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THE EVALUATION OF FIBROBLAST GROWTH FACTOR 23 (FGF-23) IN PATIENTS WITH HOMOZYGOUS FAMILIAL HYPERCHOLESTEROLAEMIA

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of Master of Medicine in the branch of Internal Medicine

Johannesburg, 2017

Declaration

I, Jarrod Mario Zamparini, do hereby declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Internal Medicine at the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____

Jarrod Mario Zamparini
Signed this 6th day of October 2017

Dedication

In loving memory of my
grandmother, May,
who lit the spark that started it all.

Abstract

The Evaluation of Fibroblast Growth Factor 23 (FGF-23) in Patients with Homozygous Familial Hypercholesterolaemia.

Background

Patients with Homozygous Familial Hypercholesterolaemia (HoFH) experience significant vascular calcification early in life, the cause of which appears to be multifactorial but is, as yet, not fully understood. Patients with chronic kidney disease (CKD) undergo similar vascular calcification, with fibroblast growth factor (FGF-23) implicated in the vascular calcification observed in these patients.

Objectives

The objectives of the study were to determine whether there was a difference in FGF-23 between patients with HoFH and age- and gender-matched controls and to determine whether there is a correlation between serum low-density lipoprotein (LDL) cholesterol and serum FGF-23 in patients with HoFH.

Methods

The study was a cross-sectional review involving 30 patients with HoFH, who follow up at the Charlotte Maxeke Johannesburg Academic Hospital Lipid Clinic, as well as 30 age- and gender-matched controls. Serum was analysed to obtain measures of FGF-23, Total Cholesterol, LDL cholesterol, calcium and phosphate. B-mode ultrasonography of the carotid arteries was carried out on the patient cohort. All data collected were analysed according to the study objectives.

Results

Thirty patients with HoFH were included in the study as well as 30 age- and gender-matched controls. There was no statistically significant difference in mean FGF-23 between the patient and control groups (62.07 ± 26.42pg/ml

versus 63.69 ± 19.84pg/ml; p=0.4621) nor was there any statistically significant correlation between FGF-23 and LDL Cholesterol (p=0.9483 and 0.8474), Total Cholesterol (p=0.9261 and 0.859), calcium (p=0.6187 and 0.4321) or phosphate (p=0.4081 and p=0.3575) for patient and control groups respectively. In the patient group, FGF-23 did not correlate significantly with any cardiovascular disease including premature coronary artery disease (p=0.4516), aortic valve replacement (p=0.4791) or carotid artery calcification (p=0.3061).

Conclusion

Serum FGF-23 is not elevated in patients with HoFH when compared to non-FH age- and gender-matched controls and there is no statistically significant correlation between serum FGF-23 and cardiovascular disease in patients with HoFH.

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Writing a research report for a Master's degree is no mean feat. The research and write-up takes not only a toll on the author but also on the family, friends and colleagues that become passengers on the ride to completion. With this in mind I'd like to thank:

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And finally, to the Good Lord. "For with God nothing shall be impossible." (Luke 1:37, KJV)

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Abbreviations and Acronyms

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
ApoB-100	Apolipoprotein B100
BMD	bone mineral density
CaAC	carotid artery calcification
CABG	coronary artery bypass graft
CIMT	carotid intima-media thickness
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CoAC	coronary artery calcification
CRIC	Chronic Renal Insufficiency Cohort
CT	computed tomography
dL	decilitre
EAS	European Atherosclerosis Society
ER	endoplasmic reticulum
ESRD	end-stage renal disease
FDA	Food and Drug Administration
FGF-23	Fibroblast Growth Factor 23
FGFR	FGF receptor
FGFs	Fibroblast Growth Factors
FH	Familial Hypercholesterolaemia
GFR	glomerular filtration rate
HeFH	heterozygous FH
HMG-CoA	hydroxy-3-methylglutaryl-coenzyme A
HoFH	homozygous FH
IDL	intermediate-density lipoprotein
L	litre
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
LDLRAP1	LDL receptor adaptor protein 1
Lp(a)	Lipoprotein (a)
LPL	lipoprotein lipase
LVH	left ventricular hypertrophy
m	metre
min	minutes
ml	millilitre
mmol	millimoles
NICE	National Institute for Health and Clinical Excellence
nm	nanometre
PCSK9	proprotein convertase subtilisin/kexin 9
pg	picogram
PIVUS	Prospective investigation of the vasculature in Uppsala seniors

PTH	parathyroid hormone
US-MEDPED	United States Make Early Diagnosis to Prevent Early Death
VLDL	very-low-density lipoprotein

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1. Introduction

1.1 Background

Familial Hypercholesterolaemia (FH) is an autosomal dominant disorder resulting from mutations that affect the function of the low-density lipoprotein receptor (LDLR). The prevalence of FH in certain South African population groups is much greater than the global prevalence.[1,2] FH can be divided into two broad categories: the more common heterozygous FH (HeFH) and the much less common but more severe homozygous FH (HoFH).[3]

HeFH patients with LDLR gene mutations express approximately half the number of LDL receptors on cell surfaces compared to non-affected individuals. They thus tend to have an elevation of their low-density lipoprotein cholesterol (LDL-C) to approximately double the normal level resulting in deposition of cholesterol in arterial walls, the skin and tendons.[3,4]

HoFH patients with mutations in both LDLR genes express few if any LDL receptors on their cell surfaces resulting in extremely elevated LDL-C levels (often five to six times normal). These patients develop premature ischaemic heart disease and, if not identified and treated, have been known to die from fatal myocardial infarctions before the age of 20.[3]

In addition to the typical signs and symptoms resulting from elevated LDL-C, patients with HoFH undergo premature vascular calcification – with calcified valvular or supra-valvular aortic stenosis representing a major complication of this process.[2] Given the young age of these patients, it is unlikely that the calcification is related to age.[4,5] Hypercholesterolaemia is known to accelerate vascular calcification by acting through vitamin D_3 to induce calcium deposition in vascular smooth muscle.[6]

Fibroblast Growth Factor 23 (FGF-23) is a hormone, derived from bone, that is important in vitamin D and phosphate metabolism. Raised FGF-23 levels have traditionally been observed in patients with CKD and this rise has been

associated with increased cardiovascular risk in these patients. Raised FGF-23 levels have been associated with "total body atherosclerosis",[7] left ventricular hypertrophy (LVH) and coronary artery calcification (CoAC).[8] However the role that FGF-23 plays in premature vascular calcification in HoFH is unknown.

1.2 Familial Hypercholesterolaemia

1.2.1 Overview

Lipoprotein metabolism is a broad subject and its importance continues to grow given the increasing prevalence of dyslipidaemia, particularly hypercholesterolaemia. A full review of lipoprotein metabolism is beyond the scope of this research but it is important to touch on one of the five key lipoproteins, that is LDL and its receptor. LDL is an 18-25nm particle that is formed when triglycerides associated with very-low-density lipoprotein (VLDL) are hydrolysed by lipoprotein lipase (LPL) in the vascular endothelium, heart, muscle and adipose tissue leaving intermediate-density lipoprotein (IDL). IDL subsequently loses more triglyceride, by hepatic lipase, rendering LDL, which can then be removed from circulation by the liver (~70%) or taken up by peripheral tissues. This is done through endocytosis of LDL via the LDLR which recognises apolipoprotein B100 (ApoB-100), a key apolipoprotein in the LDL molecule.[9]

FH is an autosomal dominant disorder that results in abnormally elevated serum LDL-C.[1] Carl Müller, a Norwegian physician, first described FH in a paper published in October 1939 where it was hypothesised that hypercholesterolaemia, tendon xanthomata and myocardial infarction were linked via an autosomal dominant "inborn error of metabolism". Twenty-five years later, in 1964, Khachadurian classified FH into its two subclasses, Heterozygous FH (HeFH) and Homozygous FH (HoFH).

HeFH is the more common subtype affecting 1 in every 200 to 500 persons worldwide. HeFH usually results from a mutation in one of the LDLR genes.

As only one LDLR gene exhibits a mutation, patients express approximately half the number of LDL receptors on cell surfaces compared to non-affected individuals and thus tend to have an elevation of their LDL-C to approximately double the normal level.[3,4] Deposition of cholesterol in the arterial walls, skin and tendons leads to the classic clinical features of HeFH, namely tendon xanthomata, cutaneous xanthelasma and arcus corneus as well as the premature coronary heart disease typical of dyslipidaemia in general.[4]

HoFH is far less common affecting 1 in 300 000 to 1 million persons worldwide. Patients effectively express few if any LDL receptors on their cell surfaces and thus have extremely elevated LDL-C levels (often five to six times normal). These patients develop the classical clinical features described above, albeit much earlier, and, if not identified and treated, suffer fatal myocardial infarctions before the age of 20 as a result of the accelerated atherosclerotic cardiovascular disease.[4]

In 1974, Goldstein and Brown demonstrated the LDL receptor for the first time, and eleven years later, in 1985, isolated the LDL receptor gene – a cell surface receptor gene located on chromosome 19. [9–11] Mutations* to this gene result in FH. By the time of publication of an article by Watts et al. in 2014, over 1200 different mutations to the LDLR gene had been described and it was estimated that approximately 20 million people worldwide were affected by FH.[12]

These 1200 plus mutations are categorised into six classes:[13,14]

Class 1: The "null allele"[13] – failure of receptor synthesis or absence of precursor protein

Class 2: Ineffective transport of receptor protein between ER and Golgi apparatus

Class 3: Normal synthesis and transport of protein but binding of LDL to the LDL receptor is ineffective

* If HoFH patients express the same mutation on both copies of the LDL receptor gene, they are said to be true homozygotes. If mutations are present on both genes but they are different the patient will be a compound homozygote.

Class 4: Inability to internalise the LDL receptor by clathrin-coated pits

Class 5: Inability to recycle receptors (results in degradation of the LDL receptor within the cell)

Class 6: Failure of the LDL receptors be sent to the basolateral membrane

Despite mutations to the LDLR gene accounting for the majority of cases of FH, mutations in other genes encoding key steps in LDL-uptake have also been described which result in a similar clinical picture, for instance, mutations of apolipoprotein B (ApoB) and proprotein convertase subtilisin/kexin 9 (PCSK9).[10] In addition to these, a mutation to the LDL receptor adaptor protein 1 (LDLRAP1) gene has also been described. This gene mutation also causes a hypercholesterolaemic clinical picture; however, its inheritance is recessive making it unique to the other mutations previously described.[15]

1.2.2 Genetics

The prevalence of FH in certain populations is much greater than the global prevalence due to the Founder Effect. This effect develops when a population is descended from a small group of initial founders that remained "genetically isolated" for a long period of time.[2,16] This leads to decreased genetic diversity and increased expression of previously rare alleles.

These population groups include Afrikaners, South African Jews and Indians, Lebanese, French Canadians, Tunisians and Finns. The prevalence of FH is much higher in these populations – up to tenfold higher in Lebanese populations, between 1:81 and 1:157 in French Canadians and approximately 1:600 in Tunisia.[17]

In South Africa, the Afrikaner population is mainly descended from the initial Dutch settlers to the country leading to a Founder Effect. The Afrikaner population grew much faster than other European populations in South Africa – as much as one thousand times in the space of three-hundred years.[18] As a

result, Afrikaners exhibit FH prevalence rates of approximately 1:100[19] although rates as high as 1:67 were described in a population in rural south-western towns in the then Cape Province.[20] The major LDL Receptor mutations in Afrikaners have been identified and subsequently named as Afrikaner-1, -2 and -3 (FH1, FH2 and FH3 respectively). Not surprisingly, all three mutations have been detected in patients with FH in the Netherlands.[18,21]

South African Jews and South African Indians also have elevated prevalence rates of FH, also quoted to be 1:100. The Jewish population in South Africa is mainly descended from a number of Lithuanian immigrants who settled in the country at the end of the 19th century and the prominent mutation in this population is FH Piscataway. This mutation is the cause of FH in most Ashkenazi Jews around the world and appears to be due to a Founder Effect in Lithuania.[18]

1.2.3 Diagnosis

The diagnosis of FH is commonly missed and often only detected after a patient suffers a cardiovascular event, such as a myocardial infarction, at a younger age than expected.[10] With regard to HoFH, patients who have had myocardial infarcts at ages as young as 4 years old have been described in the literature, as well as patients as young as 10 years old undergoing coronary artery bypass grafting.[22]

Criteria for the diagnosis of both types of FH have not been standardised worldwide resulting in the existence of various guidelines and scoring systems.[22] In a 2012 review article by Raal and Santos, more than 10 articles were tabulated, each with different guidelines on the diagnosis of HoFH.

A family history is very important in the diagnosis of FH as index patients may have family members who suffered premature cardiovascular events or that had been previously diagnosed with FH.[15] Given the heritable nature of HoFH, it is clear that both parents of a patient with HoFH will have HeFH.

Early diagnosis through screening relatives is now recommended by the National Institute for Health and Clinical Excellence (NICE) in the United Kingdom – they suggest "cascade testing" of relatives once the diagnosis is made in any patient.

Despite numerous guidelines existing, there is some consensus on the clinical findings in patients with FH – tendon and cutaneous xanthomas, and arcus cornealis [10] – and while these features are not found exclusively in patients with FH they are highly suggestive of the diagnosis.[10,15,22] Tendon xanthomas tend to occur over the calcaneal tendons as well as the dorsal surface of the metacarpophalangeal joints. Interdigital xanthomas have also been described and are pathognomonic of HoFH. The xanthomas and arcus cornealis present in the first two decades of life are easily detectable signs of HoFH and are an indicator of the cholesterol deposition that is ongoing in the rest of the body.[22] These patients tend to also have deposition of atherosclerotic plaques as well as cholesterol deposition in the aortic valve root and cusps – discussed in detail later.

Various other guidelines exist for the diagnosis of HoFH but the three most common ones are the Simon Broome Registry in the UK, the Dutch Lipid Clinic Network Criteria in the Netherlands and the United States Make Early Diagnosis to Prevent Early Death (US-MEDPED) criteria in the USA. All three of these guidelines use a combination of clinical features, genetic confirmation and lipid profiles in their criteria. They are easily available through multiple sources online.[10,15]

The general consensus in terms of biochemical diagnosis of HoFH is as follows:[10,15,22]

1. Untreated LDL-C > 13mmol/L or >11mmol/L† in children
2. Treated LDL-C > 7.7mmol/L[22] to 8mmol/L[15] (depending on guideline)
3. Non-HDL cholesterol > 8.5mmol/L

† The reason for the lower cut-off in children is due to the lower cholesterol levels in children when compared to adults – for instance up to 50% of children in the Netherlands with HoFH have an LDL cholesterol level of between 5.6 and 9.8mmol/L.[15]

It is important to note that clinical features play a significant role in the diagnostic criteria of HoFH as seen in the European Atherosclerosis Guidelines in Table 1.1 below.

Table 1.1 – European Atherosclerosis Guidelines for the Diagnosis of HoFH[10,15]

1	Two mutant alleles at the LDLR, ApoB, PCSK9 or LDLRAP1 Gene
OR	
2	Untreated LDL cholesterol >13mmol/L or treated >8mmol/L
PLUS	
3	Cutaneous or tendon xanthomas at an age earlier than 10 years
OR	
4	Untreated LDL cholesterol levels as in (2) in both parents

Most authors no longer suggest using the "treated LDL" reference due to the multitude of lipid-lowering therapies now available for patients with FH. This is made clear by a 2013 trial that had at least one patient with HoFH on a number of agents with an LDL-C of 3.9mmol/L.[23]

While genetic testing may be useful as it can provide a definitive diagnosis, it is not necessary to make the diagnosis of FH and may even miss the diagnosis of HoFH, as a patient may have a genetic mutation that has not been previously described.[15,22]

1.2.4 Treatment

The goal of treatment for both types of FH is to reduce the incidence of cardiovascular disease caused by atherosclerosis.[10]

Lifestyle modification, such as a diet low in saturated fats and cholesterol, and exercise, are important to implement in patients with FH, as is addressing additional cardiovascular risk factors (e.g. hypertension, diabetes, obesity and smoking). However, the benefit of this is not sufficient to lower LDL-C levels in patients with FH.[10,15]

Medical therapy with hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, commonly known as statins, remain the mainstay of treatment for FH and should be initiated as early as possible to delay onset of cardiovascular disease.[15,22] This is especially true for HoFH patients. Despite statins having been found to be safe at high doses, there is minimal benefit to statin therapy alone in HoFH, given that the average reduction in LDL-C levels is, on average, 15% in receptor negative HoFH patients and 26% in receptor deficient patients.[22] Nevertheless both the European Atherosclerosis Society (EAS) and the American College of Cardiology Guidelines recommend statins as first line therapy.[15,24] The reduction in LDL-C levels can be enhanced by a further 10-20% by adding the cholesterol absorption inhibitor ezetimibe to current statin therapy.[10,15,22]

Niacin is currently recommended in both the United States and Canadian guidelines although its use has declined in Europe following the publication of two trials (AIM-HIGH and HPS2-THRIVE) which both showed no benefit in the addition of niacin versus placebo. Addition of bile acid sequestrants and fibrates has also been shown to be helpful, but they are not very effective in lowering LDL-C in FH.

The Food and Drug Administration (FDA) and European Medicines Agency have recently approved Lomitapide for use for use as adjunctive therapy in patients with HoFH older than 18 years. This drug in addition to the standard of care, at open label trial, showed a reduction in LDL-C of approximately 50% at 26 weeks with sustained LDL-C reduction at one year. Mipomersen, also recently approved by the FDA, had promising reductions in LDL-C vs placebo (mean reduction of 26%) although its use is limited by its administration as a subcutaneous injection with up to 76% of patients reporting injection-site reactions. The monoclonal antibody PCSK9 inhibitors are a very recent addition to the lipid-lowering arsenal and have shown benefit in both HeFH and HoFH patients.[15]

Lipoprotein apheresis remains an important therapy for lipid-lowering in FH patients and, if available, it remains the treatment of choice for HoFH patients

in the USA, Japan and Europe.[22] The treatment needs to be undertaken weekly or fortnightly and, while LDL-C reductions of 55-70%[15] are seen after a single session of therapy, its use is limited due to availability, cost and the time-consuming nature of apheresis. The current EAS guidelines recommend apheresis treatment in all patients with HoFH starting between ages 5 and 8 years.[15]

Finally, liver transplantation is highly effective in the treatment of HoFH as it corrects the phenotypic defect in the organ that is the most active in clearing LDL-C. This is obviously limited by the availability of donors, the cost of transplantation and the need for lifelong immunosuppressive therapy.[15]

1.2.5 Complications

FH patients develop tendon xanthomas, typically in the Achilles tendon, caused by deposition of cholesterol into the tendon and its sheath. This deposition leads to thickening of the tendon, which may interfere with function and, in some cases, can lead to tendonitis and pain in the associated joints resulting in decreased quality of life. Spontaneous rupture of the xanthomatous tendon has been described, albeit rarely.[15,25] Rare cases have also been reported of cerebral, mediastinal and muscular deposition of cholesterol xanthomas.[15] By and large, the majority of complications of FH are vascular in nature.

Before the advent of therapy, FH patients would classically develop significant cardiovascular disease much earlier than non-FH patients.[4] HeFH patients would suffer from fatal myocardial infarctions and cerebrovascular events during the fourth decade of life, in males, and the fifth decade of life, in females.[10]

In FH patients, the prolonged exposure to markedly elevated LDL-C, which can be calculated as the cholesterol year score, leads to early deposition of cholesterol in vascular beds.[15] There is also a significant correlation between non-HDL cholesterol and atherosclerosis, showing a linear relationship with

an increase in atherosclerosis equal to one year of ageing with every 0.25mmol/L increase in non-HDL cholesterol.[26] The UK's Simon Broome Registry has data that revealed a standardised relative mortality rate of 48 and 125, in men and women respectively, in the third decade of life (before the advent of statin therapy). This risk is also evidenced by the fact that untreated HoFH patients rarely survive into the third decade of life.[15]

The quadrupled level of plasma LDL-C at birth in patients with HoFH leads to atherosclerosis in all arterial beds from early on in life.[14] This "accelerated atherosclerosis"[15] is not limited to the coronary vasculature though; typically, the carotid arteries, renal arteries, aortic valve cusps, aortic root and descending aorta are all affected.[14,15]

The widespread atherosclerosis poses the risk of plaque build-up with subsequent thrombosis, leading to myocardial infarction. In addition to this, the atherosclerotic plaque produces inflammatory cells and mediators that interact with vascular cells capable of osteogenic differentiation in turn leading to the vascular calcification seen in these patients.[27]

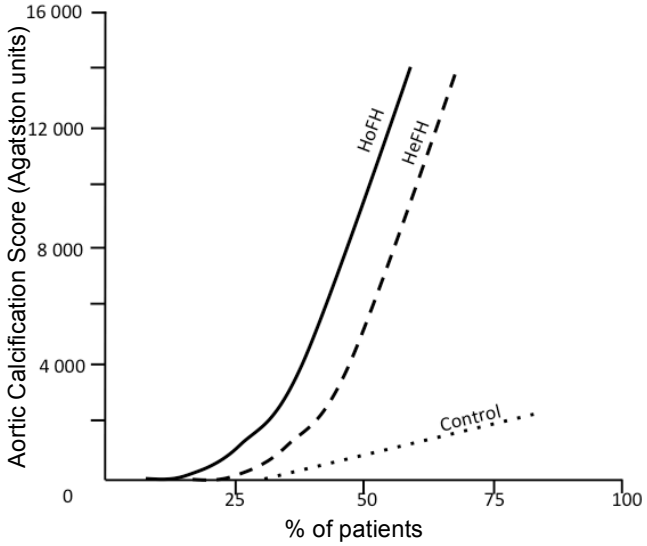
While FH is not associated with decreased bone mineral density (BMD) or disturbed calcium homeostasis, it has been hypothesised that these patients have modified endocrine bone function with reduced bone formation and reduced excretion of calcium leading to deposition of calcium in vascular tissue.[28] Importantly, the distribution of vascular calcification in these patients tends to be localised to the intima of blood vessels as opposed to the medial calcification (Monckeberg's sclerosis) seen in those with end-stage renal disease (ESRD), the elderly and diabetics.[28] Nevertheless, both types of calcification are associated with increased cardiovascular morbidity and mortality.[27]

CoAC, which can be detected and scored by computed tomography (CT) scanning, is an early marker of coronary artery atherosclerosis and, together with carotid intima-media thickness (CIMT) can be used as surrogate markers

for atherosclerosis in other vessels. Wiegman et al. report that 25% of 11- to 23-year-old patients with HeFH have coronary calcification whereas it is barely, if ever, detected in the same age group in the general population.[26]

The aorta is significantly affected by the vascular calcification seen in FH patients with an "age- and gene-dosage dependent increase in aortic calcification"[27] (Figure 1.1).

Figure 1.1 – Aortic Calcification



Aortic calcification (represented by an aortic calcification score) versus age in HoFH, HeFH and Control patients. Adapted from Fantus et al

While aortic calcium scores are strongly correlated with age, there is poor correlation with total cholesterol.[4,27] The extensive calcification seen particularly in HoFH, leads to surgical challenges at the time of aortic valve and coronary surgery, which is significant considering the need for this type of surgery in HoFH patients. In *Awan's* cohort of 25 HoFH patients, followed up for a mean of 18 years (range= 2 – 39), 45% (n = 9) required coronary artery bypass graft (CABG) surgery. In the same cohort, 11 patients had either aortic stenosis or aortic root calcification and 55% (n=6) required aortic valve replacement. All but the two youngest patients (aged 13 and 14 years) in this cohort had calcification of the aorta – 92%.[4] Similarly, all seven patients in Allen's cohort of HoFH patients had aortic root narrowing indicative of calcification.[29] This is significant considering the incidence of aortic

calcification in the general population in the Western world is only 3% in adults older than 75 years.[27]

Statins have been shown to have a procalcific effect during in vitro studies; however, the effect of statins in vascular calcification is unclear with a number of studies revealing conflicting results. A recent study, to assess the effect of statin therapy on coronary calcification, reviewed 3495 patients on either high dose, low dose or no statin therapy across eight clinical trials (all investigating the "impact of medical therapies on serial changes in coronary atheroma burden using IVUS."[30]) The study found that those in both the high-dose and low-dose statin therapy groups had greater progression of CoAC when compared to those in the no statin group.[30]

1.3 Fibroblast Growth Factor 23 (FGF-23)

1.3.1 Overview

The FGFs are a diverse group of proteins responsible for a number of functions in mammalian species. There are 22 different FGFs that have been described in humans, FGF1 – 23, which are subsequently grouped into seven subfamilies. There is no FGF-15 in humans.[31]

FGF-23 is the most recently discovered of the FGFs. Yamashita et al. originally identified it in humans and mice in 2000, when it was found to be the gene responsible for autosomal dominant hypophosphataemic rickets. It is a member of the FGF-19 subfamily, 30kDa in size and is encoded on chromosome 12 in humans.[32]

The FGFs other than FGF-23 act by binding heparin sulphate in the extracellular matrix allowing them to act in a paracrine and autocrine manner. FGF-23, however, has a difference in its heparin-binding region making it less susceptible to binding, and thus capture, in the extracellular matrix. This allows it to act as an endocrine hormone by binding to the FGF receptor (FGFR) and its co-receptor in the kidney where it exerts its effects.[31,33]

Klotho, or α-klotho, is FGF-23's co-receptor in the kidney and plays an important role in binding FGF-23 to the FGFR. It was discovered in 1997 in α-kl mutant mice. These mice expressed accelerated ageing-related disorders (e.g. hearing loss, ectopic calcium deposition, decreased BMD, etc.) and thus the gene was named Klotho – the name of one of Zeus' three daughters responsible for lifespan in Greek mythology.[31]

FGF-23 is expressed by osteocytes and osteoblasts in response to both an oral phosphate load and an increase in 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). This leads to increased expression of FGF-23 with binding to the FGFR and Klotho in the kidney resulting in decreased expression of sodium-phosphate cotransporters in the proximal renal tubule and subsequent phosphaturia. FGF-23 also decreases serum concentrations of $1,25(OH)_2D$ by decreasing concentrations of 1-α-hydroxylase (the enzyme responsible for conversion to $1,25(OH)_2D$ in the kidney).[33–35] This increase in phosphaturia and decrease in serum $1,25(OH)_2D$ results in hypocalcaemia with subsequent increase in parathyroid hormone (PTH) concentrations and further enhanced phosphaturia.[35]

In CKD, there is decreased capacity for renal excretion of phosphate and a subsequent increase in the serum concentration of FGF-23. Initially, concentrations of FGF-23 are only minimally increased but, as glomerular filtration rate (GFR) declines, FGF-23 continues to increase and can be elevated up to one thousand-fold in ESRD. This is important considering the dose dependent association between FGF-23 and cardiovascular morbidity and mortality.[33]

1.3.2 FGF-23 and Cardiovascular Disease

Elevated serum levels of FGF-23 have been shown to be an independent risk factor for both an increase in left ventricular mass and an increased prevalence of LVH. Direct induction of pathological hypertrophy in the cardiac myocytes of mice has been seen in vitro; similarly, in vivo development of LVH after both intravascular and intraventricular injection of FGF-23 in

laboratory mice has been demonstrated. In further rodent models, LVH has been noted in both Klotho-deficient mice and in FGFR-blocked mice (i.e. those with pathologically elevated FGF-23). Furthermore, in the Chronic Renal Insufficiency Cohort (CRIC) study of 3070 racially and gender diverse patients (46% female, 42% black, 13% Hispanic), FGF-23 was shown to be independently associated with LVH.[33]

Lutsey et al noted no association between cardiovascular risk and FGF-23 at serum levels < 40pg/ml; however, patients with a serum FGF-23 > 58.8pg/ml had a risk of cardiovascular disease 1.65 times greater than those with levels < 40pg/ml. This risk was still observed when cardiovascular risk was adjusted for other factors including behavioural risk‡ (1.63), classic cardiovascular risk factors§ (1.44) and GFR (1.32). In the > 58.8pg/ml group, there was a risk of cardiovascular disease associated with FGF-23 of 43% for coronary heart disease, 46% for heart failure and 51% for all-cause cardiovascular mortality independent of GFR and serum phosphate.[36]

In addition to an independent association with LVH, a positive correlation has been noted between elevated levels of FGF-23 and coronary artery stenosis as demonstrated by coronary angiography (Xiao et al.) or by whole body magnetic resonance imaging (in a subset of the PIVUS study). Subanalysis of the PIVUS study went on to demonstrate a correlation between FGF-23 and "total body atherosclerosis", defined as the sum of vascular calcification at vasculature in the neck, aorta, kidney, upper leg and lower leg.[8] However a subsequent study showed no correlation between FGF-23 and CIMT suggesting that FGF-23 does not play a role in atherosclerosis.[34]

In their 2015 study, Turan et al. demonstrated a 17% increase in risk for severe CoAC, as determined by CT scan, for every 50pg/ml increase in serum FGF-23. Of significance is that this independent correlation continued to exist in patients with a GFR > 60ml/min/1.73m^2.[34] A further independent

‡ "…education, physical activity, smoking status and BMI"

§ "…diabetes, systolic BP, hypertension medication use, diabetes medication use, LDL-C and HDL-C."

correlation between FGF-23 and aortic calcification was noted by Nasrallah et al.[37], Desjardins et al.[38] and Schoppet et al.[39] A paediatric study by Srivaths et al. (mean age 16.6 years) also found that haemodialysis patients with CoAC had significantly higher FGF-23 levels compared to those without CoAC.[40]

A statistically significant positive association between FGF-23 and CoAC was revealed in a study of 545 African American patients with type 2 diabetes mellitus implying a race-independent correlation between FGF-23 and vascular calcification. However, there was no correlation between carotid and aortic calcification and FGF-23.[41]

It should be noted that a subanalysis of the CRIC study by Scialla et al. found no correlation between FGF-23 and aortic or CoAC. While FGF-23 was associated with an increase in the prevalence of CoAC when adjusted for renal function, and "traditional cardiovascular risk factors" this association was lost when "traditional cardiovascular risk factors" were taken into account. Elevated serum phosphate was found to be associated with an increased prevalence and severity of CoAC, however.[42]

1.4 FGF-23 in FH

Subjects with FH, especially HoFH, develop atherosclerosis and vascular calcification early in life as evidenced by the presence of aortic calcification reported in over 90% of HoFH patients.[4,29]

In patients with FH, vascular calcification continues to progress despite statin therapy and reduction in LDL-C, suggesting that "the calcification process may proceed independently of cholesterol levels, once sub-endothelial damage has occurred"[27].[4,27] Fantus et al. and Awan et al. have both proposed a two-hit hypothesis in the development of vascular calcification in patients with FH.[27,28] In addition to this, Alonso et al. showed that LDL may not be the only factor responsible for the extensive vascular calcification seen in patients with FH.[43]

This suggests the possible role of another factor or factors in the development of vascular calcification in FH patients. Given that FGF-23 is an independent risk factor for vascular calcification, it could be one of the additional factors responsible for the vascular calcification seen in patients with FH.

2. Methods

2.1 Study Aim

The main aim of this study was to assess whether FGF-23 levels were elevated in patients with HoFH compared to control patients without FH. This was done in order to determine whether FGF-23 could be implicated in the pathogenesis of the severe vascular calcification seen in patients with HoFH.

2.2 Study Objectives

The objectives of this study were:

1. To measure serum FGF-23 levels in patients with HoFH and to compare the levels to those in age- and gender-matched control subjects without hypercholesterolaemia.
2. To determine whether serum FGF-23 levels are elevated in HoFH and to assess whether there is a correlation between serum LDL cholesterol levels and serum FGF-23 levels.

2.3 Study Design

This study was a cross-sectional review.

2.4 Study Location

This study was undertaken at the Carbohydrate and Lipid Metabolism Research Unit (Area 551) at Charlotte Maxeke Johannesburg Academic Hospital in Parktown, South Africa.

2.5 Study Participants

Thirty HoFH patients that follow up at the Charlotte Maxeke Johannesburg Academic Hospital Lipid Clinic as well as 30 age- and gender-matched control patients were previously recruited. These 60 patients had blood collected

between February 2011 and September 2012 for use in a previous study[**] (Appendix 6.2) and serum samples were subsequently stored at -70°C for use in future studies.

2.6 Ethics

Permission to conduct the study was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical). The Clearance Certificate (No M160537) appears as Appendix 6.1.

2.7 Data Collection

Data collected included demographic, clinical, laboratory and sonographic data as listed on the study data collection form (Appendix 6.3).

Total Cholesterol, serum calcium and serum phosphate were measured in both patient and control groups in the National Health Laboratory Service (NHLS) laboratory at CMJAH at the time of collection of blood using a Siemens Advia 1800 assay (Siemens Healthcare Pty Ltd, South Africa). Low-Density Lipoprotein Cholesterol (LDL-C) was calculated in both patient and control groups at the time of collection of blood using the Friedewald equation.

FGF-23 was measured on both patient and control groups' stored serum in the Carbohydrate and Lipid Metabolism Research Unit laboratory using an Elisa assay (Kainos Laboratories, Japan).

B-mode ultrasound measurement of the carotid arteries was carried out in order to measure CIMT and to determine if plaque or calcification was present. A standardised ultrasound technique was used, using a Toshiba Nemio Ultrasound SSA-550A (Toshiba Medical Systems Corporation, Japan). The transducer frequency was set at 11MHz for all measurements.

[**] Telomere Length of Circulating Leukocytes in Patients with Homozygous Familial Hypercholesterolaemia - Dr Tasneem Mahomedy

Measurements of the CIMT were carried out at the optimum angle of interrogation, which allows visualisation of the flow tip divider, the common carotid artery (CCA), external carotid artery (ECA) and the internal carotid artery (ICA) from a single selected angle of the carotid arteries at the bifurcation. Doppler was used to verify the identification of the ECA and ICA. The intima-medial thickness (IMT) was measured when the two echogenic lines, representing the lumen-intima interface and the media-adventitia interface, are visualised over a length of ≥1cm.

2.8 Statistics

All of the above data were collected and entered into a database using pre-assigned patient numbers for each of the patient and control samples. The data were analysed and processed to obtain median (if applicable), mean and range for variables in both the control and patient group.

Statistical analyses included:

- The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess normality of distribution of FGF-23 in patient and control groups. Since the data were not normally distributed, FGF-23 in the two groups was compared using the Mann-Whitney U Test.
- The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess normality of distribution of serum calcium and phosphate in patient and control groups. Since both variables were evenly distributed, the two groups were compared using Student's t-test.
- Assessing for a correlation between FGF-23 and various clinical and biochemical variables as well as correlation between serum calcium and phosphate with other clinical and biochemical variables was undertaken using Pearson's Correlation Coefficient.

3. Results

3.1 Study Population

Thirty patients with HoFH (including both adults and children) were recruited from the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) Lipid Clinic. All the HoFH patients were on lipid-lowering therapy at the time of sampling with 13.3% (n=4) of the patients on statin monotherapy and the remaining patients (86.7%, n=26) on a combination of a statin and ezetimibe. Mean duration of therapy was 10.9 ± 5.2 years for statins and 3.4 ± 2.3 years for combination therapy. At the same time, 30 age- and gender-matched controls without HoFH were recruited. The controls did not have hypercholesterolaemia and none were on statin or combination therapy. These 60 participants had blood drawn and serum samples were subsequently stored at -70°C at the Carbohydrate and Lipid Metabolism Research Unit in the Division of Endocrinology at CMJAH.

Patients with a Creatinine Clearance of less than 60ml/min/1.73m^2 were to be excluded given the links between FGF-23 and chronic renal failure; however, none of the patients had an abnormal Creatinine Clearance and thus no participants were excluded. Creatinine Clearance was calculated using the Cockroft-Gault formula[44] for all patients except for the four paediatric patients for whom the Haycock-Schwartz formula[45] was used.

3.2 Demographic Data

The mean age of the patient group was 29.8 years ± 13.6 (range 6 – 49 years) versus 28.8 years ± 13.0 (range 6 – 49 years) in the control group. The patient group was made up of 57% (n=17) male patients, 83.3% (n=25) white patients, 13.3% (n=4) black patients and 3.3% (n=1) Indian patients. The control group consisted of 60% (n=18) male patients, 90% (n=27) white patients and 10% (n=3) black patients.

Table 3.1 – Demographic Data

		PATIENT (n=30)	CONTROL (n=30)
	AGE	**29.8** ± 13.6	**28.8** ± 13.01
GENDER	**MALE**	**57% (n=17)**	**60% (n=18)**
	FEMALE	**43% (n=13)**	**40% (n=12)**
RACE	**WHITE**	**83.3% (n=25)**	**90% (n=27)**
	BLACK	**13.3% (n=4)**	**10% (n=3)**
	INDIAN	**3.3% (n=1)**	**0% (n=0)**

3.3 FGF-23

3.3.1 Comparison Between Groups

The mean FGF-23 in the patient group was 62.07 ± 26.42pg/ml (median = 58.1) and in the control group 63.69 ± 19.84pg/ml (median = 60.77). There was no statistically significant difference in FGF-23 between the patient and control groups (p=0.4621).

3.3.2 FGF-23 and Other Variables

There was no statistically significant correlation between FGF-23 and Total Cholesterol, LDL-C, serum calcium or serum phosphate in either the patient or control group; neither was there any correlation between FGF-23 and CIMT in the patient group. These data are represented in Table 3.2 and Figures 3.2 and 3.3.

Table 3.2 – Correlation between FGF-23 and Other Variables

	FGF-23 (Patients)		FGF-23 (Controls)	
	r-value	p-value	r-value	p-value
LDL-C	-0.0126	0.9483	-0.0367	0.8474
Total Cholesterol	-0.0180	0.9261	0.0339	0.859
Calcium	-0.0965	0.6187	-0.1490	0.4321
Phosphate	0.1596	0.4081	0.1741	0.3575
CIMT	-0.2656	0.1897	N/A	N/A

Figure 3.2 – Correlation between FGF-23 & Other Variables in Patient Group

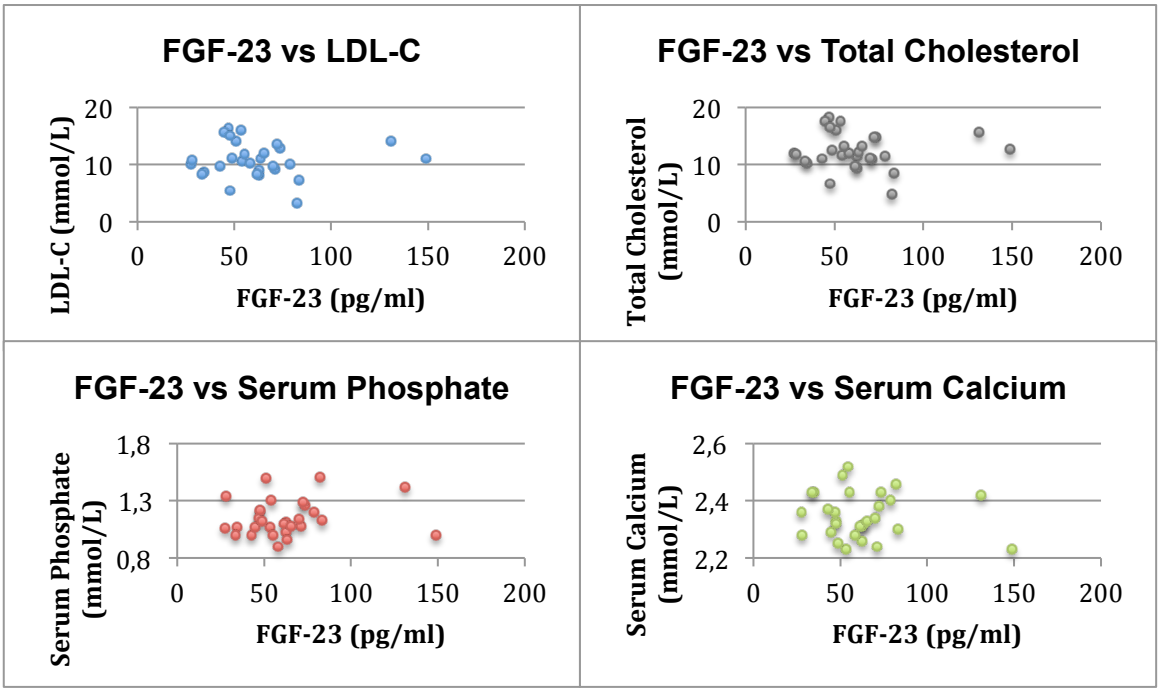
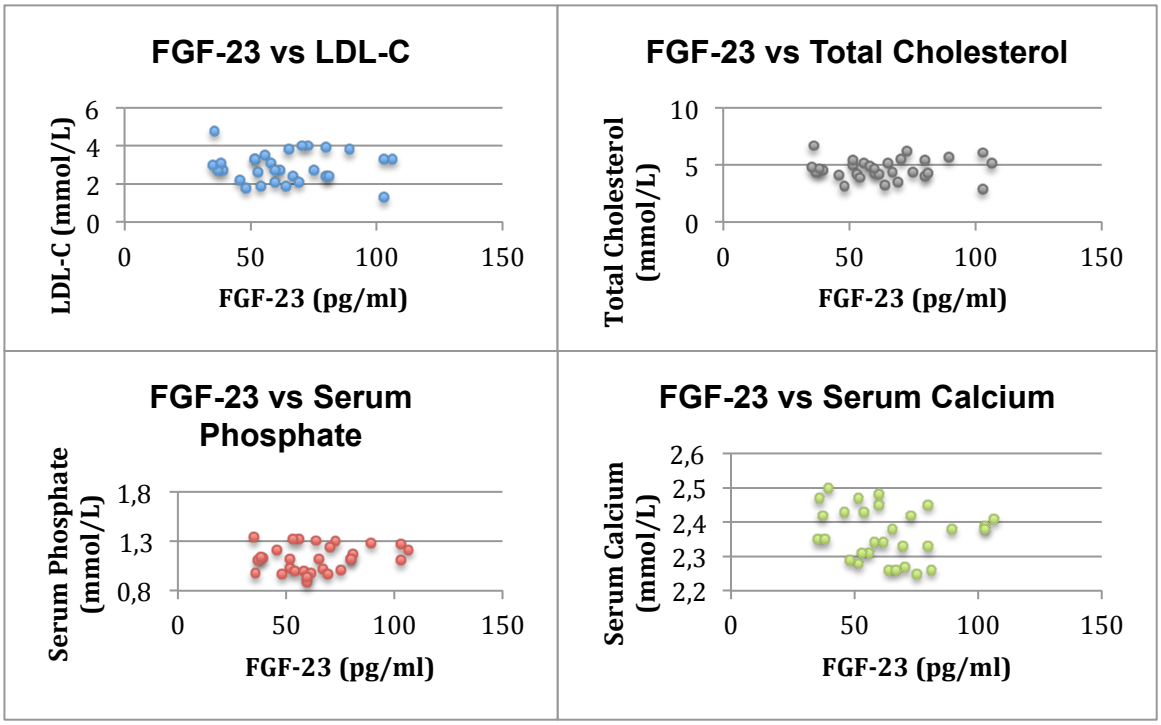


Figure 3.3 – Correlation between FGF-23 & Other Variables in Control Group



3.3.3 FGF-23 and Cardiovascular Disease

Premature Coronary Artery Disease (CAD) was present in 37% (n=11) of the patient group. The mean FGF-23 for those with CAD was 67.82pg/ml versus 59.10pg/ml in those without CAD.

History of an Aortic Valve Replacement (AVR) was present in 16.7% (n=5) of the patient group. The mean FGF-23 was 74.47pg/ml in those with previous AVR versus 59.49pg/ml in those without.

Data for the presence of Carotid Artery Calcification (CaAC) as observed sonographically was available for 27 patients. In the 62.9% (n=17) of patients with CaAC, the mean FGF-23 was 59.44pg/ml versus 70.71pg/ml in the remaining patients.

The difference in FGF-23 was not statistically significant for CAD (p=0.4516), AVR (p=0.4791) or CaAC (p=0.3061).

Table 3.3 – FGF-23 and Cardiovascular Disease

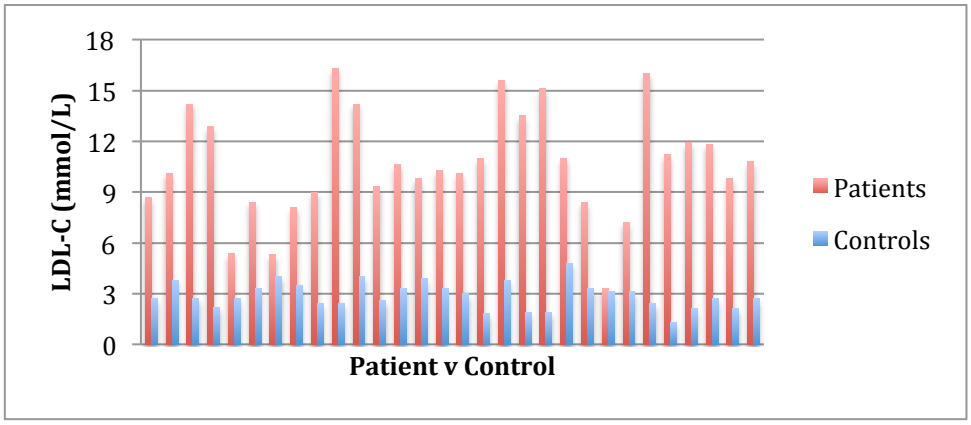
	MEAN FGF-23 (pg/ml)		
	WITH	**WITHOUT**	**p-value**
Premature CAD	**67.82**	**59.1**	**0.4516**
AVR	**74.47**	**59.49**	**0.4791**
CaAC	**59.44**	**70.71**	**0.3061**

3.4 Cholesterol

3.4.1 LDL-C

Mean LDL-C was 10.51 ± 3.31mmol/L in the patient group and 2.90 ± 0.82 in the control group. There was a statistically significant difference between LDL-C in the patient and control groups (p=<0.0001).

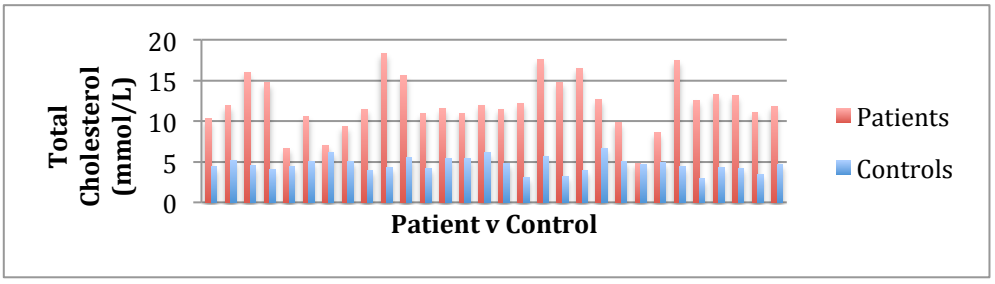
Figure 3.4 – Comparison of LDL-C in Patient and Control Groups



3.4.2 Total Cholesterol

Mean Total Cholesterol was 12.02 ± 3.35mmol/L in the patient group versus 4.68 ± 0.96 in the control group. There was a statistically significant difference (p=<0.0001) between the two groups.

Figure 3.5 – Comparison of Total Cholesterol in Patient and Control Groups

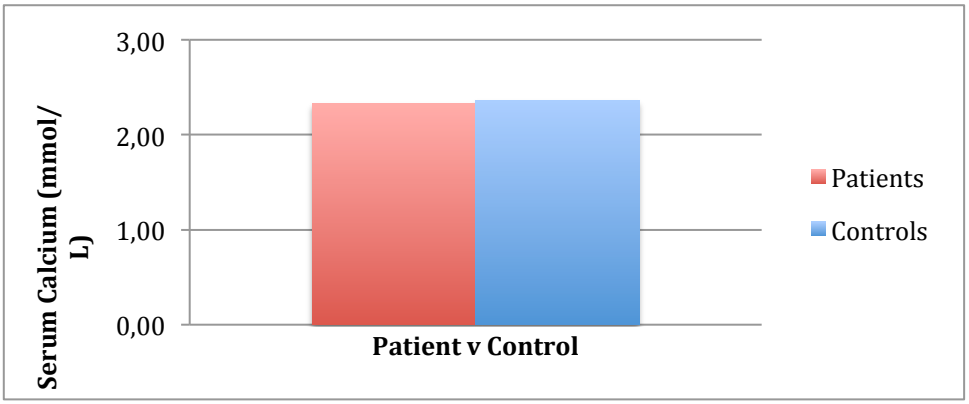


3.5 Calcium

3.5.1 Comparison between Groups

Average serum calcium in the patient group was 2.33 ± 0.07mmol/L and 2.36 ± 0.07mmol/L in the control group. There was no statistically significant difference in serum calcium between the two groups (p=0.3331).

Figure 3.6 – Comparison of Mean Serum Calcium in Patient and Control Groups



3.5.2 Calcium and Cardiovascular Disease

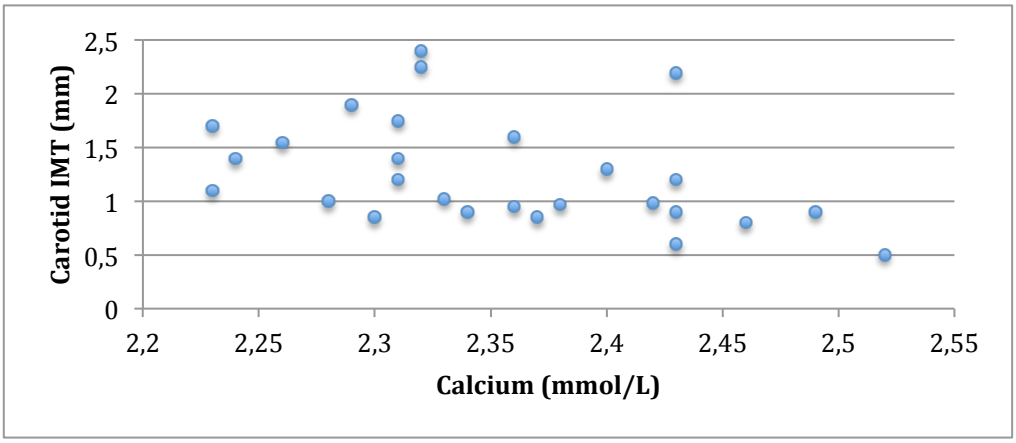
There was a statistically significant (p=0.031) negative correlation between serum calcium and Carotid IMT (r=-0.415).

Similarly, there was a statistically significant difference in serum calcium for both those patients with and without premature CAD (p=0.0014) and those with and without evidence of CaAC (p=0.0006).

Table 3.4 – Serum Calcium and Cardiovascular Disease

	MEAN SERUM CALCIUM (mmol/L)		
	WITH	**WITHOUT**	**p-value**
Premature CAD	**2.30**	**2.38**	**0.0014**
CaAC	**2.31**	**2.42**	**0.0006**

Figure 3.7 – Calcium vs Carotid IMT

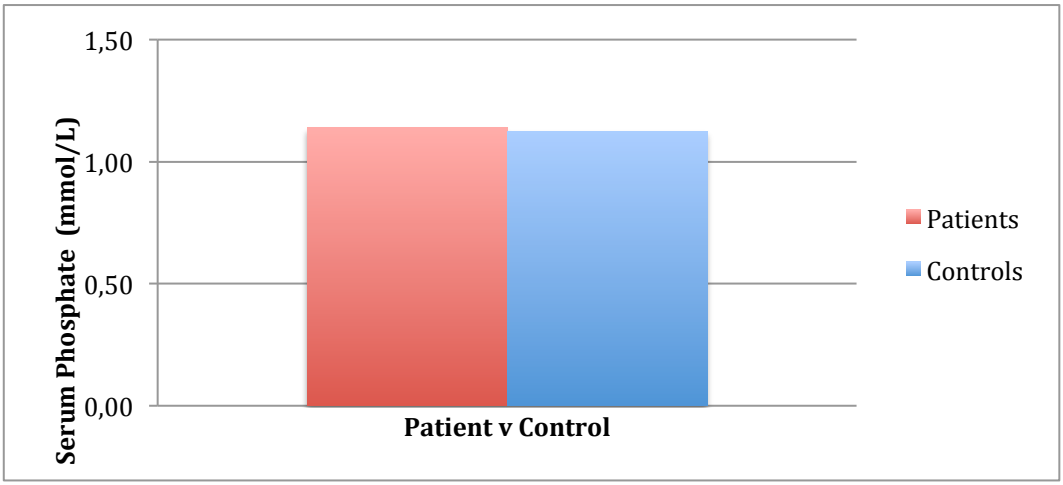


3.6 Phosphate

3.6.1 Comparison Between Groups

Average serum phosphate in the patient group was 1.14 ± 0.14mmol/L and 1.13 ± 0.14mmol/L in the control group. There was no statistically significant difference in serum phosphate between the two groups (p=0.4886).

Figure 3.8 – Comparison of Mean Serum Phosphate in Patient and Control Groups



3.6.2 Phosphate and Cardiovascular Disease

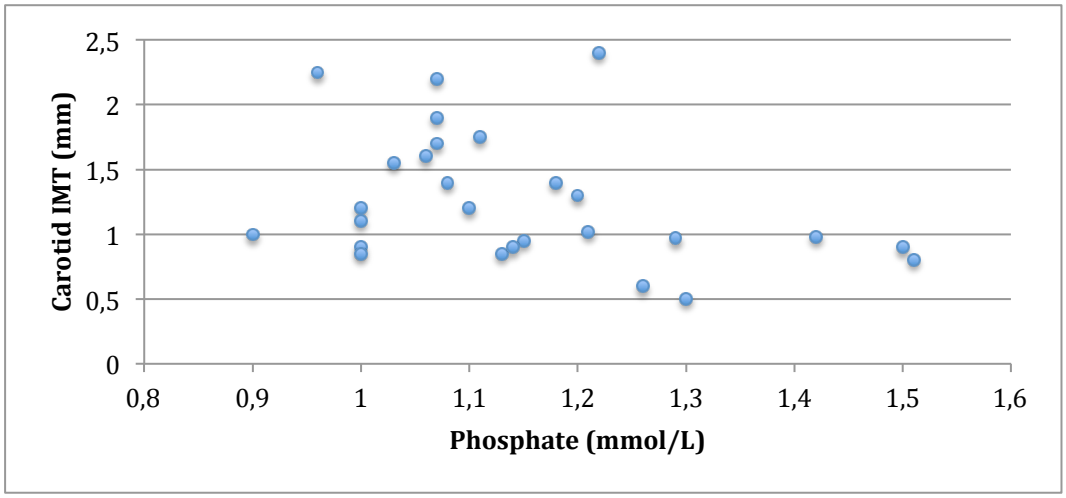
The difference in serum phosphate between those patients with and without CaAC was not statistically significant (p=0.0104) nor was the difference between serum phosphate in those with and without premature CAD statistically significant (p=0.103).

There was no statistically significant (p=0.0638) correlation between serum phosphate and IMT.

Table 3.5 – Serum Phosphate and Cardiovascular Disease

	MEAN SERUM PHOSPHATE (mmol/L)		
	WITH	**WITHOUT**	**p-value**
Premature CAD	**1.11**	**1.19**	**0.1030**
CAC	**1.09**	**1.26**	**0.0104**

Figure 3.9 – Phosphate vs Carotid IMT



4. Discussion

4.1 Overview

Patients with HoFH are known to undergo severe premature vascular calcification with fatal complications early on in life. Multiple studies have been done to assess this calcification and potential causative factors; however, no studies have been reported to date, to this author's knowledge, to assess FGF-23 in patients with FH.[4–6,10,26–29] This study aimed to fill this gap and assess whether FGF-23 might play a role in the vascular calcification seen in patients with HoFH, as observed in patients with ESRD.

4.2 Study Population and Demographics

The study population was made up of equal numbers (n=30) of patients (known with HoFH and attending the CMJAH Lipid Clinic) and age- and gender-matched controls. None of the patients recruited had evidence of renal dysfunction and thus none were excluded.

All patients were age- and gender-matched, with only one mismatch in gender between groups. The extra male patient in the control group was matched to a six-year-old female patient in the patient group due to a difficulty in recruiting gender-matched controls in a paediatric population.

Despite South Africa's population being predominantly black (79.6% at the last census in 2011),[46] this is not reflected in the demographics of the study participants. Instead the majority of the patients (83.3%, n=25) in the patient group, and thus the control group, were white. This is not surprising considering the high prevalence of FH in white population groups (viz. Afrikaners and Jews) due to the Founder Effect.[16,18,19,21]

4.3 FGF-23

The lack of a statistically significant difference in FGF-23 between patient and control groups suggests that FGF-23 does not play a major role in the

vascular calcification seen in patients with FH. This is an important finding as it shows that the pathophysiology of the vascular calcification seen in patients with FH, differs from that seen in patients with ESRD.[14,15,26,27] Vascular calcification seen in patients with renal failure occurs in the media of blood vessels and it is this vascular calcification that is associated with elevated levels of FGF-23 as opposed to the intimal calcification seen in patients with dyslipidaemia.[26–28]

Similarly, FGF-23 did not correlate with any lipid variables (in either patient or control groups) nor was there a correlation with CIMT or CaAC in the patient group. Interestingly there was no correlation between FGF-23 and phosphate in either group despite evidence of a strong correlation in previous studies.[47,48] A limitation of the study was that parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) were not measured and thus their correlation with FGF-23, serum calcium and serum phosphate could not be assessed.

Serum FGF-23 was elevated in patients with both premature CAD and history of AVR compared to those without. This difference was not statistically significant however ($p=0.4516$ for CAD and 0.4791 for AVR). Patients without CaAC had higher serum FGF-23 levels when compared to those with CaAC but, again, this result was not statistically significant ($p=0.3061$).

Interestingly serum FGF-23 was elevated in 63% of patients (n=19) and 77% of controls (n=23) when compared to the manufacturer's reference range (10 – 50pg/ml)[49] despite the absence of renal dysfunction. The reasons for this are unclear in this study; however, to fully determine the significance of this, one would need to assess the levels of serum $1,25(OH)_2D$ in both groups. Furthermore, there are no data regarding serum levels of FGF-23 in the South African population or in patients with FH.

4.4 Cholesterol

As expected, all cholesterol variables were statistically significantly higher in the patient group when compared to the control group as (see figures 3.4 and 3.5).

Statins remain the cornerstone of therapy in the management of FH as they have been shown to delay the onset of cardiovascular disease in these patients.[10,15,22,24] However, despite proven benefits in causing atherosclerotic plaque regression, multiple studies have shown no effect on aortic and coronary calcification that is already present.[4,27,50,51]

Given the lack of a statistically significant difference in FGF-23 between study groups, it is not surprising that neither Total Cholesterol nor LDL-C correlated with FGF-23. This lack of correlation seems to suggest that FGF-23 is not implicated in the two-hit hypothesis, proposed by both Fantus[27] and Awan[28], described above.

4.5 Calcium and Phosphate

The difference between serum calcium and phosphate was not statistically significant between the two groups in this study. Interestingly there was a statistically significant negative correlation between serum calcium and CIMT but not between serum phosphate and CIMT. The low-powered nature of this study makes it difficult to draw firm conclusions from this finding. Previous studies have shown that high serum phosphate is associated with an increased risk of cardiovascular disease, even in patients without CKD.[52] The evidence for the association between serum calcium and cardiovascular disease is conflicting with some studies[52] finding no association between the two and others[53] finding that patients with previous CAD had elevated levels of serum calcium compared to those without.

Considering the role of $1,25(OH)_2D$ in calcium and phosphate metabolism, it is unfortunate that there was insufficient serum to assess levels in the study

cohort. Furthermore, deficiency of vitamin D has been associated with an increased risk for cardiovascular disease and cardiovascular mortality.[54] An inverse correlation between $1,25(OH)_2D$ and vascular (both coronary and aortic) calcification has been noted in both FH and non-FH patients in the literature with the negative correlation being stronger in FH patients.[55]

4.6 Study Limitations

This study had a number of limitations that must be taken into account when interpreting the data:

- The study was conducted retrospectively with original data being collected in 2011. This led to this researcher being unable to assess for CoAC as has been done in other studies.
- The retrospective nature of the study meant that only a finite quantity of serum was available to use for further testing. This meant that other parameters, such as vitamin D and PTH, could not be assessed.
- The study had a small sample size; however, HoFH is a rare condition and this would be difficult to correct without expanding the study to a multi-centre study design.
- CIMT was not measured in the control group.

4.7 Conclusion

This study aimed to investigate Fibroblast Growth Factor 23 (FGF-23) in patients with HoFH by comparing levels in age- and gender-matched controls and assessing for the presence of any correlation between FGF-23 and LDL cholesterol. The aim of this was to determine, by extrapolation, whether FGF-23 might play a role in the devastating vascular calcification seen in these patients.

The findings of the study can be concluded as follows:

1. Serum FGF-23 is not elevated in patients with HoFH when compared to non-FH age- and gender-matched controls.

2. There is no statistically significant correlation between serum FGF-23 and LDL-C or Total Cholesterol.
3. There is no statistically significant correlation between serum FGF-23 and cardiovascular disease (including premature CAD, AVR and CaAC) in the FH patient group.

Strong correlations between FGF-23 and coronary artery stenosis as well as "total body atherosclerosis" have been shown to exist in the literature.[8] Furthermore, multiple previous studies have shown a correlation between FGF-23 and aortic calcification[37–39] as well as CoAC independent of renal dysfunction.[34,40] Despite this, from the conclusions presented above it can be extrapolated that FGF-23 does not play a role in the vascular calcification seen in patients with HoFH and thus it is not implicated in the two-hit hypothesis that has been widely discussed in this research.[27,28]

These results show that further research is needed into the pathophysiology of vascular calcification in both FH patients and all patients with lipid-related atherosclerosis. The literature review and discussion show that most research into vascular calcification has focused on that seen in ESRD, namely medial calcification, with limited data available on calcification in lipid-related atherosclerosis. The role of statins as a cause of vascular calcification has been alluded to; however, the significance of this in patients with FH needs further investigation.[7,8,30,33,34,36,37,40]

Given the known links between vitamin D and vascular calcification[54,55], it may be important to evaluate vitamin D status in this patient cohort. Furthermore there may be a role for investigating newer biomarkers such as PCSK9 and Lp(a) and their role in the vascular calcification seen in FH as has already been done by Alonso et al.[43]

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6. APPENDICES

6.1 Ethics Clearance (this study)

R14/49 Dr Jarrod Mario Zamparini

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160537

NAME: (Principal Investigator)	Dr Jarrod Mario Zamparini
DEPARTMENT:	Internal Medicine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	The Evaluation of Fibroblast Growth Factor 23 (FGT-23) in Patients with Homozygous Familial Hypercholeterolaemia
DATE CONSIDERED:	27/05/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Frederick J. Raal
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	15/07/2016

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 10004,10th floor, Senate House/2nd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the the conditions under which I am/we are authorised 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 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 review in May and will therefore be due in the month May each year.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

6.2 Ethics Clearance (previous study)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Tasneem Mahomedy

CLEARANCE CERTIFICATE	**M10M101157**
PROJECT	Telomere Length of Circulating Leukocytes in Patients with Homozygous Familial Hypercholesterolaemia
INVESTIGATORS	Dr Tasneem Mahomedy.
DEPARTMENT	Division of Endocrinology and Metabolism
DATE CONSIDERED	26/11/2010
DECISION OF THE COMMITTEE*	approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 26/11/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof FJ Raal

DECLARATION OF INVESTIGATOR(S)

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

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

6.3 Data Collection Sheet

Participant ID: _____ (□ Patient □ Control)

Demographics

Age: _____
Gender: □ Male □ Female
Race: □ White □ Black □ Coloured □ Indian

History

Smoker: □ Yes (Pack Years: _____) □ No □Ex (Stopped: _____)
Alcohol: □ Yes □ No

Cardiovascular Disease:
□ Previous MI → Age: ____
□ Aortic Valve Replacement
□ Hypertension

Height: _____m
Weight: _____ kg BMI: ___ kg/m^2

Medication

Lipid-lowering medication
□ Statin → From: dd/mm/yyyy To: dd/mm/yyyy
□ Ezetimibe → From: dd/mm/yyyy To: dd/mm/yyyy
□ Other: _____ → From: dd/mm/yyyy To: dd/mm/yyyy

Other Medication:

Laboratory Data
LIPOGRAM:
Total Cholesterol: _____ HDL: _____
Triglycerides: _____ LDL: _____

FGF-23: _____

Serum Calcium: _____
Serum Phosph: _____

Serum Creatinine: _____ eGFR:_____

Radiological Data
CIMT: _____mm
CAC: Y / N