialysis, while FGF23 was not significantly associated with all- cause mortality in this cohort. These findings further reinforce the need to continuously pay attention in addressing traditional markers of CKD- MBD.

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3. EXTENDED METHODS AND RESULTS

3.1 STUDY DESIGN, POPULATION AND SITES

This was a secondary analysis of a cohort of patients undergoing chronic haemodialysis and peritoneal dialysis at two dialysis centers in Johannesburg between October 2014 and December 2017.

3.1.1 Study sample

A total of 165 eligible patients on maintenance dialysis were enrolled into the study between October 2014 and March 2015**.** Baseline demographic, clinical characteristics, plasma FGF23 and biochemical markers of mineral bone disorders were obtained at enrollment. The obtained data were entered and saved in Excel sheet**.** Hence, secondary analysis of these data was carried out by this present study.

3.1.2 Determination of sample size

Sample size was calculated based on the general formula for survival analysis as stated below:

$N= 2E/2-P_1-P_2$

Where E= total number of events required

P_1= Survival probability in group 1

P_2= Survival probability in group 2

$E=(HR +1) \times f(\alpha\beta)/(HR-1)2$

Where $HR= \log p_1/\log P_2$

Alpha (α)= the probability of Type I error=0.05

β= 1-power

For this study, Stata was used to calculate the sample size required to achieve 80% power to detect 50% reduction in a hazard of death in the group with normal levels FGF23 using a two-sided 0.05

level log-rank test. Thus β= 1-power =1-0.8=0.2 and effect size a = 0.5. Assuming 40% in the group with high levels of FGF23 survived till the end of the study, obtained from previous study (1). Hence, using the stpower log rank 0.4 command in Stata, a total of 148 patients are required to power this study. An additional 10% of the total sample size was added to account for lost to follow-up. Therefore, a final total of 165 patients were enrolled into the study.

Additional statistical analysis

The survival function at time t is denoted by S(t) and this is the probability that a patient will survive past time t and derived mathematically from the equation; S(t) =Pr(T > t) = 1−F(t), with the assumption that survival time T is a continuous random variable with probability density function f(t) and cumulative distribution function F(t).

While the hazard function at time t is denoted by h(t), is the instantaneous risk of an event at time t, given that it had not yet occurred. This is also derived by the equation below;

$$\boldsymbol{h(t) = \lim_{\delta t \to 0} \left\{ \frac{P(t \leq T < t + \delta t | T \geq t)}{\delta t} \right\}}$$

While the Cox regression hazard model used in this study to evaluate simultaneously the impact of several factors (covariates) on survival was obtained through the equation below:

The hazard is assumed to be h(t) =h0(t) exp(β1x1+⋯+βkxk)

Where:

t is the survival time

h(t)= the hazard function which is determined by a set of covariates ($x1,x2,...,xk$) ,

while the coefficients ($\beta 1, \beta 2,..., \beta k$) measure the effect size of the covariates.

h0 is the baseline hazard and corresponds to the value of the hazard if all the *xi* are equal to zero.

To establish a possible nonlinear exposure-response relation between serum calcium and the primary outcome (all-cause mortality), fractional polynomials plot was used. Briefly; fractional polynomial (FP) assumed the mathematical equation as shown below:

$$y_i = \beta_0 + \beta_1 * x_i + \beta_2 * x_i^{(p1)} + \beta_3 * x_i^{(p2)} + u_i$$

Hence in this study, all-cause mortality= $\beta_0 + \beta_1 * x_i + \beta_2 * \text{Calcium}^{(p1)} + \beta_3 * \text{Calcium}^{(p2)} + u_i$

$p^1, p^2, ...$ were exponents obtained from {-2, -1, -0.5, 0, 0.5, 1, 2, 3}. The general rule is that $x^0 = \ln(x)$. Thus, the model FP (1, 0, -2) is $y_i = \beta_0 + \beta_1 * x_i + \beta_2 \ln * \text{Calcium} + \beta_3 * 1/\text{Calcium}^2$.

When the exposure variables were considered as continuous variables (Table 4), collinearity between the exposure variables that were included into the final model was checked using variance inflation factors through the command estat vif following regression analysis . Since the command could not be executed following Cox regression, a linear regression analysis was conducted and then the command was used to check the VIF.

All the exposure variables included the final model had their VIF less than 1.05 and the mean VIF was 1.01.

See do files in the appendix for commands used to execute all the necessary statistical analyses.

3.1.3 Study sites

The Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) is located in Johannesburg, Gauteng, South Africa. It is one of the main teaching hospitals for the University of the

Witwatersrand, Faculty of Health Sciences. The dialysis centre provides both haemodialysis and peritoneal dialysis.

Wits Donald Gordon is a private teaching hospital affiliated to the University of the Witwatersrand involved in cutting edge research and providing highly specialized services for the residents of South Africa and its environs. Most of the attending physicians at the CMJAH are also involve in the management of patients at the Wits Donald Gordon Medical center.

3.1.4 Inclusion Criteria

Patients with established ESKD on maintenance dialysis, aged eighteen years and above were enrolled into the study.

3.1.5 Exclusion criteria

Patients with active liver disease, having active malignancies or a history of parathyroidectomy were excluded.

3.2 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. An additional ethics clearance certificate was obtained for the secondary analysis of the primary data; clearance certificate number M180149.

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3.3 BLOOD COLLECTION AND LABORATORY MEASUREMENTS

Detailed blood sample collections and test methodologies as previously published (2), are further described below:

Blood samples were collected at baseline for measurements of FGF23, phosphate, serum calcium, bone specific alkaline phosphatase, total alkaline phosphatase, hemoglobin, total cholesterol,

albumin, creatinine and urea. Plasma PTH, serum calcium and phosphate were repeatedly measured at intervals of three months.

3.4 TEST METHODOLOGIES

Fibroblast growth factor 23 was measured using a sandwich enzyme-linked immunosorbent assay kit made by EMD Millipore Corporation (Billerica, MA, USA); assay lower limit of detection was 3.2 pg/ml. 50 µL of plasma samples were pipetted into wells containing 100 µL of conjugate antibody. These wells were sealed and left on microplate shaker to incubate for 2 hours. The solutions within the wells were decanted and washed off three times with a diluted buffer. Detection antibody was further added to wells and allowed to incubate for another one hour. The washing of the wells with a buffer was repeated three times following addition of 100 µL of enzyme solution with another 30 minutes incubation period. Lastly, 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).

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

Plasma 25 hydroxyvitaminD was measured using the high-performance 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).

Serum calcium, phosphate and alkaline phosphatase were usually measured using the ADVIA 1800 centaur auto analyzer (Siemens Diagnostics, Tarrytown, USA).

In alkaline medium calcium ions react 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.

Extended results

Table 1 shows a comparison of baseline participants' characteristics by the primary outcome. Patients that died had a higher mean age than the survivors (51.3 vs 44.8; p=0.008). Other baseline parameters were comparable between the two groups. As shown in Table 3.2, Patients with PTH within KDIGO recommended target values tend to be older.

Patients with serum calcium within the range of 2.38-2.51 mmol/l had significantly higher levels of plasma PTH. Other characteristics were comparable across the quartiles of serum calcium (Table 3.3).

Table 3. 1: Baseline characteristics of patients by outcome status

Variable	All(N=165)	Survived (n=119)	Died (n=46)	P Value
Age (years)	46.6±14.2	44.8±13.3	51.3±15.4	0.008
Gender n (%)				
Male	90 (54.6)	65 (54.6)	25 (54.4)	0.98
Female	75 (45.7%)	54 (45.4)	21(45.7)	
DM status n (%)	13 (7.9 %)	9(7.6)	4 (8.7)	0.06
Race n (%)				
Black	111(67.3 %)	84(70.6)	27 (58.7)	0.14
White	54(32.3)	35(29.4)	19 (41.3)	
SBP (mmHg)	143±26	142±24	146±29	0.47
Hb (g/dl)	10.8±2.1	10.9±2.3	10.5±1.8	0.35
T. Cholesterol (mmol/l)	4.30±1.43	4.28±1.44	4.36±1.43	0.79
Calcium (mmol/l)	2.21±0.28	2.18±0.25	2.28±0.33	0.05
Albumin (g/L)	36.12±6.69	36.67±7.07	34.63±5.86	0.09
Phosphate (mmol/l)	1.52±0.55	1.49±0.55	1.63±0.53	0.13
Alkaline phosphate(IU/L)	129(89-198)	129 (90-192)	123 (83-211)	0.92
BSALP (U/L)	15.8±5.3	15.6±5.6	16.4±15.1	0.39
PTH (Pg/ml)	750 (268-1359)	672 (248-1216)	842 (482-1590)	0.07
25-OH vit D (ng/ml)	27.7±13.6	27.9±13.0	27.2±15.2	0.77
FGF23 (pg/mL)	382 (145-2977)	374 (118-3152)	390 (181-2572)	0.63
CKD-MBD med n(%)				

Alfacalcidol	104(63)	77(64.7)	24(52.2)	0.23
Calcium carbonate	110(66.7)	82(68.9)	28 (60.9)	0.77
Dialysis modality n (%)				
Haemodialysis	103(62.4)	71(59.7)	32(69.6)	0.24
Peritoneal dialysis	62(37.6)	48(40.3)	14(30.4)	

T=Total; DM= diabetes mellitus; SBP= Systolic Blood pressure; Hb= Haemoglobin; FGF23= Fibroblast growth factor; BSALP=Bone specific alkaline phosphatase; PTH=Parathyroid hormone, 25-OHD= 25 -hydroxyvitamin D; CKD-MBD-Chronic kidney disease- mineral bone disease

Table 3. 2: Participants' characteristics by quartiles of intact Parathyroid hormone

Variables	All=165	Intact PTH quartiles (Pg/ml)			P Value
		<130 (n=29)	≥130-≤600 (n=45)	>600 (n=91)	
Age (years)	46.6±14.2	42.0±15.6	50.6±16.1	45.7±12.2	0.03
Gender n (%)					
Male	90 (54.5)	18 (62.1)	25 (55.6)	47 (51.6)	0.46
Female	75(45.5)	11(37.9)	20 (44.4)	44 (48.4)	
Race n (%)					
Black	111 (67.3)	18 (62.1)	22 (48.9)	71 (78.00	0.001
White	54 (32.7)	14(48.3)	23 (51.1)	48(52.7)	
Dialysis Vintage (months)	61(43-96)	85(49-123)	55(39-82)	62(42-97)	0.09
DM status n(%)	13 (7.9%)	2(6.9)	6 (1.3)	5(5.5)	0.29
Hb (g/dl)	10.8±2.1	10.6±2.3	11.0±2.2	10.7±2.1	0.66
T.Cholesterol (mmol)	4.30±1.43	3.74±1.10	4.08±1.27	4.50±1.52	0.08
FGF23 (pg/ml)	382(145-2977)	244(101-1398)	317(89-1566)	716(184-3475)	0.07
Calcium (mmol/l)	2.21±0.28	2.29±0.28	2.17±0.24	2.20±0.28	0.18
Phosphate (mmol/l)	1.52±0.55	1.52±0.51	1.39±0.54	1.59±0.56	0.15
25-OHD (ng/ml)	27.7±13.6	30.8±12.9	26.1±14.8	27.5±13.6	0.39
BSALP (U/L)	15.8±5.5	16.2±4.6	16.1±5.4	15.5±5.5	0.79
Albumin (g/L)	36.1±6.6	39.6±6.0	35.1±5.5	36.1±6.0	0.55

CKD MBD meds n (%)					
Alfa calcidol	104 (63.0)	18 (62.1)	25(55.6)	60(65.9)	0.38
Calcium carbonate	110 (66.7)	18 (62.1)	30 (66.7)	62 (68.1)	0.75

T=Total; DM= diabetes mellitus; Hb= Haemoglobin; FGF23= Fibroblast growth factor; BSALP=Bone specific alkaline phosphatase;

PTH=Parathyroid hormone, 25-OHD= 25 -hydroxyvitamin D; CKD-MBD-Chronic kidney disease- mineral bone disease, meds=medications.

Table 3. 3: Participants' characteristics by quartiles of serum calcium

		Calcium quartiles (mmol)					
Variables	All=165	<1.80 (n=13)	1.80-2.10(n=24)	2.11-2.37 (n=84)	2.38-2.51 (n=24)	>2.50 (n=20)	P Value
Age (years)	46.6±14.2	40.5±12.5	43.4±12.5	47.5±12.5	47.9±8.9	45.6±14.2	0.35
Gender n (%)							
Male	90 (54.5)	8(61.5)	15(62.5)	46(54.8)	12(50.0)	9(45.0)	0.79
Female	75(45.5)	5 (38.5)	9(37.5)	38(45.2)	12(50.0)	11(55.0)	
Race n (%)							
Black	111 (67.3)	12 (92.3)	19(79.2)	53(63.1	15(62.5)	12(60.0)	0.28
White	54 (32.7)	1 (7.7)	5 (20.8)	31(36.9)	9(37.5)	8(40.0)	
Dialysis Vintage (months)	61(43-96)	54(44-144)	53(42-90)	66(48-96)	59(39-96)	87(49-125)	0.58
DM status	13 (7.9)	1(7.7)	1(4.2)	7(8.3)	3(12.5)	1(4.2)	0.69
Hb (g/dl)		9.9±2.2	10.5±2.0	11.0±2.3	10.3±1.7	11.4±2.2	0.26
T.Cholesterol (mmol/l)	4.30±1.43	4.47±1.46	4.26±1.49	4.31±1.46	4.53±1.17	3.73±1.18	0.70
FGF23 (pg/ml)	382 (145-2977)	254(74-3881)	244(122-1242)	337(115-2977)	1707(989-3475)	603(103-36791)	0.14
PTH (pg/ml)	750(268-1359)	1097(805-1690)	719(226-1331)	689(297-986)	1718(506-1889)	129(32-1287)	0.02
Phosphate (mmol/l)	1.52±0.55	1.65±0.54	1.42±0.39	1.51±0.54	1.53±0.43	1.56±0.51	0.79
25-OHD (ng/ml)	27.7±13.6	23.4±11.4	24.7±10.0	27.1±13.2	33.5±17.6	32.0±16.2	0.09
BSALP(U/L)	15.8±5.5	16.6±7.0	16.1±4.4	15.5±5.4	16.4±4.4	15.3±3.2	0.79

Albumin (g/dl)	36.6±6.6	33.6±8.7	36.2±7.2	35.9±5.4	38.2±5.9	39.2±4.9	0.12
CKD-MBD meds n (%)							
Alfacalcidol	104(63.0)	10 (76.9)	16(66.7)	49(58.3)	15(62.5)	14(58.3)	0.65
Calcium carbonate	110(66.7)	11(84.6)	19(79.2)	50(59.5)	17(70.8)	13(54.2)	0.06

T=Total; DM= diabetes mellitus;; Hb= Haemoglobin; FGF23= Fibroblast growth factor; BSALP=Bone specific alkaline phosphatase; PTH=Parathyroid hormone, 25-OHD= 25 -hydroxyvitamin D; CKD-MBD-Chronic kidney disease- mineral bone disease, meds= medications

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4. CONCLUSIONS AND RECOMMENDATIONS

High levels of plasma PTH and serum calcium independently predicted all- cause mortality in a diverse South African population with ESKD on dialysis, while FGF23 was not significantly associated with all- cause mortality in this cohort.

In the fully adjusted Cox regression model, in comparison to the lowest quartile of FGF23 the HR for the second, third and fourth quartiles were not significantly associated with all-cause mortality. Few if any studies have evaluated the association between FGF23 and mortality in African patients on dialysis. Hence, this study has given us an insight on the possible effect of FGF23 on mortality in our South African patients on dialysis. Findings relating to the association between this novel biomarker and mortality remained inconclusive. While some studies have reported a significant association between higher levels of FGF 23 and mortality (1, 2), a contrary non-significant association was reported by other studies (3, 4).

Corrected serum calcium 2.38-2.5 mmol/l [HR 2.98 (95% CI, 1.07-8.29; P=0.04] and > 2.50 mmol/l [HR 5.50 (95% CI, 1.84-16.48; P=0.002] were independently associated with increased risk of death. Similarly, patients with intact parathyroid hormone > 600 pg/ml had a 3.46-fold higher risk of death (HR 3.46, 95% CI, 1.22-9.82 P=0.019). The biological explanation for the strong association between PTH and mortality risk may likely be through a synergetic relationship with calcium. High levels of PTH will cause mobilization of excessive calcium from the bone which will in turn accelerate arterial calcification.

These findings further reinforce the need to continuously pay attention in addressing traditional markers of CKD- MBD.

The observed significant variations in the levels of FGF23 and PTH between blacks and whites highlighted the need for race specific target ranges for these biochemical markers of CKD-MBD.

This was further supported by studies that reported racial differences in the consequences of the abnormal levels of these markers (5, 6).

Another notable finding was the association between phosphate and increased risk of death when phosphate was factored into the model as a continuous variable, but not on categories. This

further reiterates the fact that when exposure variables are analyzed only based on categories may erroneously lead to wrong conclusions. Hence, the need to further explore the relationship on a continuous scale as shown in this current study. This association is not surprising in the light of several studies that have consistently associated higher levels of phosphate with increased cardiovascular disease and all- cause mortality [32, 37]. This was reinforced by studies that reported improvement in survival with the use of phosphate binders to lower phosphate in patients with advanced CKD [39, 40]. Similarly, Sciella et al in an experimental study reported that elevated phosphate induced arterial calcification in vitro and that FGF23 played no role in the phosphate uptake during the process of the calcification [29].

Strengths and limitations of the study

The strengths and limitation of this study were highlighted broadly based on sample size, study population, confounding variables and methods of measuring exposure and outcome variables. Hence, limitations include relatively small sample size probably precluding detection of a significant association between higher levels of FGF23 and mortality risk. However, as highlighted earlier, some studies with larger sample size and more events of interest also did not detect a significant association.

A single point time measurement of FGF23 could not allow modelling of this primary exposure as a time varying covariate. However, a recent study that assessed longitudinal FGF23 trajectories and risk of mortality in CKD patients showed that plasma FGF23 levels were stable over time in most of their study cohort. Although rigorous attempt using multiple Cox regression analysis to adjust for potential confounders was used, residual confounding variables may still remain unaccounted for. Finally, we could not ascertain specific causes of death. On the other hand, the strengths our study include the diverse study population involving both black and white South African populations, and patients on both peritoneal and haemodialysis. Hence, allowing comparison of data not only between Black Africans and Black Americans but also

between Whites in Africa and USA/Europe. In addition, previous studies relating to FGF23 were largely from US, Europe and Asia and thus this current study has given insights on the burden of FGF23 and traditional markers of CKD-MBD in an African dialysis population.

Repeated measurements of the traditional markers of CKD-MBD have allowed us to account for variations of these markers on the impact of our primary endpoint over a period. Furthermore, modelling our explanatory variables on a continuous scale has further reinforced our derived findings based on categorical variables.

4.1 RECOMMENDATIONS

Attention should be paid to controlling calcium, phosphate and PTH levels in our chronic dialysis patients. In an attempt to control phosphate levels in these patients most of them are on calcium containing phosphate binders which in turn predispose them to hypercalcaemia. Therefore, we recommend avoidance of these medications in patients with hypercalcaemia.

We further re emphasize regular measurements of serum calcium, phosphate and PTH levels. This will allow early identification of derangements of these markers of CKD-MBD leading to prompt management and improvement of our patients' survival.

Larger studies are recommended through multi center studies to further evaluate the non - significant association between FGF23 and all-cause mortality in African chronic dialysis patients. Furthermore, these larger multi center studies from Africa may lead to change in practice patterns rather than adopting global guidelines which were largely derived based on studies from Europe, America and Asia.

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APPENDICES

Plagiarism Form



PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Bala Waziri_____ (Student number: 1111800) am a student

registered for the degree of ____Master of Science_____ in the academic year __2017_____.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

Signature:  Date: 28/03/2019

Ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Dr Bala Waziri

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M180149

NAME: (Principal Investigator)	Dr Bala Wazini
DEPARTMENT:	Epidemiology and Biostatistics School of Public Health Charlotte Maxeke Johannesburg Academic Hospital Wits Donald Gordon Medical Centre (WDGMC)
PROJECT TITLE:	Association between Fibroblast Growth Factor 23 and Mortality in South African Chronic Kidney Disease Patients on Haemodialysis
DATE CONSIDERED:	26/01/2018
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Eustasius Musenge
APPROVED BY:	Professor CB Pepper, Chairperson, HREC (Medical)
DATE OF APPROVAL:	31/01/2018

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 Research Office Secretary in Room 301, Third Floor, Faculty of Health Sciences, Philip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. 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**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in January and will therefore be due in the month of January each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Patients Data sheet

Project: Association between fibroblast growth factor 23 and mortality inSouth African chronic kidney disease patients on haemodialysis

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 _____

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

8. Presence of 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. Smoking cigarette? (a) Yes (b) No

12.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) _____

14. lost to follow up (a) Yes (b) No

15. Had kidney transplant (a) Yes (b) No, if yes when _____

Clinical Parameters

1) Weight (Kg) _____

2) Height **(**m**)** _____

3) BMI (Kg/m^2) _____

4) Blood Pressure (mmHg)_____

Investigations biochemical parameters

Parathyroid hormone (PTH)_____

Fibroblast growth factor 23 (FGF23) _____

Albumin_____Calcium_____

Phosphate_____ Ca× PO_4_____

Bone specific alkaline phosphate (BAP) _____

Osteocalcin _____ 1, 25-OH vitamin D_____

25-OH vitamin D_____ Serum Creatinine (µmol/L)_____

Urea (mmol/L)_____Total Cholesterol_____

LDL_____HDL_____TG _____

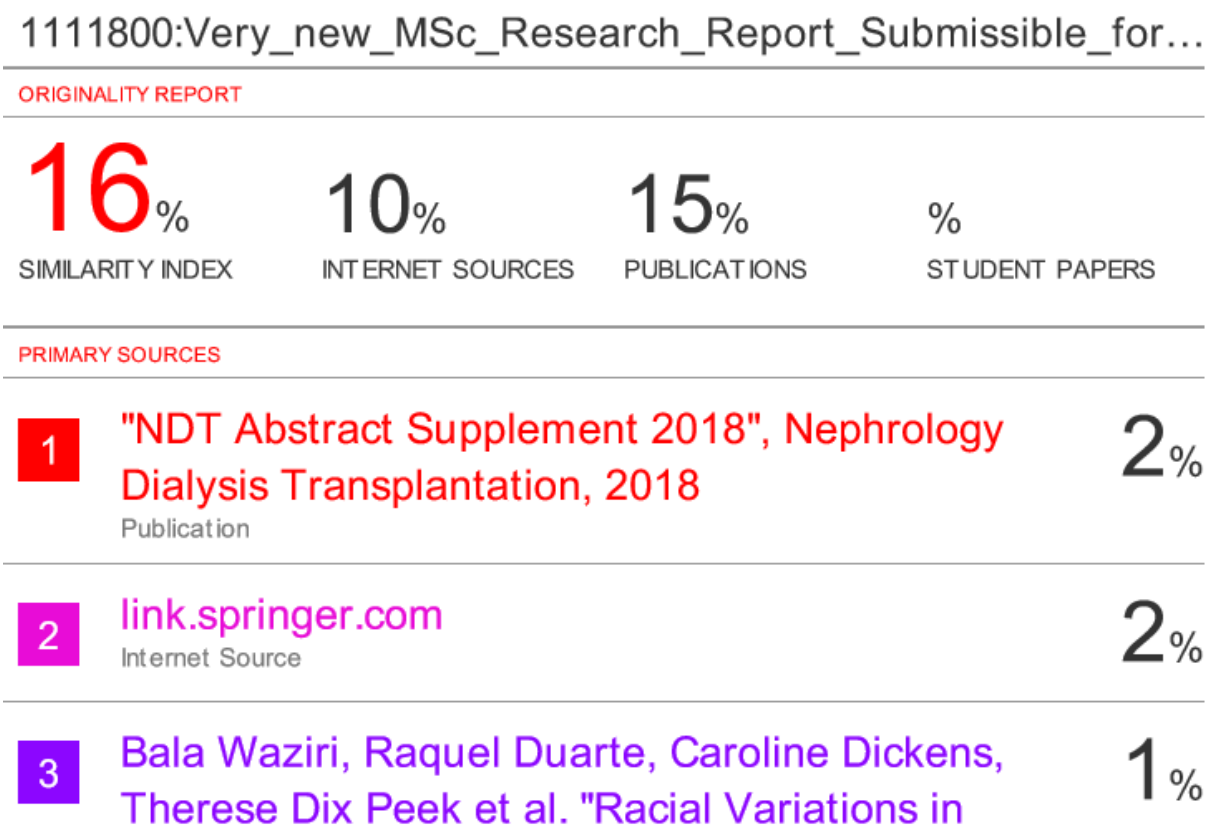
Estimated GFR: _____

Primary outcome

Dead :_____**,** date certified_____,

cause of death_____"

Turnitin report



1111800:Very_new_MSc_Research_Report_Submissible_for…

ORIGINALITY REPORT

16%	10%	15%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	"NDT Abstract Supplement 2018", Nephrology Dialysis Transplantation, 2018 Publication	2%
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8	Yang, Hongliu, Han Luo, Xi Tang, Xiaoxi Zeng, Yang Yu, Liang Ma, and Ping Fu. "Prognostic value of FGF23 among patients with end-stage renal disease: a systematic review and meta-analysis", Biomarkers in Medicine, 2016. Publication	<1%

Journal guidelines

Style and Format

File format	Manuscript files can be in the following formats: DOC, DOCX, or RTF. Microsoft Word documents should not be locked or protected. LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.
Length	Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information. We encourage you to present and discuss your findings concisely.
Font	Use a standard font size and any standard font, except for the font named "Symbol". To add symbols to the manuscript, use the Insert → Symbol function in your word processor or paste in the appropriate Unicode character.
Headings	Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.
Layout and spacing	Manuscript text should be double-spaced. Do not format text in multiple columns.
Page and line numbers	Include page numbers and line numbers in the manuscript file. Use continuous line numbers (do not restart the numbering on each page).
Footnotes	Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.

Language	Manuscripts must be submitted in English. You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.
Abbreviations	Define abbreviations upon first appearance in the text. Do not use non-standard abbreviations unless they appear at least three times in the text. Keep abbreviations to a minimum.
Reference style	PLOS uses "Vancouver" style, as outlined in the ICMJE sample references. See reference formatting examples and additional instructions below.
Equations	We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor or Microsoft's Insert→Equation function is acceptable. Avoid using MathType, Equation Editor, or the Insert→Equation function to insert single variables (e.g., "$a^2 + b^2 = c^2$"), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values. Do not use MathType, Equation Editor, or the Insert→Equation function for only a portion of an equation. Rather, ensure that the entire equation is included. Equations should not contain a mix of different equation tools. Avoid "hybrid" inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor.
Nomenclature	Use correct and established nomenclature wherever possible.
Units of measurement	Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.

Do File

```
*generating mortality status*
 gen mortality=.
 replace mortality=1 if status=="yes"
 replace mortality=0 if status=="no"
 tab mortality
 *Declariing d data as survival data**
stset outcomedate, fail( mortality) enter(Dateofenrolment )  scale(30.25) origin( Dateofenrolment)
sts graph , risktable
*summarizing FGF 23 and age*
summarize FGF23, detail
sum Age, detail
sum IPTH2, detail



*generating dialysis modalities*
gen dialysiscat=.
replace dialysiscat=1 if HD=="yes"
replace dialysiscat=2 if PD=="yes"

*Coding FGF 23 into 4 quartiles*
recode  FGF23 (min/147.9=1)(148/382=2)(382.1/2977=3)(2977.1/max=4), gen (FGF23cat)
*Coding FGF23 into three quartiles*
recode  FGF23 (min/382=1)(382.1/2977=2)(2977.1/max=3), gen (FGF23cat2)
```

```
*coding Intact parathyroidhormone into categories*

recode  IPTH2 (min/268=1)(268.1/755=2)(755.1/1359=3)(1359.1/max=4), gen(IPTHcat)

* re categorize IPTH2 using a different reference point*

recode  IPTH2 (min/268=2)(268.1/755=1)(755.1/1359=3)(1359.1/max=4), gen(IPTHcat2)

*Plotting Kaplan Meire graph by FGF23 quartiles*

sts graph, by(FGF23cat )

*univariable analysis.

stcox i. FGF23cat

stcox i.FGF23cat2

stcox i. BSALP2cat

stcox i. calciumcat

*Mutivariable analyses*

stcox  i.FGF23cat  calciumcat Phos IPTH2 Age

stcox  i.FGF23cat  i.calciumcat i.phoscat i.IPTHcat Age

stcox  i.FGF23cat  i.calciumcat i.phoscat i.IPTHcat Age i.BSALPcat

*checking for assumption of Cox proportional hazard model*

stcox  i.FGF23cat  i.calciumcat i.phoscat i.IPTHcat Age

estat phtest , det

*comparing models*

stcox  i.FGF23cat  i.calciumcat i.phoscat i.IPTHcat Age

est stor A

stcox  i.FGF23cat  i.calciumcat i.phoscat i.IPTHcat

est stor B

lrtest A B
```

** test of significance for survival accross the quartiles of FGF 23 & log Rank**

sts test FGF23cat

**mortality rate*

strate , per(100)

strate FGF23cat, per(100)

categorizing calcium into quartiles

recode Calcium(min/2.10=2)(2.11/2.37=1)(2.38/max=3), gen(calciumcat)

multivariable analyses for calcium levels

stcox i. calciumcat Phos IPTH2 FGF23 BSALP2 Age

stcox i. calciumcat i. IPTHcat i.FGF23cat i.BSALPcat Age

categorizing Bonespecific alkaline phosphate(BSALP2) into quartiles

recode BSALP2 (min/11.9=2)(12/15=1)(15.1/18=3)(18.1/max=4), gen(BSALPcat3)

stcox i. BSALPcat3

real calcium for analysis

recode Calcium(min/1.79=2)(1.80/2.10=3)(2.11/2.37=1)(2.38/2.50=4)(2.51/max=5), gen(calciumcatreal3)

PTH categories based on KDIGO

recode IPTH2 (min/64.9=2)(65/129.9=3)(130/600=1)(600/max=4), gen(IPTHcatkdgo)

Coding BSALP

recode BSALP2 (min/11.9=2)(12/15=1)(15.1/max=3), gen(BSALP7new)

*multiple reg

stcox i.BSALP7new Age i.calciumcatreal3 i. FGF23cat i. IPTHcatkdgo i. phosquar vitaminDbi i.dialysiscat

recode vitamin d

recode OHD (min/9.9=3)(10/29.9=2)(30/max=1), gen(vitaminDcut)

Model B

stcox i.BSALP7new Age i.calciumcatreal3 i. FGF23cat2 i. IPTHcatkdgo i. phosquar vitaminDbi i.dialysiscat i. alfacat i.DMcat i.racecat gendercat i.titrallcat

* linefit with 95 %ci)

graph twoway (lfitci Age systolicBP) (scatter Age systolicBP)

Fractional polynomial

fracpoly: stcox FGF23 IPTH2 Age BSALP2 OHD Phos

* FRAC PLOT*

fracplot, ylab(`=log(0.25)' "0.25" `=log(0.5)' "0.5" 0 "1" `=log(2)' "2" `=log

(5)' "4") ytitle(Hazard ratio)

Adding an additional plot

fracplot, ylab(`=log(0.25)' "0.25" `=log(0.5)' "0.5" 0 "1" `=log(2)' "2" `=log(5)' "4") ytitle(Hazard ratio)addplot (histogram Calcium, frequency yaxis(2))

Analyses for calcium as time varying c

recode calcium2(min/1.79=2)(1.80/2.10=3)(2.11/2.37=1)(2.38/2.50=4)(2.51/max=5), gen(calcium2tvc)

* multiple cox for time varying*

stcox i.BSALP7new Age i. calcium2tvc i. FGF23cat i. IPTH4CF2 i. phosquarreal OHD i.dialysiscat i. alfacat i.racecat gendercat i.titrallcat

checking for interaction between FGF23 and race

stcox i.BSALP7new Age i.calciumcatreal3 i. IPTHcatkdgo i. phosquar vitaminDbi i.dialysiscat i. alfacat i.DMcat gendercat i.titrallcat FGF23catbi#racecat

fracpoly: stcox Calcium IPTH2 , deg(2) compare

*coding pth into three categories *

recode IPTH2 (min/129.9=2)(130.1/600=1)(600.1/max=3), gen(IPTHcat3)

* label define*

label define calciumcat 1 "2.11-2.37mmol/l" 2 "<=2.10mmol/l" 3">=2.38mmol/l"

Plos One Reviewers' Comments and Point to Point responses

The Editor

PLOS ONE

4h Marh 2019.

Sir/Ma,

Re- Manuscript ID PONE -18-35338 R1

We are pleased to resubmit for publication the revised version of our manuscript entitled "Associations of Plasma Fibroblast Growth Factor 23, Calcium and Parathyroid hormone with All-Cause Mortality in South African Patients on maintenance dialysis: A 3-year Prospective Cohort Study"

We appreciate the time and insightful suggestions provided by each reviewer and have implemented the suggested changes into the manuscript to the best of our ability. Kindly find below our point-by-point responses to each of the comments.

Reviewer 1:

1. The background part should be more connected with the whole manuscript. And the introduction of aim should be more natural.

Response: Other makers of chronic kidney disease mineral bone disease (CKD -MBD) such 25-hydroxyvitamin D, PTH and Calcium are now briefly mentioned in the background. Hence, making it more connected to the whole manuscript.

2. Since there was no actual proof that suggesting Plasma FGF23 level had a positive effect on all-cause mortality, the levels lower than the 25 percentile should not simply used as reference value.

Response: The majority of the studies have linked higher levels of FGF 23 to all- cause mortality and have utilized lower levels of FGF23 as their reference point. Therefore, we have

also utilized lower levels of FGF 23 as our reference which would allow us to compare our results with previous studies. Thus, we have referenced these studies in our methodology.

:

3. In the background authors trying to discuss the unclear relationship between mortality and plasma FGF23 level in different race, this should be a main part to discuss, another table should be intake to describe these data. And group design should be redone between white and black groups to complete the FGF23 levels in different race directly.

Response: Although the main objective was not to compare mortality between white and black participants, we have now included another table that also highlights differences in the levels of FGF23 between Blacks and Whites (see Table2). This is further discussed in the discussion section see line 337-347.

Reviewer 2:

1. Consideration should be given to changing the title to reflect that associations are reported (i.e., use the word "association" instead of "effect") and that the data are prospective and longitudinal, which is a strength of the study. Also, the title only reflects the association between FGF23 and mortality, when the authors report the association of various markers of mineral metabolism and mortality.

Response: The initial primary objective and hypothesis of this study was to evaluate the association between FGF23 and all- cause mortality. While the secondary objective was also to determine the impact of traditional markers of mineral metabolism on mortality and adjust for them as potential confounders to the effects of FGF23 on mortality. However, based on your recommendation we have rephrased the title as "Associations of Plasma Fibroblast Growth Factor 23, Calcium and Parathyroid hormone with All-Cause Mortality in South African Patients on Maintenance Dialysis: A 3-Year Prospective Cohort Study".

2. The authors should include a more detailed discussion regarding the notion that discrepancies in outcomes studies may be due to FGF23 assay type (c-terminal vs. intact), race, and/or an interaction between FGF23 assay type and race. Lines 69-71, pg 4 (2nd paragraph of introduction) are vague. At the very least, the differences in assay should be described and how

the discrepancies were related to each assay. These ideas should also include relevant citations. As written, there is not enough detail to justify the phrase “intriguing complexity.”

Response: The assays’ methodology is briefly highlighted with citation to it. This is further discussed under discussion section with relevant citations see lines 322-343.

3. In the 3rd paragraph of the Introduction, the authors should give examples of what they mean by “traditional risk factors” for mortality in dialysis patients. They should also cite the studies that examine these risk factors in African patients. Please also cite the “few” studies that have examined the relationship between FGF23 and mortality in African patients on dialysis.

Response : example of traditional risk factors are now highlighted and few studies that examined these risk factors are now cited. However, to the best of our knowledge, no study from Africa has examined the association between plasma FGF23 and mortality in an African dialysis population. Hence, unable to cite a study from Africa.

4. There is no hypothesis included in the Introduction

Response: Hypothesis is now included in the introduction section.

Methods

5. Why was dialysis vintage not included in the collected baseline demographics and considered in the multivariable analysis.

Response: Thanks for reminding us on this, though dialysis vintage was part of the data collected, this was an oversight in reporting. This is now included as part of the baseline characteristics and also adjusted for in the multivariable analysis leading to some subtle changes in the adjusted HR as shown in Table 3. However, because of strong collinearity between some important continuous exposure variables we could not factor in dialysis vintage when these exposure variables were considered on a continuous scale. Hence, Table 4 remains the same.

6. The inter- and intra-assay coefficients of variation should be provided for FGF23, PTH, and 25(OH)D.

Response: The inter- and intra-assay coefficients of variation for FGF23, PTH, and 25(OH)D are now documented see methods.

7. Explanation should be given as to why bone specific alkaline phosphatase was categorized at the 25, 50, and 75 percentile and the interquartile range used as the reference value.

Response: This explanation with relevant references is now highlighted in the method section see methods lines 143-147.

8. A more detailed description of how PTH, calcium, and phosphate were categorized should be given and it should be explicitly stated that KDIGO target ranges were used as the reference.

Response: Suggestion noted and effected, we have now highlighted a detailed explanation with relevant refences cited on how PTH, calcium and phosphate were categorized.

9. Variables included in each model should be explicitly stated.

Response: These variables were stated under the methods, results, and below the tables 3 and 4.

Results

10. While the Methods section states that comorbidities were collected at baseline and that models were adjusted for comorbidities, in the results section, there is no mention of comorbidities such as prevalent cardiovascular disease, heart failure, diabetes, which are important confounders for mortality in ESKD. These comorbidities should be included in Tables 1 and 2. If there were none, this should be explicitly stated.

Response: Diabetes mellitus which is one of the co morbidities collected is being documented, included in Table 1 and 2 and adjusted for. Although, we excluded patients with other co morbidities such as active malignancies, infection and patients with unstable clinical conditions at baseline, we could not establish development of cardiovascular events during the follow up period. This is highlighted as one of the limitations of our study. Specific causes of death such as cardiovascular which is likely the leading cause of death could not be documented and this is also stated as part of our study limitations.

11. How many subjects were transplanted and lost to follow-up?

Response : Observation noted and effected under the results section

12. Please explicitly state which variables were included in Models 1 and Models 2.

Response

These variables were highlighted and mentioned in the methods and statistical analysis sections

13. When each mineral metabolite was used as the predictor variable, was it excluded as an adjustment variable? This is unclear from the Methods and Results description.

Response: In this study in which Cox regression analysis was used to determine our primary outcome, it is not possible to factor into the model exposure variables twice. Multivariable analysis which is used to determine predictors of outcome also adjusted for potential confounders. While on a continuous scale when a variable is factored into a model, Stata will automatically omit as a repeated variable.

14. Why was the use of continuous variables not described in the Methods section.

Response. This was described under statistical analysis. However, based on this recommendation we have highlighted it again in the methods section.

15. The explanatory variables are explored as potential non-linear variables by using the normal range as the refence and using fractional polynomials, but they are also explored on a continuous scale, which assumes a linear relationship. When describing the relationship between calcium and death, the authors suggest both a linear relationship and a U-shaped relationship. Including a hypothesis would be helpful to choose the appropriate analysis for all explanatory variables.

Response

Although categorization of continuous variables like calcium levels is done to allow ease of interpretation, it is associated with loss of information. When calcium was categorized in this study we saw a drop in hazard ratios from 2.56 for calcium < 1.80 mmol which then dropped to 0.96 for calcium 1.80-2.10 and rose again and plateau to 5.66 for calcium > 2.50 mmol. To

graphically represent this relationship and confirm the U-shaped pattern fractional polynomial was used. In addition, arbitrary assuming that the relationship between serum calcium and mortality based on linear regression may not be sufficient.

16. Why were figures only included for FGF23, calcium, and PTH?

Response: To avoid plenty figures we choose to include figures of the exposure variables that were significantly associated with the primary outcome. We included FGF23 despite not significantly associated with the outcome, since it is our primary exposure variable of interest.

Discussion

17. When the authors discuss their findings that higher calcium is associated with mortality, they do not mention if models were adjusted for use of calcium carbonate and alfacalcidiol.

Response : In the fully adjusted model 2 we mentioned that we adjusted for use of calcium carbonate and alfacalcidol. This is now further stated in the discussion section.

18. The authors statement that the proposed mechanism for PTH and mortality should be considered in the context of the EVOLVE study needs further explanation (pg 18-19, lines 344-347).

Response : This now re worded and further explained. However, this was just to highlight the fact that despite recommendations to maintain PTH at a certain range which was based on observational studies that showed increased mortality risk with high levels of PTH, an available randomized controlled trial failed to show a significant improvement in outcome with lowering of PTH. Thus, more randomized controlled trial are needed to prove the benefit of lowering PTH levels to recommend targets.

Minor comments:

1. Add the work “factor” on line 51, pg 3 to complete the phrase, “fibroblast growth factor.”

Response : Correction noted and effected.

2. The Fixed Hazard Ratio section of Table 3 should be explicitly labeled.

Response Suggestion effected see Table 3.

3. Figure 3 survival curve for PTH categories should come after Figure 5 HR for all-cause mortality and time-dependent calcium

Response: Correction noted and effected.

Reviewer #3:

1) Drop table 1. Typically baseline demographics are only shown by the main covariate of interest.

Response: This is noted and table 1 dropped

2) Why did you show results from time varying covariates if there was no violation of proportional hazards and the variables were only measured at one time point?

Response : The time varying covariates analysis was utilized to account for possible variations in the markers of CKD-MBD that were repeatedly measured over time. Serum calcium, plasma parathyroid hormone and phosphate were measured in our dialysis patients every 3 months. Since these exposure variables were known to fluctuate over time, it is necessary to make our findings more robust by updating these exposure variables in time varying analysis. Unfortunately, we could not repeat measurement of plasma FGF23 and this was highlighted as one of our study limitations.

3) see 1 reported Schoenfeld global test chi-square, yet several Cox models were run, so there should be one for each model.

Response : The individual Schoenfeld test have P Value greater 0.05 and this is now stated in the statistical analysis section.

4) In table 2, please include the categorical variables for calcium, ipth, phosphate, and BSALP,.. similar to how they were included in the Cox models.

Response: Since Table 2 is already lengthy and adding more variables may make it difficult for readers to follow. We have similarly assessed the quartiles of these variables that were significantly associated with mortality and presented the results in separate tables, see

supplementary S1 Table and S2 Table.

5) In table 3, please describe the variables in Models 1 and 2 in the footnote. Also please add a line so that there is a clear separation between the fixed and time varying hazard ratios. Also what time point was used for the time dependent hazard ratios?

Response: The variables in the models are now mentioned in the foot note of the table 3 and 4.

A line has now been added to separate between the fixed and time varying hazard ratios.

6) Just wondering if you might see a stronger effect with a longer follow-up-- 5 years instead of 3?

Response: A possibility that we could not ascertain at 3 years. We may re evaluate the outcome at 5 years to confirm this.

7) Kaplan Meier curves should show the number of patients at risk over time. These counts can be given at the bottom of the figure.

Response : This suggestion is noted and effected as recommended. See revised Kaplan Meier curves.

We hope our accompanying responses and effected corrections have reinforced our manuscript and made it suitable for publication in your esteemed journal.

Yours sincerely,



Waziri Bala

Informed Consent Form

Informed Consent Form: General (patient)

I confirm that I have been informed about the study by 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 (printed name)……..

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

Witness (printed name)……

Signature……...Date…….

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

STUDY INVESTIGATOR

…….. Signature……Date……..

Printed Name