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Association of genetic polymorphisms in the Fibroblast Growth Factor 23 genes with fibroblast growth factor 23 levels in South African patients with chronic kidney disease

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A research report
submitted to the University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Medicine.

TITLE:

Association of genetic polymorphisms in the Fibroblast Growth Factor 23 genes with fibroblast growth factor 23 levels in South African patients with chronic kidney disease.

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DECLARATION

I, Allyson Peddle, declare that this research report is my own unaided work. The research report is being submitted for the degree Master of Medicine, in Internal Medicine, at the University of Witwatersrand, Johannesburg. It is being submitted in the submissible article format, with my protocol and an extended literature review. It has not been submitted before, for any degree or examination, at this or any other University.

_____

Allyson Peddle

Signed __6th_____day of____June_____2023

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I would like to acknowledge the assistance of my supervisors as well as Dr Brett Mansfield in completing this research report.

Abstract

Introduction

It is now understood that FGF23 plays a fundamental role in calcium, phosphate and vitamin D homeostasis and that it can be used as an early biomarker of chronic kidney disease mineral and bone disorders (CKD-MBD). The aim of this study is to investigate genetic polymorphisms in South African patients with CKD-MBD which have been associated with circulating FGF23 levels in Caucasian patients in order to improve our understanding of its role in CKD in a South African population.

Methods

Study participants included 293 patients with CKD stage 3-5 and 90 apparently normal participants. Whole blood samples were previously collected and FGF23 levels measured using a sandwich based enzyme-linked immunosorbent assay kit. TaqMan® minor allele groove-binding based allelic discrimination assays were used to genotype the three single nucleotide polymorphisms (rs17216707, rs11741640 and rs4075958).

Results

No statistically significant difference was seen in the prevalence of rs4075958 and rs17216707 in participants with CKD compared to controls. There was a significant difference (p=0.03) when comparing the circulating levels of FGF23 in CKD between Black African and Caucasian participants. There was no association between any of the three SNP's and circulating FGF23 level (all p-values > 0.1). There was a significant association between FGF23 and phosphate levels (p=0.00).

Conclusion

The lack of correlation is possibly due to low levels of homozygosity in the minor trait in our sample suggesting other factors play a role in the development of CKD and CKD-MBD. This

study has added to existing data supporting the association between hypertension, increased phosphate levels and FGF23.

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Abbreviations

CKD – Chronic Kidney Disease

GFR – Glomerular Filtration Rate

ESKD – End Stage Kidney Disease

RTT – Renal Replacement Therapy

CKD-MBD – Chronic Kidney Disease Mineral and Bone Disorder

FGF23 – Fibroblast Growth Factor 23

PTH – Parathyroid Hormone

GWAS – Genome-Wide Association Studies

SNP – Single Nucleotide Polymorphism

1: PROTOCOL WITH EXTENDED LITERATURE REVIEW

Introduction

Chronic kidney disease (CKD) is defined as structural or functional abnormalities in the kidney present for - 3 months or more, irrespective of diagnosis (1). Based on the estimated glomerular filtration rate (GFR), CKD is staged from stage 1 to stage 5 as shown in Table 1 (1). Accurate international data is lacking for the true prevalence of CKD and the use of different formulae to define CKD likely contribute to these variations; however, the estimated global prevalence is placed between 11 and 13% (2). A recent meta-analysis conducted to estimate the burden of CKD in Africa suggested that 4.6% of adults living on the continent have moderate or severe decreases in renal function (i.e CKD 3 to 5) (3).

Africa faces the double burden of a rise in both communicable as well as non-communicable diseases (4). By 2030 estimates place 70% of patients with CKD in low-income countries (5). In 2006 the reported prevalence of end-stage kidney disease (ESKD) in South Africa was approximately 500 per million, with an incidence of 100 per million (6). The high financial cost of renal replacement therapy (RRT) and the lack of expertise limits access to a large percentage of patients living with CKD, thus the numbers of patients accepted for RRT fall far below the reported prevalence (6).

Chronic kidney disease should be viewed as a syndrome resulting from the progressive decline in GFR and which is associated with multi-organ dysfunction (2). As the renal function worsens with progressive loss of nephron mass there is an accumulation of circulating waste products, disruptions in electrolyte homeostasis, pH and fluid balance as well as in the metabolism and production of hormones (2).

Table 1. CKD GFR stages (1)

GFR Categories (mL/min/1.73m^2)		
Description		**Range**
G1	Normal or high	≥90
G2	Mildly decreased	60-89
G3a	Mildly to moderately decreased	45-59
G3b	Moderately to severely decrease	30-44
G4	Severely decreased	15-29
G5	Kidney failure	<15

The definition of chronic kidney disease-mineral and bone disease (CKD-MBD) is that of a bone and mineral metabolism disorders due to CKD. It is as a consequence of one or a combination of three potential abnormalities, which include 1) metabolism of calcium, phosphorus, parathyroid hormone (PTH) or Vitamin D; 2) aspect issues affecting the homeostasis, the strength or the growth of bone and 3) calcification of vascular or soft tissue structures (7). The gold standard for the diagnosis and differentiation of the types of renal osteodystrophy, the skeletal component of CKD-MBD, is that of a bone biopsy (1,8). However, biochemical abnormalities in calcium, phosphate, PTH and alkaline phosphatase are the primary indicators by which the diagnosis of CKD-MBD is conventionally made. Whilst a bone biopsy provides direct information about bone turnover, mineralization, and volume it is an invasive procedure that carries a risk of complications (1). It is also not practical to perform bone biopsies on all CKD-MBD patients, as it would be expensive and time-consuming.

As a result, CKD-MBD diagnosis is often made using biochemical parameters such as serum levels of calcium, phosphate, parathyroid hormone (PTH), and alkaline phosphatase (ALP).

These tests are less invasive, less costly, and readily available, making them the preferred diagnostic approach for CKD-MBD. These changes are most commonly seen in patients with CKD stages 3 to 5 (7).

Normal Electrolyte physiology

Extra-cellular calcium is the primary source for mineralisation of bone(15). Its regulation is governed by parathyroid hormone (PTH) and 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) as well as Fibroblast Growth Factor 23 (FGF23). This circulating extra-cellular calcium is present at concentrations close to saturation (16)(7). Efflux and influx of calcium into the extracellular fluid is a result of flow from the gut, bone and urine. Absorption of calcium from the gut is the only available route by which total body calcium stores can be replenished (17). People with no abnormalities have the ability to protect against calcium overload by decreasing intestinal absorption, through the actions of PTH and $1,25(OH)_2D$ as well as possibly FGF23 and are able to increase renal excretion of calcium (18). This ability is, however, lost in patients with CKD (7).

In the kidney, calcium which is filtered through the basement membrane into the tubule fluid is mostly reabsorbed passively in the proximal tubule, a process driven by a gradient generated by sodium and water reabsorption. The calcium absorbed in the distal convoluted tubule is regulated by $1,25(OH)_2D$ which upregulates the transport protein resulting in calcium reabsorption into the serum (7). Parathyroid hormone stimulates calcium reabsorption in the thick ascending limb of the loop of Henle (17).

Phosphorus is primarily thought to be absorbed in the duodenum and the jejunum (17). The kidney is responsible for excreting the net amount of phosphate absorbed which is freely

filtered by the glomerulus and about 70-80% reabsorbed in the proximal tubule. The primary transporters are thought to be downregulated by PTH and FGF23, with its co-transporter Klotho (17).

Vitamin D is derived from two primary sources, the diet and exposure to sunlight (15). In the liver 25-hydroxylation by cytochrome P450-like enzymes alter vitamin D. The 25(OH)D then undergoes 1α hydroxylation to form 1,25$(OH)_2D_3$ (calcitriol) in the kidney which is a tightly regulated process (15). PTH and hypophosphatemia induce hydroxylation whilst calcium, and FGF23 supress it. Calcitriol results in increased calcium absorption in the intestine and in the kidney and the suppression of PTH transcription in the parathyroid glands (15)(19).

Parathyroid hormone is synthesised in the parathyroid gland chief cells. Its increased secretion is controlled by the serum ionised calcium level as well as phosphates levels (13). Its secretion is stimulated by hypocalcaemia, hyperphosphataemia and elevated FGF23 levels as well as calcitriol deficiency (7). Its primary function is to maintain calcium homeostasis and this is achieved by several mechanisms. It results in the increased conversion of 25(OH) D to 1,25$(OH)_2D$ with an indirect increase in intestinal absorption of calcium by its activity on calcitriol synthesis (7).

Chronic Kidney Disease

The Kidney Disease Outcomes Quality Initiative (KDOQI) developed a conceptual model describing the development, progression as well as the complications that arise from CKD (9). In this conceptual model kidney damage precedes a decrease in GFR which eventually ends in kidney failure and death as the final stages. The progression to kidney failure typically

evolves over an extended period of time with intervention in the earlier stages possibly slowing down or preventing progression (9). This model emphasises that progressive decline in kidney function is not the only process affected but that it is a multi-systemic disease.

Accurate data on the causes of CKD, especially in the developing world, are scarce. In 2013 the estimated number of deaths directly attributable to CKD was estimated at 956 200, which is an increase of 134.6% from 1990 (10). This number is most likely an underestimation as it probably represents deaths as a result of ESKD, which according to data from the US represents less than 2% of CKD patients (11). When assessing the causes of CKD the most common in the developed world are attributable to diabetes mellitus, hypertension and glomerulonephritis (12).

Most forms of injury to the kidney over a period of time result in the nephron being replaced by fibrotic tissue. This non-specific response to injury results in loss of renal function (13). These pathological changes are characterised by glomerulosclerosis and tubulointerstitial fibrosis; when the damage is longstanding and advanced it may not be possible to determine the underlying primary renal disease (13).

The ureamic syndrome reflects the multi-organ dysfunction which is the result of a progressive decline in GFR and refers to the group of signs and symptoms that is the consequence of this process (2). The majority of these non-specific symptoms are frequently only seen from CKD stage 4 and reflect the dysfunction in the specific organ systems affected by the decline in renal function (13). The most prominent complications of advancing renal dysfunction are the abnormalities in calcium, phosphorus, and mineral-regulating hormones

such as $1,25(OH)_2D_3$ (calcitriol), parathyroid hormone and FGF23 as well as anaemia and abnormalities in sodium, potassium, water and acid-base homeostasis (14).

Pathogenesis of CKD-MBD

Risk factors for increased mortality in patients who are receiving dialysis include an elevated PTH and hyperphosphatemia both of which are integral components in CKD-MBD (20). The relationship between calcium, phosphate, FGF23 and calcitriol is complex in the manner in which they result in the development of secondary hyperparathyroidism (7). Secondary hyperparathyroidism is a prominent feature of CKD-MBD and it begins from CKD stage 3. The main abnormalities that result in the development of secondary hyperparathyroidism are: phosphate retention, decreased ionised calcium levels, decreased calcitriol levels, and an increase in FGF23 (19). Initially the circulating calcium and phosphate levels are within normal as a result of the increased PTH, which is elevated from the time the GFR is below 60mL/min/1.73m^2 (19). Chronic Kidney Disease results in progressive calcitriol deficiency via increasing FGF23 serum concentration, which results in decreased calcium absorption in the intestine as well as decreased bone sensitivity to PTH as well as hyperphosphatemia (16).

FGF23, and the FGF23-Klotho signalling axis

The discovery of FGF23 in the year 2000 revolutionised our understanding of vitamin D and phosphate homeostasis (21). In autosomal dominant hypophosphatemic rickets (ADHR), FGF23 was first described as a phosphaturic hormone (22). It has now been established that the interaction between FGF23, PTH and calcitriol is a complex multiorgan feed-back system (23). There are more than 10 disorders of altered phosphate homeostasis which are associated with abnormalities in FGF23 physiology (23).

Fibroblast Growth Factor 23 is secreted by osteocytes in response to hyperphosphatemia and calcitriol and it requires Klotho to bind to the FGF receptors in the kidneys and parathyroid glands (21). Klotho's main sites of expression are in the proximal and distal tubules of the kidney which are also the most likely sources of circulating Klotho (21). Fibroblast Growth Factor 23 decreases calcitriol by reducing 1αhydroxylase activity in a Klotho-dependant manner in the kidney which results in reduced intestinal calcium absorption (21). It also reduces expression of the sodium phosphate (NaPi) transporters in the apical luminal membrane in the kidney, which normally facilitates the excretion of phosphates (16). More recently, there is evidence to suggest that FGF23 has a direct effect on calcium reabsorption in distal nephron (21).

FGF23 Gene

The *FGF23* gene is located on chromosome 12p13 (24) Several rare and inherited conditions have been associated with dysregulated FGF23 metabolism, however, only two involve direct mutations of the *FGF23* gene itself, suggesting that there are other genetic components that are associated with its regulation (25). In a recent meta-analysis of genome-wide association studies (GWAS) of circulating FGF23 levels, 5 loci on the chromosomes 5, 9, 16 and 20 were found to be significantly associated with increased circulating FGF23 concentrations (25).

A single nucleotide polymorphism (SNP) is a variation in a single base pair in the DNA. There is approximately one SNP every 1000 base pairs and they account for approximately 90% of all sequence variations and are thus a major source of genetic heterogeneity (26).

The SNP with the strongest association for circulating FGF23 levels found in GWAS was SNP rs17216707, which encodes the primary catabolic enzyme for calcitriol, as well as two other SNPs (rs11741640 and rs4075958) which are both located on the Regulator of G Protein Signalling 14 gene, *RGS14*, which encodes for the sodium phosphate cotransporter (Robinson-Cohen, Bartz, et al., 2018). A recently published GWAS identified SNP rs17216707 as being a variant driving an association between eGFR and risk of CKD as well as on the presence hypertension (27). This is in keeping with a similar finding in the study conducted by Robinson-Cohen *et al* which found an association between the SNP rs17216707 and eGFR and CKD (28).

Relationship of FGF23 with Morbidity and Mortality in CKD and CKD-MBD

Increasing levels of FGF23 are found prior to the development of secondary hyperparathyroidism in patients with CKD and is a compensatory mechanism for the decreasing calcium levels and rising phosphate found in the pathology of CKD (15). The Homocysteine study showed that raised levels of FGF23 was a significant predictor of cardiovascular mortality (29). FGF23 binds to FGFR4 in a Klotho dependant manner in the heart and directly induces progressive left ventricular hypertrophy which results in an increase in cardiovascular mortality (30). One hypothesis as to the mechanism of action that brings about hypertension and left ventricular hypertrophy is the induction of expression and function of the sodium-chloride cotransporters in the distal renal tubule which results in reduced sodium excretion and increased water retention (31).

Recent work by Schwantes-An *et al* showed evidence that certain SNPs in the FGF23 gene are associated with cardiovascular mortality and heart failure but not specifically with circulating FGF23 levels, possibly indicating that these SNPs on the FGF23 gene do not modulate the serum levels (30).

3. Rationale and Study Objectives

3.1.1 Hypothesis

Genetic polymorphisms in the *FGF23* gene are associated with circulating FGF23 levels in South African patients with CKD.

3.1.2 Aim

To investigate the role of genetic polymorphisms in the *FGF23* gene in South African participants with CKD-MBD and their interactions with circulating FGF23 levels.

3.1.3 Objectives

1. To determine the association between SNP rs17216707, rs11741640, and rs4075958 in the *FGF23* gene with circulating FGF23 levels
2. To determine the correlation between circulating FGF23 levels and estimated GFR as well as markers of CKD-MBD
3. To compare circulating FGF23 levels with different causes of CKD

4 Methods and Materials

4.1 Study Design

This is a cross-sectional multicentre study using samples which have been pre-collected at the Charlotte Maxeke Johannesburg Academic Hospital and Donald Gordon Renal Units in Johannesburg. The samples were collected as part of the study conducted by Dr Bala titled "Biochemical and Genetic Markers of Mineral Bone Disease in South African Patients with Chronic Kidney Disease", ethics clearance M141016 (Appendix 1). This study is thus a sub-study of that project.

4.2 Study Participants

The study participants include 293 patients with established CKD stage 3-5, ≥ 18 years. The control group included 90 apparently normal participants. Exclusion criteria included CKD patients with chronic liver disease, malignancy or on treatment with steroids and or bisphosphonates.

4.3 Data Collection

4.3.1 Demographic Data

Demographic details of all participants and the determination of race was self-reported. Demographic data used included the sex, age, race, weight and height as well as the presence of specific co-morbidities (hypertension, diabetes mellitus). Additional information collected included aetiology of CKD, modality of renal replacement therapy as well as duration of dialysis, if applicable.

4.3.2 Biochemical Data

Biochemical data already collected as part of Dr Bala's study include the following:

- Calcium, phosphate, vitamin D, PTH, alkaline phosphatase, serum creatinine, with estimation of GFR and FGF23 levels (Appendix 2)

4.4 Genetic polymorphisms in FGF 23 gene

- Using already extracted DNA, for this study the genotyping of the following *FGF23* SNPs will be performed (using: TaqMan® SNP genotyping assays; ThermoFisher Scientific, Waltham, MA, USA):

  SNP rs17216707

SNP rs11741640

SNP rs4075958

5. Statistical Assessment

Data was collated on an Excel spreadsheet and analysed using the Statistica™ software package. The continuous variables will be expressed as means ± standard deviations (SD) for normally distributed data or as medians and interquartile ranges (IQR) and discrete variables reported as percentages. A Shapiro-Wilk test will be used to test for normality.

Frequency distribution of *FGF23* SNPs was compared between the CKD group and control group using the Fisher's exact test or Chi square test as appropriate. An independent t-test or Wilcoxon rank-sum test was used to compare variables between two groups and for more than two groups, either an one-way ANOVA or a Kruskal-Wallis test will be used, depending on the distribution of the data.

6. Ethics

Permission to use data and samples already collected by Dr Bala has been obtained (Appendix 3). Ethical clearance has been obtained from the University of the Witwatersrand Human Research Ethics Committee Clearance Certificate Number: M190576 (Appendix 3).

7. Funding

Dr Waziri received funds from Astra Zeneca Research which were used for this analysis. The Department of Molecular Biology Wits Health Laboratory, under Prof Raquel Duarte, will provided additional funds that were required.

Table 1. Cost of Reagents

	Number of Reactions	Unit Price	Total
TaqMan® Gene Expression Assay	360 per unit per SNP	R5518.41 (incl VAT)	R33110.47

8. Data Sharing

The data will be used for the degree purpose of the Master of Medicine (Internal Medicine). It will also be submitted to a suitable peer reviewed journal for publication, which will also be made available to the Department of Internal Medicine, University of the Witwatersrand.

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CHAPTER TWO: SUBMISSABLE ARTICLE

Introduction

Chronic Kidney Disease (CKD) is a syndrome that results from a progressive decline in glomerular filtration rate (GFR). The associated accumulation of waste products disrupts the metabolism and production of hormones which results in organ dysfunction [1]. The alterations in the mineral and bone metabolism that are found in CKD are collectively referred to as chronic kidney disease mineral and bone disorders (CKD-MBD) [2]. The discovery of FGF23 in the year 2000 revolutionised our understanding of vitamin D and phosphate homeostasis and it has now been established that the interaction between FGF23, PTH and calcitriol form a complex multiorgan feed-back system [3] [4].

Increasing levels of FGF23 are found prior to the development of secondary hyperparathyroidism in patients with CKD and is a compensatory mechanism for the decreasing calcium levels and rising phosphate found in the pathology of CKD [5]. For this reason, serum FGF23 measurement can be considered an early biomarker for the detection of CKD-MBD. In a Brazilian study involving patients with type 2 Diabetes Mellitus, the serum FGF23 levels were an independent predictor of death, worsening serum creatinine and/or dialysis requirement after adjusting for creatinine clearance and parathyroid hormone [6].

In a large multi-centre prospective study Chronic Renal Insufficiency Cohort (CRIC) involving 3879 CKD stages 2–4 patients with a median follow- up of 3.5 years, high FGF23 levels were independently associated with poor renal outcome. Few if any studies have investigated the role of FGF23 in African CKD patients.

The *FGF23* gene is located on chromosome 12p13. Several rare and inherited conditions have been associated with dysregulated FGF23 metabolism, however, only two involve direct mutations of the *FGF23* gene itself, suggesting that there are other genetic components that

are associated with its regulation [7]. In a recent meta-analysis of genome-wide association studies (GWAS) of circulating FGF23 levels, 5 loci on the chromosomes 5, 9, 16 and 20 were found to be significantly associated with increased circulating FGF23 concentrations [7].

The aim of this study is to investigate genetic polymorphisms in South African patients with CKD-MBD which have been associated with circulating FGF23 levels in Caucasian patients in developed countries.

Materials and Methods

Study Population

Ethical clearance was granted for this cross-sectional multicentre study by the Human Research Ethics Committee of the University of the Witwatersrand, South Africa (Clearance certificates M141016 and M190576). The study participants included 293 patients with CKD stage 3-5 and 90 apparently normal participants. A minimal sample size was determined using Fisher's statistical formula, where the prevalence of CKD-MBD is 88.29% based on the findings of Gosh *et al*[8]. Therefore a minimum of 158 patients with CKD were required with 80 healthy controls in a ratio of 2:1. The general characteristics of the patients enrolled where previously determined using a structured questionnaire detailing demographics, blood pressure, co-morbidities and underlying aetiology of CKD (Appendix 1). Race determination was self-reported. Patients with chronic liver disease, malignancy or on treatment with steroids or bisphosphonates were excluded. The plasma samples used were collected previously as well as the determination of serum electrolytes and measured by an accredited laboratory at Charlotte Maxeke Johannesburg Academic Hospital. The serum FGF23 levels were also previously determined using a sandwich based enzyme-linked immunosorbent assay kit from EMD Millipore Corporation (Billerica, MA, USA)50 µL of plasma samples were added to the

appropriate wells in addition to 100 µL of conjugate antibody. The plate was covered with a sealer and allowed to incubate for 2 hours on a microliter plate shaker. The solutions were then decanted from the wells and washed three times with a diluted wash buffer. 100 µL of detection antibody was then added to all wells and further incubated for 1 hour on a plate shaker. The solutions were decanted from the wells and washed three times with a diluted wash buffer. The washing step was repeated after adding 100 µL of enzyme solution with 30 minutes incubation period. Finally, 100 µL of substrate solution was added to each well to stop the reaction. Optical density at 450 nm was measured on an ELx800 microplate reader (BioTek, Winooski, VT, USA).

Single Nucleotide Polymorphism selection

A literature review was conducted of genome-wide association studies (GWAS) to find the single nucleotide polymorphism (SNP) with the strongest associations with circulating FGF23 levels. After selection of the three most promising candidate SNPs they were checked in the SNP database system of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/snp) and the Sanger Institute Biobank (http://www.ensembl.org/index.html). The minor allele frequency (MAF) for the three SNP's was determined in the Yoruba of Nigeria, as these were the only publicly available data comparable to a South African population, based on this the final three SNPs where then selected (Table 1).

Table 1 Selected SNPs

SNPs	Minor Allele	Major Allele	MAF (Yoruba)	Location	Function domain
rs17216707	C	T	0.028	20:54115823	Intergenic variant
rs11741640	A	G	0.032	5:177365742	Intron variant
rs4075958	A	G	0.060	5:177357511	Tf binding site variant

MAF = Minor Allele Frequency

Genotyping

DNA was previously extracted from whole blood using the Maxwell DNA purification kit (Promega AS1010, USA). The DNA concentrations and purity were determined using NanoDropTM 2000 spectrophotometer (Thermo Scientific, USA) at A260 and A260/A280 ratios. Salting out method was used to re-extract DNA from samples with low yield (<10ng/ µL). TaqMan® minor allele groove-binding (MGB) based allelic discrimination (AD) assays (Thermofisher Scientific, Waltham, USA) were used to genotype each participant for the presence of the three SNPs in the CYP24A1 and the Regulator of G Protein Signalling 14 gene, RGS14 (rs17216707, rs11741640 and rs4075958). The TaqMan® AD Assay has the advantage of being accurate, robust, highly reproducible and with minimal post-PCR handling to reduce contamination. The primer sequences for the specific TaqMan® SNPs were designed by ThermoFisher Scientific. The ThermoFisher website was used to retrieve the context sequences (Table 2), however the primer sequences used are proprietary of TaqMan. The G allele was detected with FAM® dye, whilst the A allele was detected with the VIC® dye. The samples were processed using the TaqMan Gene Expression Assay protocol available from ThermoFisher. The samples, with a total reaction volume of 20ng of genomic DNA, were added into a 96-well plate for real-time polymerase chain reaction (PCR) amplification and the reactions were run for 40 cycles; each cycle consisted of 15s at 95^{o}C for denaturation and 1 min at 60^{o}C for annealing or extension. In order to obtain no-template controls 3µL of DNase-free water was placed into each well instead of DNA. All amplification reactions were performed on the PikoReal Real-time PCR system (Thermo Scientific, Leicestershire, England). Post-PCR plate read and analysis was performed using the allelic discrimination analysis option on the SDS version 2.3 sequence detection software. Scatter plots were created with allele 1 vs allele 2. Each well of the 96-well plate was represented as an individual plot point.

Table 2. Context sequences for TaqMan® SNP genotyping assays

SNP	Gene Name	Chromosome	Context Sequence
rs17216707	CYP24A1	Chr.20: 54115823 on GRCh38	TCAAAATAACTGATCAACTCAGCTA**[C/T]**TTTAATACCAG CGATTCTCACCTAA
rs11741640	RGS14	Chr.5: 177365742 on GRCh38	GGGCTGGGGGAGGAGGGAACCAGGG**[G/A]**CTGCTGGA AGGTACCTGCCATAAGT
rs4075958	RGS14	Chr.5: 177357511 on GRCh38	AGTAGGTATGAGGTAAGTGTGTGCC**[A/G]**AGTAAAGTAC ACGTGTGGATACACA

Single nucleotide polymorphism (SNP)

Statistical analysis

Data was collated on an Excel spreadsheet and analysed using the Statistica™ software package. The continuous variables were expressed as means ± standard deviations (SD) or as medians and interquartile ranges (IQR) and discrete variables reported as percentages.

Frequency distribution of the SNPs was compared between the black and white CKD groups and the control group using the Fisher's exact test or Chi square as appropriate. An independent t-test or Wilcoxon rank-sum test was used to compare variables between the groups and for more than two groups, either an one-way ANOVA or a Kruskal-Wallis test was used. A p <0.05 was considered to be statistically significant.

Results

The sample population of patients with CKD included 178 participants of Black African descent and 72 Caucasian participants. Black African participants were, on average, younger with a mean age of 48 years, whereas Caucasian patients had a mean age of 58 years. Gender groups were similarly represented in both ethnicities in the control as well as CKD participant groups (Table 3).

Hypertension was the most common aetiology for CKD (59.1%) followed by diabetes mellitus (20.9%). The majority of participants with CKD (62%) had stage 5 CKD or ESKD with the rest split between stages 3 and 4.

Since FGF-23 values were not normally distributed, non-parametric tests were used to evaluate differences between groups. FGF-23 values in Black African participants were compared with those of their Caucasian counterparts by means of a two-sample Mann-Whitney U test (Tables 4 and 5). Black African patients had a lower FGF-23 than Caucasian patients (p=0.001), however, although this was significant, the observed standardized effect size was found to be small.

Table 3. CKD Group and Control group characteristics

CKD Group and Control Group Characteristics					
	CKD Group		**Controls**		**P-value**
	N	**%**	**N**	**%**	
Race					P=0.00
Black	178	71	46	54	
Caucasian	72	29	39	46	
Sex					P=0.05
Male	136	54	35	41	
Black African	96	53.9	20	57.1	
Caucasian	40	56.3	15	42.9	
Female	113	45	48	56	
Black African	82	46.1	26	54.2	
Caucasian	31	43.7	22	45.8	
Unspecified	1	0	2	2	
	N	**Mean (SD)**	**N**	**Mean (SD)**	
Age	249	50.7 (14.4)	72	45 (15.6)	P=0.01
Black African	178	48 (12.1)	40	42 (13.0)	
Caucasian	71	58 (11.5)	32	47 (17.8)	

Aetiology of CKD	N	%
Hypertension	130	59.1
Diabetes Mellitus	46	20.9
Idiopathic	14	6.4
Glomerulonephritis	11	5.0
ADPKD	8	3.6
Drug or Toxin related	6	2.7
Obstructive	2	0.9
Myeloma	2	0.9
Schistosomiasis	1	0.5

CKD – Chronic Kidney Disease, N – Sample number, ADPKD – Autosomal dominant polycystic kidney disease.

Table 4. FGF-23 measurements in Black African and Caucasian participants with CKD

	Black African	Caucasian
N	178	71
Females n, (%)	82 (46.1)	31 (43.7%)
Median	100.29ng/mL	235.70* ng/mL
Q1;Q3	35.05 ng/mL;621.2 ng/mL	80.36 ng/mL;1370.5 ng/mL
IQR	586.15 ng/mL	1290.14 ng/mL

*P=0.03, N – sample number, IQR – Interquartile range, Q - Quartile

Table 5. FGF-23 measurements in Black African and Caucasian control participants

	Black African	Caucasian
N	46	39
Females n, (%)	82 (46.1)	31 (43.7%)
Median	20.81 ng/mL	22.87 ng/mL
Q1;Q3	12.56 ng/mL;26.19 ng/mL	15.59 ng/mL;34.12m ng/mL
IQR	13.63 ng/mL	18.53 ng/mL

P=0.20, N – sample number, IQR – Interquartile range, Q - Quartile

Black African and Caucasian participants without CKD had similar circulating levels of FGF-23 but Caucasian individuals with CKD had an FGF-23 level more than two-fold greater than that of their black African counterparts (Figure 1 and 2).

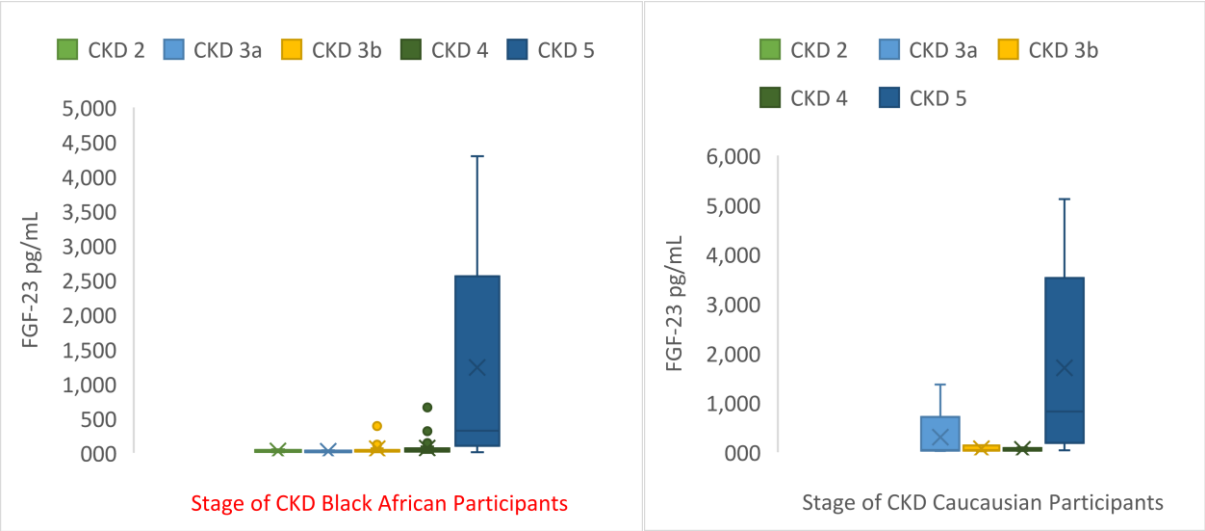


Figure 1. Box and whisker plot showing FGF-23 levels according to stage of CKD in Black African and Caucasian participants.

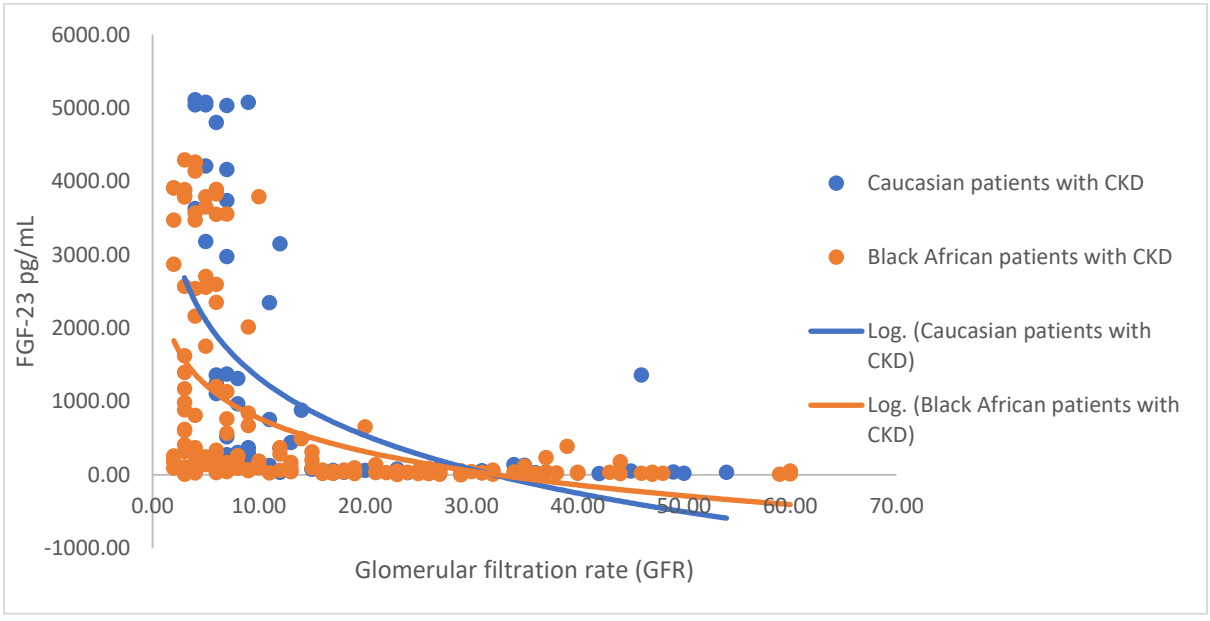


Figure 2. Scatter plot with logarithmic fit lines displaying FGF-23 levels with respect to GFR in Caucasian and Black African individuals with CKD.

When using Spearman's rank order to assess correlation between circulating FGF23 levels and progressively decreasing estimated GFR there was a statistically significant negative correlation in all participants with CKD (Rs= -0.67, p=0.00). There were significant differences in the FGF23 levels by CKD stage (p=0.00), as the estimated GFR worsened there was a corresponding increase in the circulating FGF23 level (Table 6).

There was a significant difference (p=0.03) when comparing the circulating levels of FGF23 between the 3 most prevalent aetiologies of CKD (hypertension, diabetes and idiopathic). The highest median level was for hypertension (176pg/mL), followed by patients with idiopathic aetiology (161 pg/mL) and lastly patients with diabetes had the lowest median level (75 pg/mL).

Table 6 FGF23(ng/mL) levels by CKD stage in all participants.

CKD Stage	N	Median	Inter quartile range	
			25th	**75th**
3a	11	32.2 ng/mL	25.2 ng/mL	350 ng/mL
3b	29	35.05 ng/mL	27.5 ng/mL	60.67 ng/mL
4	51	39.84 ng/mL	30.33 ng/mL	55.23 ng/mL
5	149	372.6 ng/mL	350.25 ng/mL	700.28 ng/mL

Kruskal-Wallis test on FGF23 level at various stages of CKD p=0.00, df 3 *(p<0.05).*
N = sample number, FGF23 – Fibroblast growth factor 23

No statistically significant difference was seen in the prevalence of rs4075958 and rs17216707 in all participants with CKD compared to controls by means of a Chi-Squared test (Table 7 and 9). The SNP rs11741640 was more prevalent in those with CKD compared to those without (Table 9; p=0.04).

Two hundred and forty seven (176 black African and 71 Caucasian) participants with CKD were evaluated for the heterozygous or homozygous minor trait prevalence of single nuclear polymorphisms rs4075958, rs11741640 and rs17216707.

The majority (n=174; 91.6%) of 190 Black African participants with CKD carried the homozygous major alleles for rs4075958. This is in comparison to a smaller proportion (n=48; 66.7%) of 72 Caucasian individuals with CKD with this SNP. This difference was statistically significant by way of a Fisher's Exact Test ($p<0.05$). The odds of being homozygous for the minor allele in this SNP in the Black African group are 5.44 times higher than the odds of being homozygous in the Caucasian group, suggesting a strong association between ethnicity and homozygosity for r4075958.

However, homozygosity for the minor allele in SNP rs4075958 was also more prevalent among the Black African control group (n=43; 87.8%) compared to the Caucasian controls (n=22; 59.5%) (Fisher's Exact Test statistic = 0.0046; $p<0.05$). These SNP prevalence data were also similar to that of the subjects with CKD ($p=0.41$).

Heterozygosity for rs4075958 was present in 36 of 263 individuals with CKD (13.7%) compared to controls (17 of 86; 19.7%). Heterozygosity in Black African and Caucasian controls were no different (Fisher's Exact Test statistic = 0.0573; $p>0.05$), however, a difference was seen between the ethnicities in those with CKD ($p<0.05$); 27.8% (n=20) of Caucasian patients compared to 8.4% (n=16) of Black Africans.

Only 4 Caucasian participants with CKD possessed a minor homozygous trait (0.06%) for rs4075958. This was also present in 4 Caucasian control participants representing a greater proportion (10.8%) of all Caucasian controls but this prevalence was not statistically significantly different ($p=0.45$). No Black African individuals, whether control or with CKD, were found to have this trait.

For the rs11741640 SNP, no study subjects were identified to have a minor homozygous trait. One hundred and seventy (96%) of Black African patients with CKD possessed the major

homozygous trait. Fewer Caucasian individuals (n=41; 63%) with CKD possessed the major homozygous trait (Fisher exact test statistic < 0.00001. The result is significant at p <0.05).

Heterozygosity for rs11741640 was more prevalent among Caucasian patients with CKD (n=22; 33.8%) than Black African patients (n=10; 5.6%) (Fisher exact test statistic < 0.00001; p <0.05). Differences seen between ethnicities were observed in a similar manner among control groups with 23 (60.5%) of Caucasian controls and 41 (91%) of Black African possessing the major homozygous trait. Fifteen (39.5%) Caucasian controls and 4 (8.9%) Black African controls displayed heterozygosity. There no cases of the minor homozygous traits among any groups.

Among individuals with CKD, SNP rs17216707 homozygosity for the minor allele was more prevalent in Black African (n=128; 68.8%) than Caucasian patients (n=22; 35.5%) (Fisher exact test statistic < 0.00001; p < 0.05), whereas heterozygosity was more prevalent in Caucasian (n=36; 58.1%) than in black African (n=57; 30.6%) patients (Fisher exact test statistic 0.0002; p < 0.05).

In control subjects, homozygosity for the minor allele in rs17216707 was, again, found more commonly in Black African patients (n=35; 76.1%) than in their Caucasian counterparts (n=11; 28.2%) (Fisher exact test statistic 0; p < 0.05). There was no difference between the two ethnicities for heterozygous status (p=0.052).

Table 7. SNP rs4075958

		SNP rs4075958		
		Major Allele Homozygous (GG)	Heterozygous (AG)	Minor Allele Homozygous (AA)
Overall CKD Group	N	211	36	4
	%	84%	14%	2%
Control	N	62	17	4
	%	75%	20%	5%
Total	N	273	53	8
	%	82%	16%	2%

Chi-Square Tests

	Value	df	Sig.
Pearson Chi-Square	4,86	2	0,09

Table 8. SNP rs11741640

		SNP rs11741640	
		Major Allele Homozygous (GG)	Heterozygous (AA)
Overall CKD Group	N	209	32
	%	87%	13%
Control	N	65	19
	%	77%	23%
Total	N	274	51
	%	84%	16%

Chi-Square Tests

	Value	df	Sig.
Pearson Chi-Square	4,11	1	0,04

Table 9. SNP rs17216707

		SNP rs17216707		
		Major Allele Homozygous (TT)	Heterozygous (CT)	Minor Allele Homozygous (CC)
Overall CKD Group	N	149	93	5
	%	60%	38%	2%
Control	N	44	38	1
	%	53%	46%	1%
Total	N	193	131	6
	%	58%	40%	2%

Chi-Square Tests

	Value	df	Sig.
Pearson Chi-Square	1,83	2,00	0,40

Single nucleotide polymorphism - SNP, CKD – Chronic kidney disease, N – sample number

The analysis of all three of the SNPs within the CKD group, demonstrated that there were very few cases of homozygosity in the minor trait overall.

The following biochemical markers including the serum levels of calcium, magnesium, phosphate and 25-hydroxyvitamin D were used to asses for association with FGF23 levels. Of all these biochemical markers the only one found to have a significant association with FGF23 levels was phosphate (p=0.00).

Discussion

Chronic kidney disease is an important cause of mortality and a major public health problem worldwide [9]. Of the individuals living with end-stage kidney disease, world-wide, 70% of them are expected to be residing in a low-income country by 2030 [10]. Making it an important area of focus for research in Africa. Our current understanding of the inter-relationship between calcium, phosphate, FGF23, klotho and PTH is limited by the complex nature of the system as well as the rapid changes that occur when one aspect is altered [11]. The secondary hyperparathyroidism, which is a fundamental aspect of CKD-MBD, as well as the raised serum FGF23 levels that occur in patients with declining renal function are important risk factors for cardiovascular disease and mortality [3].

As renal function deteriorates there is a corresponding increase in the prevalence of hypertension, this may be attributed to the rising phosphate as well as rising FGF23 levels [12]. The ARIC study found an increased risk in the development of hypertension with rising FGF23 levels which was independent of renal function [13]. Thus, rising FGF23 levels can be considered a causal factor in the development of hypertension. In our study we found that hypertension, as an aetiology for CKD, was correlated with the highest median levels of

FGF23. This supports the existing data regarding this relationship and emphasises the importance of targeting FGF23 as a therapeutic approach in reducing cardiovascular morbidity in all patients with CKD.

In a recently reported GWAS, 5 novel loci were identified and found to have an association with FGF23 serum levels suggesting that factors outside of the FGF23 gene itself or factors directly affecting phosphate, calcium, vitamin D and PTH pathways may be involved [7]. The minor allele frequencies for three of the statistically significant SNPs (rs17216707, rs4075958, rs11741640) identified in the GWAS were found to be 0.028, 0.06 and 0.056 respectively in the Yoruba population of Nigeria. Of the three identified SNPs two were more prevalent in the Black African participants, however, there was no significant difference between the CKD group and the control groups. The number of individuals, whether Black African or Caucasian, in our sample who were homozygotes in the minor alleles for any of the three SNPs was too small to find a statistically significant association between its presence and the circulating FGF23 levels, neither was there an association with heterozygosity in any of the three SNPs. This finding is not in keeping with a previous study done by Robinson-Cohen *et al* that found an association between two SNPs and FGF23 levels, although the sample group was made up entirely of patients of White European ancestry [14]. In a GWAS done in a wholly indigenous Taiwanese population the SNPs found in the Robinson-Cohen *et al* study were also not found to be associated with CKD [15].

This suggests that in the South African Black population other factors, or SNPs, are more likely to be involved in the development of CKD-MBD. The geolocation of patients may have an impact on epigenetic expression that could provide a mechanistic basis to understand how several different SNPs are associated with the development of CKD in different areas. As race

in itself is unlikely to be the only explanation for the differences found between the two GWAS studies and our results.

Our results showed that FGF23 levels in Black African participants were lower than what was found in Caucasian participants. To further investigate the role played by FGF23 in our population and CKD-MBD the correlation between serum FGF23 levels and biomarkers of CKD-MBD were explored. A significant association was found between FGF23 and phosphate, which supports our existing understanding of the role FGF23 plays in phosphate excretion in the kidney and the resulting inhibition of FGF23 mediated reduction in phosphate reabsorption in the late stages of renal failure [12] [4]. This was further extended to include calcium and 25-hydroxyvitamin D but interestingly no association was found with either. A significant association was found between FGF23 levels and serum creatinine which is also in keeping with existing knowledge with regards to this relationship. When it was further evaluated according to CKD stage it was noted that as the eGFR declines there was a progressive increase in FGF23 levels, the highest noted at CKD stage 5. This result is explained by the fact that FGF23 is excreted via the kidneys thus increasing serum FGF23 levels will occur as eGFR worsens. The number of patients within the CKD stage 5 group consisted of 37.3% of the total number of CKD patients studied. The total number of patients on dialysis in the CKD group was 57.2% the majority of which were in the CKD stage 5 group. The current understanding of the elimination of FGF23 is via enzymatic degradation [4], this result raises the possibility that dialysis may have an influence on circulating FGF23 levels and warrants further investigation.

Limitations

There are several limitations to this study, most importantly is the lack of a definitive bone biopsy to concretely establish the diagnosis of CKD-MBD in our patients. Additionally, as this

is a cross sectional study the temporal relationship between FGF23 and CKD-MBD and its relationship to genetic polymorphisms cannot be established. A longitudinal study would thus be needed to further assess this relationship.

Conclusions

In attempting to further understand the complex relationship governing the development of CKD-MBD this study investigated 3 SNPs previously identified as being associated with FGF23 levels in the serum of Caucasian patients of European descent. In this study no correlation was found, suggesting other factors play a role in the development of CKD. This study has further added to the existing data supporting the association between hypertension, increased phosphate levels and FGF23 emphasising the importance of targeting FGF23 as a biomarker for assisting in therapeutic intervention decision making in patients with worsening of renal function.

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Chapter 3: Appendices

Appendix 1

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

Patients Data Sheet Serial Number: ____

Hospital No: _____

Phone No._____

1. Age at last Birthday (Years) _____
2. Sex: Male ( ) Female ( )
3. Ethnicity _____ Black ( ) White ( )
4. Marital status:Single ( )Married ( ) Widow/Widower ( ) Separated/Divorced ( )
5. Occupation _____
6. History of bone pain in the last one month (a) Yes (b) No
7. Presence of Hypertension: (a) Yes (b) No
8. Do you have diabetes mellitus? (a) Yes (b) No
9. Drug history :

(a) Calcium supplement _____ i.Duration (months)._____Dose____

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

(c) Others_____

10. Aetiology of CKD_____
11. Do you smoke cigarette? (a) Yes (b) No
12. Are you on dialysis (a) Yes (b) No
13. If answer to 12) is yes what type

(a) Peritoneal (a.i) duration (months)_____

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

Clinical Parameters

1) Weight (Kg) _____
2) Height (m) _____
3) BMI (Kg/m2) _____
4) Blood Pressure (mmHg) _____

Investigations

Parathyroid hormone (PTH)_____

Fibroblast growth factor 23 (FGF23) _____

Albumin _____

Calcium _____

Phosphate _____

Ca× PO4 _____

Total alkaline phosphate (TAP) _____

25-OH vitaminD_____

Serum Creatinine (µmol/L) _____

Urea (mmol/L) _____

Total Cholesterol_____

LDL _____ HDL_____ TG _____

Estimated GFR: _____

Appendix 2
Ethics Clearance form M141016

R14/49 Dr Bala Waziri

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M141016

NAME: (Principal Investigator)	Dr Bala Waziri
DEPARTMENT:	Internal Medicine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Markers of mineral bone disease in Black South Africans with chronic kidney disease
DATE CONSIDERED:	31/10/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Saraladevi Naiker
APPROVED BY:	Professor PE Cleaton-Jones Chairperson, HREC (Medical)
DATE OF APPROVAL:	05/12/2014

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix 3
Ethics Clearance form M190576

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Allyson Peddle

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190576

NAME: (Principal Investigator)	Dr Allyson Peddle
DEPARTMENT:	Internal Medicine Department of Molecular Medicine Laboratory
PROJECT TITLE:	Association of genetic polymorphisms in the fibroblast growth factor 23 gene with fibroblast growth factor 23 levels in South African patients with chronic kidney disease
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	A Sub-study under Primary Study M141016 Dr Bala Waziri
SUPERVISOR:	Dr NU Nqebelele
APPROVED BY:	Dr. CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	30/05/2019

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 on the Third Floor, Faculty of Health Sciences, Phillip 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 **May** and will therefore be due in the month of **May** 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

Appendix 4
Author Guidelines for the South African Medical Journal

Guidelines to Research articles
Main article
All articles are to include the following main sections: Introduction/Background, Methods, Results, Discussion, Conclusions.
The following are additional heading or section options that may appear within these:
• Objectives (within Introduction/Background): a clear statement of the main aim of the study
and the major hypothesis tested or research question posed
• Design (within Methods): including factors such as prospective, randomisation, blinding, placebo control, case control, crossover, criterion standards for diagnostic tests, etc.
• Setting (within Methods): level of care, e.g. primary, secondary, number of participating centres.
• Participants (instead of patients or subjects; within Methods): numbers entering and completing the study, sex, age and any other biological, behavioural, social or cultural factors
(e.g. smoking status, socioeconomic group, educational attainment, co-existing disease indicators, etc)that may have an impact on the study results. Clearly define how participants were enrolled, and describe selection and exclusion criteria.
• Interventions (within Methods): what, how, when and for how long. Typically for randomised
controlled trials, crossover trials, and before and after studies.
• Main outcome measures (within Methods): those as planned in the protocol, and those ultimately measured. Explain differences, if any.
Results
• Start with description of the population and sample. Include key characteristics of comparison
groups.
• Main results with (for quantitative studies) 95% confidence intervals and, where appropriate,
the exact level of statistical significance and the number need to treat/harm. Whenever possible, state absolute rather than relative risks.
• Do not replicate data in tables and in text.
• If presenting mean and standard deviations, specify this clearly. Our house style is to present
this as follows:
• E.g.: The mean (SD) birth weight was 2 500 (1 210) g. Do not use the ± symbol for mean (SD).
• Leave interpretation to the Discussion section. The Results section should just report the findings as per the Methods section.
Discussion
Please ensure that the discussion is concise and follows this overall structure – sub-headings are not needed:
• Statement of principal findings
• Strengths and weaknesses of the study

• Contribution to the body of knowledge
• Strengths and weaknesses in relation to other studies
• The meaning of the study – e.g. what this study means to clinicians and policymakers
• Unanswered questions and recommendations for future research

Conclusions

This may be the only section readers look at, therefore write it carefully. Include primary conclusions and their implications, suggesting areas for further research if appropriate. Do not go beyond the data in the article