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The role of fibroblast growth factor-23 in chronic kidney disease-mineral and bone disorder

MAJD A. I. MIRZA

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Abstract
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Fibroblast growth factor-23 (FGF23) was initially identified as the causative factor of autosomal dominant hypophosphatemic rickets. Further studies confirmed that FGF23 is predominantly expressed in the osteocytes and osteoblasts of bone and that circulating FGF23 acts on the kidney to inhibit renal phosphate reabsorption and $1,25(OH)_2D_3$ hydroxylation.

With the progression of chronic kidney disease (CKD), the kidneys become insufficient to maintain a normal systemic mineral homeostasis, resulting in various abnormalities of bone and mineral metabolism, generally referred to as Chronic Kidney Disease – Mineral and Bone Disorders (CKD-MBD). FGF23 increases early in the course of CKD in order to maintain normal serum phosphate levels; long before a significant increase in serum phosphate can be detected. Recent studies suggest that increased FGF23 levels are associated with progression of CKD, mortality, and the development of refractory secondary hyperparathyroidism. Because FGF23 is the very earliest marker of CKD-MBD, it is of particular interest to evaluate the relation between FGF23 and CKD-MBD abnormalities, in the setting of early CKD and also in individuals with normal renal function.

In the present work, we show that FGF23 is linked to several dynamic measurements of vascular function, including endothelial dysfunction, arterial stiffness, and atherosclerosis. FGF23 is also positively associated with left ventricular mass index and an increased risk of having left ventricular hypertrophy. All associations were independent of serum phosphate and were strengthened in subjects with diminished renal function. Furthermore, we found significant evidence for an association between higher FGF23 and increased fat mass and dyslipidemia, which could represent a novel pathway linking FGF23 to cardiovascular disease. Finally, we show that FGF23 is a significant predictor of future fracture risk.

Although these associations could be reflecting the increased risk associated with hyperphosphatemia and calcitriol deficiency, current evidence points towards FGF23 being more than an innocent bystander. At the very least, FGF23 holds promise of being a biomarker of cardiovascular status and phosphate-related toxicity both in CKD and in the general population, and might be a therapeutic target that could improve the fatal prognosis in CKD patients.

Keywords: FGF23, FGF-23, CKD, CKD-MBD, cardiovascular disease, vascular function, atherosclerosis, hypertrophy, LVMI, LVH, fractures, bone, fat mass, obesity, apolipoproteins, cholesterol, lipids

Majd A. I. Mirza, Internal Medicine, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

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Fibroblast growth factor-23 in chronic kidney disease-mineral and bone disorder (CKD-MBD)

Majd A. I. Mirza

The role of fibroblast growth factor-23 in chronic kidney disease-mineral and bone disorder

Majid A. I. Mirza

I dedicate this thesis to my family,
for your love and support over the years.
Thank you all.

Majd Ayman Ibrahim Mirza

September 9, 2010

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Mirza MA**, Larsson A, Lind L, Larsson TE. Circulating fibroblast growth factor-23 is associated with vascular dysfunction in the community. *Atherosclerosis* 2009, 205(2):385-390.

II **Mirza MA**, Hansen T, Johansson L, Ahlstrom H, Larsson A, Lind L, Larsson TE. Relationship between circulating FGF23 and total body atherosclerosis in the community. *Nephrol Dial Transplant* 2009, 24(10):3125-3131.

III **Mirza MA**, Larsson A, Melhus H, Lind L, Larsson TE. Serum intact FGF23 associate with left ventricular mass, hypertrophy and geometry in an elderly population. *Atherosclerosis* 2009, 207(2):546-551.

IV Marsell R*, **Mirza MA***, Mallmin H, Karlsson M, Mellstrom D, Orwoll E, Ohlsson C, Jonsson KB, Ljunggren O, Larsson TE. Relation between fibroblast growth factor-23, body weight and bone mineral density in elderly men. *Osteoporos Int* 2009, 20(7):1167-1173.
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V **Mirza MA**, Karlsson MK, Mellström D, Orwoll E, Ohlsson C, Ljunggren Ö, Larsson TE. Serum Fibroblast Growth Factor-23 (FGF23) and Fracture Risk in Elderly Men. *J Bone Miner Res* 2010. *In Press.*

VI **Mirza MA**, Alsiö J, Hammarstedt A, Erben RG, Michaëlsson K, Tivesten Å, Marsell R, Orwoll E, Karlsson MK, Ljunggren Ö, Mellström D, Lind L, Ohlsson C, Larsson TE. Circulating FGF23 is associated with fat mass and dyslipidemia in two independent cohorts of elderly individuals. *ATVB* 2010. *In Press.*

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Abbreviations

$1,25(OH)_2D$	1,25-dihidroxy vitamin D (calcitriol) – the active form of vitamin D
ADHR	Autosomal dominant hypophosphatemic rickets
ANCOVA	Analysis of co-variance
apoA1	Apolipoprotein A1
apoB	Apolipoprotein B
ARHR	Autosomal recessive hypophosphatemic rickets
AS	Atherosclerosis score
ATP	Adenosine triphosphate
BMD	Bone mineral density
Cbfa-1	Core-binding factor-1
cDNA	Complementary DNA
CI	Confidence interval
CKD	Chronic kidney disease
CKD-MBD	Chronic kidney disease – mineral and bone disorder
CV	Cardiovascular
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
EDV	Endothelium-dependent vasodilation
eGFR	Estimated Glomerular filtration rate
EIDV	Endothelium-independent vasodilation
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-stage renal disease
FGF	Fibroblast growth factor
FGF19	Fibroblast growth factor-19
FGF21	Fibroblast growth factor-21
FGF23	Fibroblast growth factor-23
FGFR	Fibroblast growth factor receptor
FTC	Familial tumoral calcinosis
GAGs	Glycosaminoglycans
GALNT3	UDP-N-acetyl-alpha-D-galactosamine-polypeptide N-acetylgalactosaminyltransferase 3
GFR	Glomerular filtration rate
HDL	High-density lipoprotein
IQR	Inter-quartile range

LDL	Low-density lipoprotein
LV	Left ventricular
LVH	Left ventricular hypertrophy
MAPK	Mitogen-activated protein kinase
mg	Milligram
MrOS	Osteoporotic fractures in men study
NaPi IIa	See NPT2a
NPT2a	Sodium-dependent phosphate co-transporter type 2 a
NPT2b	Sodium-dependent phosphate co-transporter type 2 b
NPT2c	Sodium-dependent phosphate co-transporter type 2 c
PHEX	Phosphate regulating gene with homologies to endopeptidases
PIVUS	Prospective investigation of the vasculature of Uppsala seniors
PTH	Parathyroid hormone
RI	Reflection index
RNA	Ribonucleic acid
RU	Reference units
SD	Standard deviation
sHPT	Secondary hyperparathyroidism
TIO	Tumor-induced osteomalacia
VDR	Vitamin D receptor
VDRE	Vitamin D responsive element
WBMRA	Whole-body magnetic resonance angiography
XLH	X-linked hypophosphatemia

Introduction

Phosphate metabolism

Phosphate (P_i)

Phosphorous (P) is the 6th most common element in the body and is a nutrient essential for many biological processes including skeletal mineralization and energy provision in the form of ATP [1]. Phosphorous is also an integral molecule in DNA and RNA, a major constituent of cell membranes and intracellular organelles, and serves as the substrate for kinase and phosphatase regulation of intracellular signaling. Total body phosphorous is 500-700 g, whereof 85% comprises the hydroxypatite crystals of the skeleton together with calcium, 15% is located in the cellular compartments and less than 1% of the total body phosphorous is located in the extracellular fluids (Figure 1). Phosphorous in the blood is free from binding proteins and exists in several reduced forms of phosphate like $H_2PO_4^-$, HPO_4^{-2} and PO_4^{-3}, therefore, circulating phosphorous is often denoted as inorganic phosphate or P_i. Normal serum P_i concentration is 0.7 – 1.5 mmol/L (2.2 – 4.5 mg/dL) and is maintained through a complex interplay between the intestine, kidneys, bone and parathyroid glands.

Phosphate homeostasis

Dietary intake of phosphorous is approximately 1200 mg per day and metabolic studies indicate that 70% is absorbed from the intestinal lumen (Figure 1). Intestinal absorption of phosphorous is directly proportional to dietary intake (passive absorption) but is to some extent regulated by active transport through the sodium-dependent phosphate co-transporter type 2 b (NPT2b). Phosphate is buffered in the mineralized skeleton in the form of hydroxyapatite crystals and there is a daily exchange of approximately 150-300 mg phosphate between bone and extra-cellular fluid.

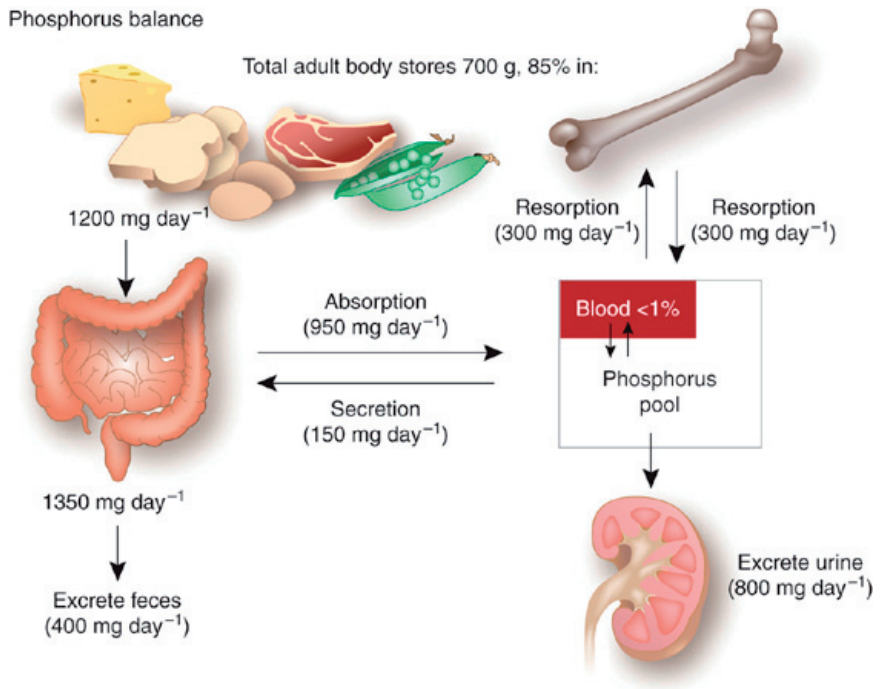

Figure 1. Phosphorous balance in normal physiology.
The kidney is the main regulator of human phosphate homeostasis and the skeleton is a storage depot for P_i and contains 85% of the total body phosphorus. Reproduced from [2], with kind permission from the International Society of Nephrology.

The kidney is the main regulatory organ for maintenance of phosphate balance. When phosphate balance is positive, the reabsorption of filtered phosphate in the kidney proximal tubules is diminished leading to increased phosphaturia and lowering of serum phosphate levels and vice versa. The renal reabsorption of phosphate is mediated by NPT2a and NPT2c, which are expressed in the proximal epithelial cells [1, 3].

Phosphate homeostasis is under extensive hormonal control. Intestinal uptake is stimulated by vitamin D, the movement of phosphate into and out of bone mineral is regulated by vitamin D and parathyroid hormone (PTH), and PTH causes renal phosphaturia by decreasing the number of NPT2a co-transporters on the apical surface of the proximal tubular cell and by decreasing its synthesis over longer periods [4, 5]. Thus, the expression of NPT2a and NPT2c in the kidney is the main determinant of serum phosphate levels when renal function is normal. Hyperphosphatemia usually occurs as a result of renal failure and reduced ability of the kidney to excrete a phosphate load.

Fibroblast growth factor-23 – a phosphate-regulating hormone

Fibroblast growth factor-23 (FGF23) was originally discovered through positional cloning, which revealed missense mutations in the *FGF23* gene as the causative factor of autosomal dominant hypophosphatemic rickets (ADHR)[6]. ADHR is a phosphate wasting disorder that is characterized by short stature, bone pain, fracture and lower extremity deformity [7, 8]. The ADHR phenotype is caused by gain-of-function mutations (R176Q, R179Q and R179W) leading to FGF23 resistance to proteolytic cleavage, thereby elevating circulating FGF23 concentrations, leading to phosphate wasting [6, 9, 10]. Circulating FGF23 concentrations are also elevated in patients with X-linked hypophosphatemia (XLH) [11-13], which is characterized by a skeletal and a renal phenotype [14]. The skeletal abnormalities are characterized by defective calcification of cartilage and bone, leading to rickets, osteomalacia, and growth retardation. The renal disorders include impaired renal tubular reabsorption of phosphate and aberrant regulation of $1,25(OH)_2D$ production, leading to hypophosphatemia that is commonly resistant to phosphorous and vitamin D therapy [14].

Simultaneously, FGF23 was shown to be an ectopically over-expressed phosphaturic factor in patients with tumor-induced osteomalacia (TIO) [15-18]. TIO, also called oncogenic osteomalacia, is an acquired paraneoplastic disease characterized by hypophosphatemia caused by renal phosphate wasting [19]. Patients with TIO share biochemical and clinical similarities with ADHR and XLH patients including hypophosphatemia, low or inappropriately normal $1,25(OH)_2D$ levels and osteomalacia [20]. Removal of the tumor leads to reductions in circulating FGF23 concentrations and correction of the hypophosphatemia [11, 12].

In contrast, inactivating mutations in the *FGF23* gene, leading to rapid degradation of FGF23 into inactive C-terminal fragments, causes familial tumoral calcinocis (FTC, OMIM # 211900). FTC is characterized by a reversed biochemical phenotype including hyperphosphatemia, elevated $1,25(OH)_2D$ levels and extensive soft tissue and/or vascular calcifications [21-23].

Thus, several human syndromes causing either FGF23 excess or deficiency point to FGF23 as a central hormonal regulator of phosphate homeostasis.

FGF23 characteristics

Fibroblast growth factors (FGFs) comprise a family of polypeptides that share a common core region containing approximately 120 highly conserved amino acid residues, with variable flanking N- and C-terminal residues. There are seven subfamilies of human FGFs [24-28].

FGF23 is the 23rd discovered FGF [29]. The *FGF23* gene is located on chromosome 12p13 and is composed of 3 exons spanning 10kb of genomic sequence [6]. FGF23 is a secreted 32kDa (251 amino acids) protein with a 24 amino acid hydrophobic terminus that acts as a signal sequence (Figure 2) [6, 29]. In order to be transported from the Golgi apparatus through the cytoplasm and finally secreted, FGF23 requires O-linked glycosylation of the threonine residue at position 178 [9]. The enzyme responsible for the post-translational modification of FGF23 is GALNT3 [30]. Inactivating mutations in *GALNT3* causes FTC and hyperphosphatemia due to a defective post-translational processing of FGF23.

Phylogenic and sequence analyses have shown that FGF23 shares common structural and biological features with FGF19 and FGF21, which are members of the FGF19 subfamily [28]. The FGF19 subfamily members have low affinity for heparin, and can therefore be distributed in the bloodstream throughout the body to mediate their systemic functions [31]. In contrast, members of other FGF subfamilies bind to heparin sulfate present on the cell surface of producing cells, resulting in their capture and explaining their paracrine function [32].

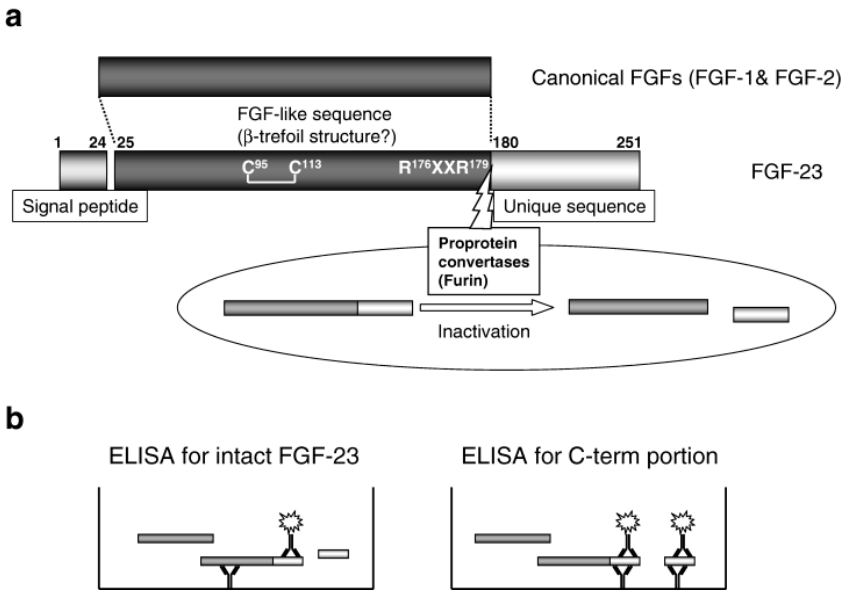

Figure 2.
(a) Schematic structure of FGF23. FGF23 has a disulfide bond in the FGF-like sequence and an internal cleavage site immediately after the RXXR consensus sequence. (b) Schematics of two types of sandwich ELISAs for intact mature form of FGF23 and its C-terminal portion. Reproduced from [32], with kind permission from Blackwell Publishing.

The half-life of intact FGF23 in the circulation of healthy individuals has been estimated to be 58 minutes [33]. The biologically active protein can be cleaved at its 176RXX179R motif by a furin-like enzyme, which generates biologically inactive N- and C-terminal fragments (Figure 2) [9, 15, 34-36]. Two assays for measurement of human FGF23 are commercially available. Full-length FGF23 levels can be determined with a sandwich ELISA technique, in which two kinds of monoclonal antibodies detect the simultaneous presence of both the N-terminal and C-terminal portions of FGF23 [12, 37]. In contrast, the C-terminal assay recognizes both full-length and processed C-terminal fragments of FGF23 (Figure 2).

Normal FGF23 physiology

The FGF19 subfamily members, FGF19, FGF21, and FGF23, function as hormones that act on specific target organs and regulate diverse metabolic processes [27, 38]. FGF19 expression is induced upon feeding in intestinal epithelial cells in response to bile acid released into the intestinal lumen. FGF19 then acts on hepatocytes and on the gall bladder as an essential component in a postprandial negative feedback loop for bile acid synthesis and release [39, 40]. In contrast, FGF21 expression is induced upon fasting in the liver [41, 42]. FGF21 stimulates glucose uptake in adipocytes [43] and reduces fat storage through its activity that stimulates lipolysis [41, 42].

FGF23 is expressed in bone (primarily in osteoblasts and osteocytes) [34, 36, 44] and is important in mineral metabolism, where it exerts three distinct functions. First, full-length FGF23 is a phosphaturic hormone [15, 45, 46]. FGF23 induces phosphaturia through decreased expression and endocytosis of the sodium-phosphate co-transporters NPT2a and NPT2c in the kidney proximal tubule. This is accomplished by activation of the mitogen-activated protein kinase (MAPK) pathway [12]. Second, as opposed to PTH-induced phosphaturia, FGF23-induced phosphaturia does not lead to upregulation of $1,25(OH)_2D$ production. In contrast, FGF23 suppresses renal 1-alpha-hydroxylase (1α-OHase), leading to decreased conversion of 25-hydroxyvitamin D (25(OH)D) to its active metabolite $1,25(OH)_2D$. FGF23 further reduces 25(OH)D and $1,25(OH)_2D$ levels by stimulating 24-hydroxylase, which is responsible for vitamin D degradation [47]. Third, in the parathyroid, FGF23 decreases PTH expression and secretion [48, 49], and increases 1α-hyroxylase mRNA levels, which contrasts with the negative effects of FGF23 on 1α-OHase expression in the kidney [49].

Administration or transgenic expression of FGF23 in mice reproduces the common clinical features of ADHR and TIO such as hypophosphatemia due to increased renal phosphate wasting, inappropriately low serum $1,25(OH)_2D$ levels and rachitic bone [9, 45, 50-53]. In addition, clear growth retardation, a disorganized and widened growth plate and reduced bone mineral density (BMD) are observed in the transgenic mice. These changes are independent

of vitamin D/vitamin D receptor (VDR) system as a rapid bolus injection of FGF23 to *VDR null* mice further decreases the serum phosphate level in these mice, and reduces NPT2a protein abundance and renal 1α-OHase expression [54].

In contrast, *Fgf23 null* mice are characterized by hyperphosphatemia with increased renal phosphate reabsorption, hypercalcemia, suppressed PTH expression and secretion, sustained elevation of 1α-OHase expression, and high serum $1,25(OH)_2D$ levels [55, 56]. These are accompanied by skeletal abnormalities, decreased BMD, and excessive mineralization in soft tissues, including heart and kidney. Of note, administration of anti-FGF23 neutralizing antibodies to normal adult mice, evoked a significant elevation in serum phosphate and $1,25(OH)_2D$ levels. However, these mice did not suffer from other phenotypes observed in *Fgf23 null* mice indicating that these are secondary phenomena that could arise from excess $1,25(OH)_2D$ or due to other unknown factors [57, 58].

FGF23 regulation

Although the regulation of FGF23 expression in bone remains somewhat controversial, there are several factors that directly or indirectly appear to influence serum FGF23 levels.

Phosphate

High-media phosphate concentrations increase *Fgf23* promoter activity *in vitro* [59]. *In vivo* studies show a consistent increase in FGF23 levels in response to phosphate loading [60, 61]. In contrast, both diet-induced hypophosphatemia and hypophosphatemia due to secondary hyperparathyroidism in *VDR null* mice result in significantly lower FGF23 levels [62]. Normalization of serum phosphate by diet in *VDR null* mice, which on a normal diet have undetectable FGF23 levels, increases FGF23 [62].

In human, FGF23 levels are affected by changes in dietary phosphorus [63-65] and increase in response to increased serum phosphate levels in patients with chronic hypoparathyroidism [66]. In healthy male volunteers, acute increase and decrease of serum phosphate level did not affect circulatory FGF23 levels, which suggests that FGF23 is not involved in the rapid adaptation of phosphate homeostasis [67, 68]. Furthermore, while dietary phosphorous load affects FGF23 levels, FGF23 levels were unchanged by non-dietary interventions that increased serum FGF23 levels such as intravenous phosphorous infusion [68]. Thus, it is still unclear how phosphate in sensed; the phosphate sensor is yet to be discovered and it is still not fully understood what triggers FGF23 actions.

Vitamin D

Direct exposure of osteoblast cultures to $1,25(OH)_2D$ and *in vivo* administration of calcitriol both stimulate the production of FGF23 in bone and osteoblasts independent of serum phosphate and PTH [61, 69, 70]. This action is mediated by a vitamin D responsive element (VDRE) in the *FGF23* promoter [69]. In contrast, *VDR null* mice have very low serum FGF23 and do not respond to the $1,25(OH)_2D$ administration [54, 61, 62, 71]. In human, intravenous calcitriol therapy increases serum concentrations of FGF23 [72].

PTH

FGF23 levels are elevated in an animal model of primary hyperparathyroidism, and decrease after parathyroidectomy [73]. However, the effect of PTH on circulating FGF23 in human is still unclear and contradicting results have been reported [74-78]. Although, in healthy male volunteers, an 24-hour intravenous infusion of PTH lead to a significant increase in FGF23, this effect could be attributed to the simultaneous increase in $1,25(OH)_2D$ [79].

Estrogen

In vitro, estrogens lead to increased transcription and translational upregulation of FGF23 in osteoblast-like cells in a time- and concentration-dependent manner [80]. Exogenous administration of estrogens to an ovariectomized rat model of chronic kidney disease, decreases serum $1,25(OH)_2D$ and phosphate levels and significantly increases FGF23 mRNA and serum levels [80].

Genetic factors

Genetic defects can either directly or indirectly result in overstimulation of serum FGF23 levels as is the case in ADHR [6, 9, 10], ARHR [81, 82] and XLH [11-13, 36, 83].

For example, XLH is caused by inactivating mutations of *PHEX*, a phosphate-regulating gene with homologies to endopeptidases on the X chromosome [14, 84-86]. The mouse cDNA sequence of *PHEX* is highly homologous to that of humans, and deletion of the *PHEX* gene in the *Hyp* mouse results in an animal model of XLH [87, 88]. Mutations in *PHEX* result in elevated FGF23 production by osteocytes in bone [36, 83], which is evident by elevated FGF23 levels in XLH patients [11-13] and also in the *Hyp* mouse [89]. Inhibiting the FGF23 action in these mice by crossing the *Hyp* mice with the *Fgf23 null* mice [56], or by administration of anti-FGF23 neutralizing antibodies to *Hyp* mice [90], corrects the hypophosphatemia and inappropriately normal serum $1,25(OH)_2D$ levels in these mice and ameliorates the rachitic bone phenotypes typically observed in *Hyp* mice, such as impaired longitudinal elongation, defective mineralization, and

abnormal cartilage development. Thus, excess actions of FGF23 underlie hypophosphatemic rickets observed in *Hyp* mice.

Fibroblast growth factor receptors and FGF23 signaling

The search for the physiologically relevant FGF23-receptor has been a larger challenge than one at first envisioned. Many initial *in vitro* studies failed to reproduce the indisputable effects of FGF23 on mineral metabolism. However, binding studies supported a low-affinity binding to FGFR1c, FGFR2c, FGFR3c, and FGFR4 in the presence of highly sulfated heparins and the expression of specific cell-surface glycosaminoglycans (GAGs) [91].

Now it stands clear that FGF23 signaling was not understood until the discovery of type I membrane-bound alpha-Klotho (Klotho). Klotho directly binds to multiple FGFRs forming a Klotho-FGFR complex, which binds to FGF23 with higher affinity than FGFR or Klotho alone [92, 93]. Klotho signaling enhances the ability of FGF23 to induce phosphorylation of FGF receptor substrate and ERK in various types of cells [92]. The target specificity of all the FGF19 subfamily members is determined by the tissue distribution of Klotho [94]. However, in contrast to FGF23, FGF19 and FGF21 require beta-Klotho for their action [95-97].

The *KLOTHO* gene encodes a 130 kDa single-pass transmembrane protein with a short cytoplasmic domain (10 amino acids) and is expressed predominantly in the kidney. Mice carrying a loss-of-function mutation in the *KLOTHO* gene develop a syndrome resembling human aging, including a shortened life span, skin atrophy, muscle atrophy, osteoporosis, arteriosclerosis, and pulmonary emphysema [98]. Conversely, overexpression of the *KLOTHO* gene extends the life span and increases resistance to oxidative stress in mice [99-102]. Klotho is a permissive and critical FGF-receptor co-factor for FGF23, which is evidenced by common phenotypes developed by Klotho-deficient and FGF23-deficient mice, including shortened life-span, growth retardation, infertility, muscle atrophy, hypoglycemia, and vascular calcification in the kidneys. Notably, they both have increased serum levels of phosphate [55, 103]. Finally, injecting wild-type mice with an anti-Klotho monoclonal antibody induces FGF23 incompetence [93].

Chronic Kidney Disease

Chronic kidney disease (CKD) is a worldwide public health problem affecting 5-10% of the world population [104]. Etiological causes of impaired kidney function are typically hypertension, diabetic angiopathy, polycystic kidney disease and various inflammatory and systemic disorders.

Glomerular filtration rate (GFR) is commonly used to measure the level of kidney function and determine the stage of kidney disease. GFR represents the flow rate of produced urine per unit area (normal >100 mL/min/1.73 m^2), and corresponds to the percent of kidney function available. Measurements of endogenous creatinine or cystatine C in blood are most commonly used by clinicians for GFR approximations.

CKD is defined as either kidney damage or GFR <60 mL/min/1.73 m^2 for ≥3 months [105]. All individuals with GFR <60 mL/min/1.73 m^2 for ≥3 months are classified as having chronic kidney disease, irrespective of the presence or absence of kidney damage. The rationale for including these individuals is that reduction in kidney function to this level or lower represents loss of half or more of the adult level of kidney function, which may be associated by a number of complications such as hypertension, hypocalcemia, hyperphosphatemia and low serum hemoglobin and albumin levels. The degree of renal deficiency can be divided into five CKD stages according to the GFR level.

I. Stage 1, GFR ≥90 mL/min/1.73 m^2: kidney damage with normal or high GFR (slightly diminished kidney function). Kidney damage is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine test or imaging studies.
II. Stage 2, GFR 60-89 mL/min/1.73 m^2: mild reduction in GFR with kidney damage.
III. Stage 3, GFR 30-59 mL/min/1.73 m^2: moderate reduction in GFR.
IV. Stage 4, GFR 15-29 mL/min/1.73 m^2: severe reduction in GFR.
V. Stage 5, GFR <15 mL/min/1.73 m^2: established kidney failure or permanent renal replacement therapy (RRT).

Chronic Kidney Disease – Mineral and Bone Disorder

With the progression of CKD, the kidneys become insufficient to maintain a normal systemic mineral homeostasis, resulting in various abnormalities of bone and mineral metabolism, generally referred to as Chronic Kidney Disease – Mineral and Bone Disorder (CKD-MBD) [106, 107].

Table 1. Definition of CKD-MBD [106].

Definition of CKD-MBD
A systemic disorder of mineral and bone metabolism due to CKD manifested by either one or a combination of the following: ▪ Abnormalities of calcium, phosphorous, PTH and/or vitamin D metabolism. ▪ Abnormalities in bone turnover, volume, linear growth, and/or strength. ▪ Vascular and/or other calcifications.

The Kidney Disease: Improving Global Outcomes (KDIGO) has supplied a definition for the entity "CKD mineral and bone disorder (CKD-MBD)" (Table 1) [106]. According to this definition, CKD-MBD is characterized by a broad clinical syndrome; that is, it is a systemic disorder of mineral and bone metabolism due to CKD, which is manifested by abnormalities in bone and mineral metabolism and/or extraskeletal calcifications. Abnormalities in bone mineral metabolism have been implicated in bone fragility and in various cardiovascular disorders, including cardiovascular calcifications, hypertension, and left ventricular hypertrophy in CKD. Abnormal mineral metabolism occurs early in the course of CKD, which can result in significant consequences even in patients not yet on dialysis [108-110]. In addition, CKD-MBD might also contribute to the progression of kidney injury. Thus, if optimal management of CKD-MBD is established, it may be possible to decrease cardiovascular morbidity and mortality and to inhibit CKD progression.

CKD-MBD – Changes in mineral metabolism and secondary hyperparathyroidism

As kidney function declines, there is a progressive deterioration in mineral homeostasis with a disruption of normal serum and tissue concentrations of phosphorus and calcium, and changes in the circulating levels of PTH, vitamin D, and FGF23. Hyperphosphatemia develops due to impaired renal phosphate excretion [111]. At early CKD stages, hyperphosphatemia is compensated for by an increase in serum FGF23 and PTH, which results in increased phosphate excretion. However, in CKD stage 4 and 5, hyperphosphatemia becomes fixed because of insufficient renal excretion despite high FGF23 and PTH [112]. Diminished renal function also leads to decreased $1,25(OH)_2D$ production, which decreases calcium absorption and leads to hypocalcaemia [112].

Secondary hyperparathyroidism (sHPT) is the first CKD-related metabolic complication, often occurring before the onset of anemia, acidosis, hypocalcemia, and hyperphosphatemia [113]. Phosphate retention, hypocalcemia, and a progressive decline in $1,25(OH)_2D$ are main factors for abnormal PTH secretion [114-116]. As a result, hypertrophy and proliferation of the parathyroid cells lead to nodular hyperplasia. Importantly, the increase in FGF23 levels in CKD precedes the decrease of serum $1,25(OH)_2D$ concentrations, suggesting an important role of FGF23 in the development of sHPT in patients with CKD [117]. Thus, FGF23 may be a central mediator in the early pathogenesis of sHPT in CKD as increased FGF23 levels may help maintain normal serum phosphate levels but at the cost of suppressing calcitriol levels and worsening sHPT because of decreased feedback inhibition by calcitriol [117].

Along with diminishing kidney function, hyperphosphatemia, hypocalcemia, elevated PTH, and vitamin D deficiency develop. These abnormalities have been associated with high all-cause and cardiovascular morbidity and mortality in CKD [118-124]. Hyperphosphatemia is thought to be an important risk factor for the development of complications commonly seen in CKD-MBD [108, 123, 125-128]. It has been known for decades that hyperphosphatemia causes soft tissue and vascular calcification [125]. Block *et al.* found that in dialysis patients, survival was significantly less when the pre-dialysis serum phosphate concentration exceeded 6.5 mg/dL [123]. Hence, restriction of oral phosphorous intake in addition to medical treatment with phosphate-binders is often used in the treatment of CKD patients [129]. The adverse effects of phosphate are however not restricted to patients with CKD. Tonelli *et al,* found that serum phosphate levels (even within the upper normal range) were associated with adverse cardiovascular outcomes [108]. Thus, even minor changes in phosphate metabolism within the normal range may contribute to cardiovascular complications in subjects with a normal renal function as well as in patients with CKD.

CKD-MBD – Bone disease

The earliest histological abnormalities of bone in CKD-MBD are seen after a relatively mild reduction in GFR (CKD stage 2) [130]. By CKD stage 5, the skeletal abnormalities are found in virtually all patients and can present themselves as (1) low bone turnover, which encompasses osteomalacia and adynamic bone disease, (2) high bone turnover due to the elevated PTH levels (hyperparathyroidism) [131-134]. In osteomalacia, the rate of mineralization is slower than the rate of collagen synthesis, which results in defective bone mineralization and an excessive accumulation of unmineralized osteoid. In adynamic bone disease there is a marked reduction in the rate of mineralization and of collagen synthesis. Finally, in high bone turnover, the number and size of the bone resorbing osteoclasts are increased, as are the number and depth of the resorption lacunae formed by the osteoclasts. The deposition of collagen by the osteoblasts is less ordered, and the rate of bone formation is increased.

The prevalence of osteoporosis in the population with CKD exceeds the prevalence in the general population [135, 136] and may be observed with either high turnover [137, 138] or low turnover forms of osteodystrophy [139], even before dialysis is required [137]. Patients in early and end-stage CKD are also at an increased risk for fragility fractures [135, 140-145]. Fracture risk in CKD patients increases at a younger age than the typical individual with senile or postmenopausal osteoporosis [139, 146].

In subjects without kidney disease, the measurement of the amount of mineralized bone mass by dual energy X-ray absorptiometry (DXA) is an established clinical tool for discriminating among those with and without

prevalent fractures and for identifying those who are at an increased risk for incident fractures [147]. However, DXA measurements can be low, normal, or high, in each of the three major forms of kidney bone disease; hyperparathyroidism, adynamic bone disease and osteomalacia [137, 148, 149]. Furthermore, DXA measurements do not discriminate between those with and without prevalent fractures [149]. The lack of predictive value of DXA for fracture in the setting of kidney failure is likely because metabolic abnormalities that accompany kidney disease affect the cortical and trabecular compartments of bone differently.

Given the many potential mechanisms by which CKD could decrease bone strength [106] and increase fracture risk, it is important to identify those CKD patients who are at an increased risk for fractures and develop therapeutic strategies to decrease fracture risk in this population.

CKD-MBD – Cardiovascular disease

The marked and excessively high mortality in end-stage renal disease (ESRD) is largely attributable to the increased cardiovascular morbidity and mortality [150, 151]. In patients with ESRD on hemodialysis, adjusted cardiovascular mortality is 10-20 times higher than in the general population [152]. Furthermore, the presence and extent of vascular calcifications are strong predictors of cardiovascular and all-cause mortality in ESRD patients [153]. In addition to classic cardiovascular risk factors (e.g. arterial hypertension, smoking, dyslipidemia, family history), disturbances in calcium-phosphate metabolism contribute to vascular calcification and higher cardiovascular mortality in CKD patients [154, 155].

Calcification is already present in patients new to hemodialysis [156-158], and affects the survival of dialysis patients [159]. Calcification progression is more prominent in CKD patients, who also experience more frequent fatal and nonfatal cardiovascular events, compared with subjects with normal renal function [160]. Hence, the “illness” in CKD is not only dependent on the development of end stage renal disease (ESRD) and the need for maintenance dialysis, but it is important to manage the cardiovascular events associated with diminished renal function and reduce the cardiovascular burden in this population.

Vascular calcium accumulates when there is a net calcium efflux from bone [161, 162] and is often associated with osteoporosis [163, 164]. Vascular calcification involves two different parts of the vessel wall. Intimal calcification is often associated with inflammation and atherosclerosis [165], whereas medial calcification occurs in smooth muscle cells [166]. Risks for the development of medial calcinosis include renal failure, diabetes and aging and proceeds to processes similar to bone formation [167-169]. Dialysis patients presented with medial calcification are nearly 20 years younger on average and predominantly characterized by a longer duration of

dialysis treatment and derangements in their Ca × P_i balance [155]. In contrast, intimal calcifications are found in older patients characterized by a history of traditional risk factors (e.g. smoking, dyslipidemia) and occur at sites of atherosclerotic plaques combined with cholesterol deposition, inflammation and cellular necrosis [170-172].

Importantly, vascular calcification in CKD is not just passive precipitation but an active regulated process cause by differentiation of the vascular smooth muscle cells into osteoblast-like cells as evident from the immunohistochemical detection of bone matrix proteins (cbfa-1, alkaline phosphatase and osteopontin) in calcified epigastric arteries [173, 174] (For a review see [175, 176]). *In vitro* studies have shown that human smooth muscle cells accumulate very little calcium mineral in media containing normal serum phosphate levels (1.4 mmol/L). In contrast, in the presence of 2 mmol/L phosphate, calcium deposition dramatically increased in a time- and dose-dependent manner [177]. Calcium deposition was dependent on cells and cell-derived matrix, especially the involvement of the type III sodium-dependent phosphate co-transporter, Pit-1. Hence, under hyperphosphatemic conditions similar to those associated with ESRD, uptake of phosphate by Pit-1 is triggered and intracellular phosphate is increased. As a result, a cellular matrix more prone to mineralization is produced as supported by the expression of both Cbfa-1 and osteocalcin in the smooth muscle cells [177]. However, while this is true *in vitro*, the pathology of vascular calcification in CKD *in vivo* is far from being explained only by increased phosphate and Ca^{2+} levels, suggesting the involvement of other counter-regulatory mechanisms.

Treatment of CKD-MBD

Treatment of CKD-MBD requires a careful evaluation and all therapeutic options need to be individualized. Hyperphosphatemia is treated with dietary phosphorous restriction, oral phosphate binders and dialysis. Hypocalcemia and vitamin D insufficiency are treated with calcium supplementation and VDR activators. Secondary hyperparathyroidism is treated with calcimimetics, dialysis and in some cases with parathyroidectomy. Finally, renal replacement therapy (kidney transplantation) improves renal function and generally leads to significant improvements of CKD-MBD.

The question that remains is when should we treat patients with CKD? Should we treat patients when their serum phosphate levels become abnormal? Or should we treat patients when their response to a phosphorous load becomes abnormal looking at their fractional excretion of phosphate? Or do we need a more informative marker, which is associated with the adverse outcomes in CKD, in order to better inform about when and how to treat each and every individual patient.

FGF23 and pathophysiology of CKD-MBD

In CKD, the failing kidney is unable to adequately maintain mineral homeostasis, which initiates a series of events that inevitably lead to biochemical changes in serum, altered bone metabolism, vascular calcification and increased morbidity and mortality. The question that remains unanswered is what sets off this entire process in the earliest phase of CKD.

Circulating FGF23 levels gradually increase with declining renal function [117, 178]. During early stages of CKD, normal serum phosphate levels are maintained despite a declining nephron mass, in part by a progressive increase in FGF23 levels, which stimulate greater excretion of phosphate through the remaining nephrons and limit the absorption of dietary phosphorous by inhibiting the synthesis of $1,25(OH)_2D$ [117, 127, 178, 179]. Hyperphosphatemia is thus only observed with advanced renal disease (GFR <30 mL/min/1.73 m^2) [180], whereas FGF23 increases in earlier CKD stages (Figure 3) [117, 181]. By the time patients reach dialysis, FGF23 levels can be up to 1000-fold higher than in healthy individuals [178]. Note that, increased FGF23 concentrations in CKD are not due to decreased renal clearance but due to increased FGF23 secretion [182]. Increased serum FGF23 in CKD can also be attributed to end-organ resistance to the phosphaturic stimulus of FGF23. This can arise because of a deficiency of the necessary Klotho cofactor. In support, Klotho mRNA expression is significantly reduced in kidney biopsy specimens of CKD patients [183].

Although elevated serum phosphate, low calcitriol, and high PTH levels are each independently associated with future cardiovascular events and mortality in CKD [184, 185], the traditional pathophysiological concept of a deranged calcium-phosphate metabolism in CKD has been challenged by the recent discovery of FGF23, in particular three studies in which increased FGF23 levels were shown to be independently associated with CKD progression, future risk for refractory sHPT, and mortality.

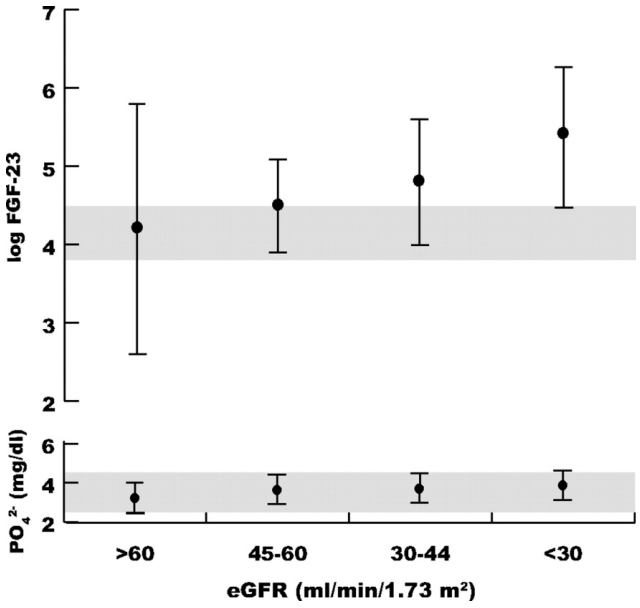

Figure 3. Mean concentrations of log FGF-23 and phosphate according to level of kidney function.
Mean phosphate and log FGF-23 concentrations significantly increased with decreasing levels of eGFR (P for trend <0.001 for both); however, the absolute difference in mean serum phosphate concentrations between the lowest and highest eGFR groups was only 0.6 mg/dL (relative difference, 14%). Furthermore, the mean serum phosphate concentration was within the normal range in all CKD stages, including the group with the most severe CKD (eGFR <30 mL/min/1.73 m^2). In contrast, the analogous percent increase in mean log FGF-23 concentrations was substantially larger (68%) and levels were already above the normal range in patients with mild to moderate CKD (eGFR 30 to 60 mL/min/1.73 m^2). Bars represent SDs; shaded areas, normal ranges for each analyte. Reproduced from [181], with kind permission from the American Heart Association.

Fliser et al. investigated the association between FGF23 and renal survival [186]. 177 non-diabetic CKD patients were followed prospectively for a median of 53 months to assess progression of renal disease. The primary end-point was defined as doubling of baseline serum creatinine and/or terminal renal failure necessitating renal replacement therapy. Both C-terminal and intact FGF23 independently predicted renal disease progression after adjustment for age, GFR, proteinuria and serum levels of calcium, phosphate and PTH. FGF23 was even a better predictor than phosphate, which lost its predictive value after adjusting for FGF23. Patients with high baseline serum FGF23 experienced a faster renal progression than subjects with low baseline FGF23 (Figure 4).

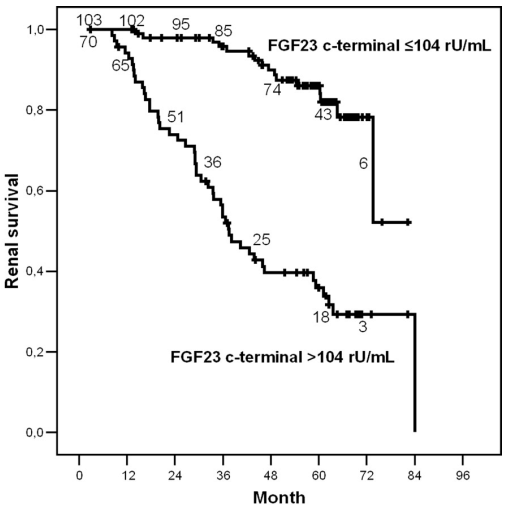

Figure 4. Kaplan-Meier curve of renal end-point in patients with below and above optimal cutoff of plasma C-terminal FGF23 concentrations.
In patients with C-terminal FGF23 levels above the optimal cutoff (>104 RU/mL), progression was significantly faster (log-rank test, $P < 0.0001$. Numbers near the survival curves represent the number of patients at risk with FGF23 levels below and above the optimal cutoff at the times 0, 12, 24, 36, 48, 60, and 72 mo. Reproduced from [186] with kind permission from the American Society of Nephrology.

Nakanishi et al, studied the prospective relation between FGF23 and the future development of refractory secondary hyperparathyroidism [187]. One hundred and three non-diabetic hemodialysis patients with serum PTH levels <300 pg/mL were included. The primary endpoint was refractory sHPT defined as either retaining intact PTH levels >300 pg/mL at 2-years after baseline, or having received parathyroid intervention treatment, including surgical parathyroidectomy. At 2 years after baseline, only serum levels of

FGF23 were significantly associated with the future progression of sHPT, independently of serum phosphate and PTH. Patients who were judged to have refractory sHPT had significantly higher serum levels of FGF23 at baseline, compared to subjects with medically controlled sHPT.

Gutierrez et al. studied the relation between FGF23 and mortality in a prospective case-controlled study of incident hemodialysis patients [188]. They investigated 200 cases and 200 controls from the total cohort of >10 000 patients. Cases were defined as individuals who died within 1 year on hemodialysis and controls were individuals who survived the first year of dialysis. Increased FGF23 was dose-dependently associated with an increased risk for mortality. In a fully multivariable adjusted model including PTH and phosphorus, subjects in the highest quartile of FGF23 had almost a 6-fold increased risk of dying within the first year of dialysis compared to subjects in the lowest FGF23 quartile (Figure 5). Again, FGF23 was a stronger predictor of mortality than serum phosphate levels; FGF23 levels were most informative when serum phosphate levels were relatively low, whereas the most extremely phosphate levels were only modestly associated with mortality.

Thus, FGF23 is an independent predictor of kidney disease progression, the development of refractory sHPT, and mortality in CKD patients. Hyperphosphatemia and calcitriol deficiency are both associated with increased risk for cardiovascular and bone disease and increased fracture risk. Because FGF23 is both a potent regulator of phosphate and vitamin D, and the very earliest marker of CKD-MBD, it is of particular interest to evaluate the relation between FGF23 and these outcomes in the setting of early CKD and also in individuals with normal renal function.

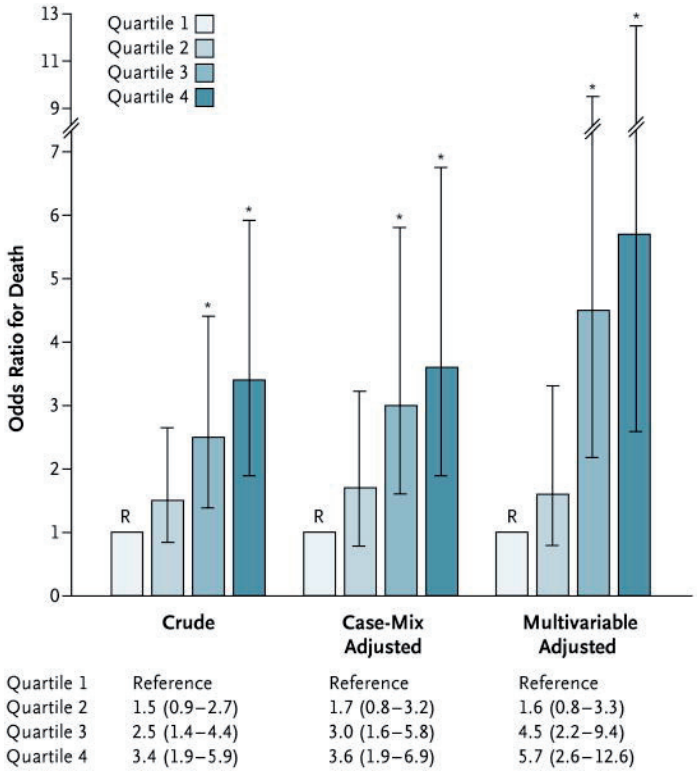

Figure 5. Odds Ratios (and 95% CIs) for death according to quartile of C-terminal FGF23 levels.
Crude, case-mix adjusted, and multivariable adjusted odds ratios for death are shown according to quartile of cFGF-23 levels (quartile 1, <1090 reference units [RU] per milliliter; quartile 2, 1090 to 1750 RU per milliliter; quartile 3, 1751 to 4010 RU per milliliter; quartile 4, >4010 RU per milliliter). The case-mix adjusted analysis included the following variables: age, sex, race or ethnic group, blood pressure, body-mass index, facility-specific standardized mortality rate, vascular access at initiation of dialysis (fistula, graft, or catheter), cause of renal failure, urea reduction ratio, and coexisting conditions. The multivariable adjusted analysis included the case-mix variables plus phosphate, calcium, log parathyroid hormone, albumin, creatinine, and ferritin levels. Quartile 1 was the reference group in all models. Asterisks indicate P<0.05. R denotes reference. Reproduced from [188] with kind permission from the Massachusetts Medical Society.

Aims of the current investigation

The overall aim of the included papers was to investigate the relation between serum FGF23 and cardiovascular and bone phenotypes in the setting of early CKD and also in subjects with a normal renal function.

Specific aims

I To investigate the relation between FGF23 and endothelium function (vasodilation and arterial stiffness).

II To investigate the relation between FGF23 and atherosclerosis.

III To investigate the relation between FGF23 and echocardiographic measurements of the left ventricle.

IV To investigate the relation between FGF23 and bone mineral density.

V To investigate the predictive power of FGF23 for future fracture risk.

VI To investigate the relation between FGF23 and traditional cardiovascular risk factors, including anthropomorphic measurements of obesity, fat mass, serum lipids, and the metabolic syndrome.

Material and Methods

The PIVUS population

Subjects

The Prospective Investigation of the Vasculature in Uppsala Seniors (the PIVUS study) was initiated with the primary aim to evaluate three different tests of vasoreactivity in more than 1000 subjects aged 70 living in the community of Uppsala, Sweden [189]. Eligible were all subjects aged 70 living in the community of Uppsala, Sweden. The subjects were chosen from the register of community living and were invited in a randomized order. The subjects received an invitation by letter within 2 months of their 70th birthday. Of the 2025 subjects invited, 1016 subjects participated giving a participation rate of 50.1%. The Ethics Committee of the University of Uppsala approved the study and the participants gave informed consent.

Basic investigation

The participants were asked to answer a questionnaire about their medical history, smoking habits and regular medication.

All subjects were investigated in the morning after an over-night fast. No medication or smoking was allowed after midnight. After recordings of height, weight, abdominal and hip circumference, an arterial cannula was inserted in the brachial artery for blood sampling and later regional infusions of vasodilators. During the investigation, the subjects were supine in a quiet room maintained at a constant temperature. The total investigation took 4 hours. The *invasive forearm model* was carried out first followed by *flow-mediated dilatation (FMD)* and then the *pulse wave based* technique. At least 30 min passed between the different tests.

Blood pressure was measured by a calibrated mercury sphygmomanometer in the non-cannulated arm to nearest mmHg after at least 30 min of rest and the average of three recordings was used. Lipid variables and fasting blood glucose were measured by standard laboratory techniques.

Basic risk factors characteristics, medical history and regular medication are given in Table 2 and Table 3.

Table 2. Basic characteristics and major cardiovascular risk factors in the total sample and in a cardiovascular healthy reference groups.

	Total sample	PIVUS healthy reference group	Young healthy reference group
N	1016	131	20
Females (%)	50.2	44.3	50
Height (cm)	169 (9.1)	171 (10)	174 (8.7)
Weight (kg)	77 (14)	72 (12)	67 (9.4)
Waist circumference (cm)	91 (12)	86 (9.0)	77 (5.2)
BMI (kg/m2)	27.0 (4.3)	24.6 (2.8)	22.2 (1.7)
Waist/hip ratio	0.90 (0.075)	0.88 (0.066)	0.81 (0.042)
SBP (mmHg)	150 (23)	125 (9.8)	113 (13)
DBP (mmHg)	79 (10)	72 (7.2)	68 (12)
Heart rate (beats/min)	62 (8.7)	61 (9.0)	64 (13)
Serum cholesterol (mmol/l)	5.4 (1.0)	5.2 (0.74)	3.8 (0.7)
LDL-cholesterol (mmol/l)	3.3 (0.88)	3.1 (0.60)	2.1(0.5)
HDL-cholesterol (mmol/l)	1.5 (0.42)	1.6 (0.48)	1.4 (0.3)
Serum triglycerides (mmol/l)	1.3 (0.60)	1.0 (0.42)	0.8 (0.3)
Fasting blood glucose (mmol/l)	5.3 (1.6)	4.8 (0.5)	4.9 (0.4)
Current smoking (%)	11	0	0

Means are given with SD in parenthesis. SBP= Systolic blood pressure. DBP= Diastolic blood pressure. BMI= Body mass index.

Healthy reference group

A group with no cardiovascular diagnosis or major risk factors were identified. The exclusion criteria were: History of any cardiovascular diagnosis or medication, Obesity (BMI>30 kg/m2), Hypertension (antihypertensive treatment or blood pressure > 140/90 mmHg), Diabetes (antidiabetic treatment including diet or fasting blood glucose > 6.1 mmol/l), Hyperlipidemia (antihyperlipidemic treatment, LDL-cholesterol > 3.5 mmol/l or serum triglycerides > 1.7 mmol/l) and current smoking. This group was denoted the PIVUS cardiovascular healthy reference group.

To give a descriptive young reference group, 10 young men and 10 young women (age 20-25 years) with the same exclusion criteria were investigated with an identical protocol. This group was denoted the young cardiovascular healthy reference group.

As the participation rate in this cohort was only 50%, we carried out an evaluation of cardiovascular disorders and medications in 100 consecutive non-participants. The prevalence of cardiovascular drug intake, history of myocardial infarction, coronary revascularization, antihypertensive medication, statin use and insulin treatment were similar to those in the investigated sample, while the prevalence of diabetes, congestive heart

failure and stroke tended to be higher among the non-participants (see Table 3).

Table 3. Self-reported history of cardiovascular (CV) disorders and regular drug intake given in percentage in the investigated sample and in one hundred non-attendees.

	Total investigated sample	**Not attending**
n	1016	100
Myocardial infarction	7.1	7.9
Stroke	3.7	6.7
Angina pectoris	8.1	13.8
CABG/PTCA	5.3	5.6
Congestive heart failure	3.8	6.9
Diabetes	8.7	16.9
Any regular drug	70	64
Any CV drug	45	52
Any antihypertensive medication	32	36
Beta-blockers	22	26
Calcium antagonists	11	12
Diuretics	13	19
ACE-inhibitors	8.5	11
Angiotensin II-blockers	8.3	9.1
GTN	3.0	3.4
Digoxin	2.1	9.2
Statins	15	17
Other antihyperlipidemic drugs	1.2	4.5
Insulin	1.8	1.1
Oral antidiabetic drugs	6.1	12
Warfarin	3.2	6.8
Aspirin/Clopidogrel	18	21
Other antiarrythmic drugs	0.2	0

CABG/PTCA= coronary revascularisation. GTN= any nitroglycerine preparation.

Serum biochemistries

Plasma cystatin C (reagent: 1014, Gentian, Moss, Norway), creatinine (reagent: 14.3600.01, Synermed International, Westfield, IN, USA), phosphate (reagent: 7D71-30) and urea (reagent: 7D75-20) measurements were performed on an Architect Ci8200 analyzer (Abbott Laboratories, Abbott Park, IL, USA) and reported using SI units. Estimated glomerular filtration rate (eGFR) was derived from cystatin C, by the formula $eGFR_{CystC}=79.901x^{-1.4389}$. The assay has a very low total imprecision, good linearity and strong correlation with iohexol clearance ($R^2=0.956$) [190]. Impaired renal function was defined as eGFR<60 mL/min/1.73 m^2. The LIAISON 25(OH)D_3 assay (DiaSorin Inc., Saluggia, Italy) was performed on the LIAISON analyzer according to the manufacturer's instructions. PTH levels were analyzed using the Immulite 2000 Intact PTH Assay (Diagnostic

Products Corporation, Los Angeles, CA, USA). Lipid variables, calcium, and fasting blood glucose were measured by standard laboratory techniques at Uppsala University Hospital. Smoking status, current smoking versus no-smoking, was obtained form a questionnaire. Previous cardiovascular disease was defined as history of any cardiovascular disease including: myocardial infarction, stroke, heart failure or angina pectoris.

Serum lipids

Serum total cholesterol, triglycerides and HDL were assayed by enzymatic techniques. LDL was calculated by Friedewald´s formula. ApoA1 and apoB were determined by a two-site immuno-radiometric assay, using commercial kits from Pharmacia (Uppsala, Sweden).

Insulin resistance, diabetes and the metabolic syndrome

Serum insulin was measured by an enzymatic-immunological assay (Boehringer Mannheim). HOMA insulin resistance index was defined as in [191] and was not evaluated in subjects on insulin treatment. Diabetes mellitus was defined as a self-reported history of diabetes or fasting blood glucose of 6.2 mmol/L or above.

Metabolic syndrome was defined by the NECP/ATP III criteria [192]. Three of the following five criteria should be fulfilled: blood pressure >130/85 mmHg or antihypertensive treatment, fasting blood glucose >5.6 mmol/L, serum triglycerides >1.7 mmol/L, waist circumference >102 cm in men and >88 cm in women, HDL-cholesterol <1.0 mmol/L in men and <1.3 in women.

The invasive forearm technique

Forearm blood flow (FBF) was measured by venous occlusion plethysmography (Elektromedicin, Kullavik). A mercury in-silastic strain-gauge was placed at the upper third of the forearm, which rested comfortably slightly above the level of the heart. The strain-gauge was connected to a calibrated plethysmograph. Venous occlusion was achieved by a blood pressure cuff applied proximal to the elbow and inflated to 50 mm Hg by a rapid cuff inflator. Evaluations of FBF were made by calculations of the mean of at least five consecutive recordings. An arterial cannula was placed in the brachial artery. No more than one attempt to insert the cannula in each arm was allowed. Resting FBF was measured 30 min after cannula insertion. After evaluation of resting FBF, local intraarterial drug-infusions were given during 5 min for each dose with a 20 min wash-out period between the drugs. The infused dosages were 25 and 50 µg/min for Acetylcholine (Clin-Alpha) to evaluate endothelium-dependent vasodilation (EDV-Ach) and 5

and 10 µg/min for SNP (Nitropress, Abbot) to evaluate endothelium-independent vasodilation (EIDV-SNP). The dosages of these drugs have been chosen to result in FBFs on the steep part of the dose-response curve without giving systemic effects. The drugs were given in a random order at a maximal rate of 1 mL/min. In the present study only data from the highest doses of Acetylcholine and SNP were used. EDV-Ach was defined as FBF during infusion of 50 µg/min of Acetylcholine minus resting FBF divided by resting FBF. EIDV-SNP was defined as FBF during infusion of 10 µg/min of SNP minus resting FBF divided by resting FBF. We did not use any distal cuff to exclude the hand circulation to avoid the discomfort and increase in sympathetic drive caused by this procedure. We have previously shown the reproducibility (coefficient of variation) for EDV-Ach and EIDV-SNP to be 8% to 10% [193].

Pulse wave analysis

A micromanometer tipped probe (Sphygmocor, Pulse Wave Medical Ltd) was applied to the surface of the skin overlying the radial artery and the peripheral radial pulse wave was continuously recorded. The mean values of ≈10 pulse waves were used for analyses. Recordings were regarded as satisfactory if the variations in the systolic peak and the diastolic peak were 5% or below. Only three attempts to achieve a satisfactory recording were allowed. The maximal systolic peak and the reflected waves were identified by the calculations of the first and second derivative of the pulse curve. After a baseline recording, terbutaline was administered subcutaneously (0.25 mg in the upper part of the arm), and a reevaluation of the pulse wave was performed after 15 and 20 min. We had previously found that the maximal alterations in the pulse waveform occurred after 15 min in young healthy subjects [194], but we performed a measurement also after 20 min in this sample of elderly subjects. Thus, the maximal change occurring at either 15 or 20 min was used for calculations. The relative height of diastolic reflected wave (d/a, here denoted reflection index, RI) has been validated previously [194]. Here, we report this variable as relative changes from baseline after terbutaline (change in RI; ΔRI). Thus, a large reduction of the pulse wave indices indicates a good response. We have previously shown the reproducibility (coefficient of variation) for ΔRI to be 9.4 % [194].

Whole body magnetic resonance angiography (WBMRA)

The WBMRA method is described in detail in [195, 196]. In brief, a gadolinium contrast agent was administered intravenously and the WBMRA examination was divided into four stations. The 1st station included the supra-aortic arteries and the thoracic aorta. The 2nd station contained the abdominal aorta, including the renal arteries, and the 3rd station started at

the external iliac arteries and continued to the popliteal arteries. The 4th and last station continued for a varying distance below the ankle. An overlap of 3 cm between each station gave a maximum total length of coverage of 171 cm. Vessels that were overlapped were assessed on both stations and there was no difference when assessing the degree of stenosis in a vessel that was overlapped. The resulting images were manually evaluated and the degree of stenosis was measured with callipers on a work station. The atherosclerosis score (AS) was defined as the sum of vascular abnormalities for each of the five different vascular territories; neck, aorta, kidney, upper leg and lower leg, was assessed. A normal vessel segment received null points, <50% stenosis one point, and ≥50% stenosis and occlusion received two points. The sum for each territory was then divided by the maximum achievable sum for that territory and the ratio was multiplied by 100. For each segment, only the most severe stenosis or occlusion was noted. Total body AS is the sum of the AS for all five vascular territories divided by five. Approximately one third of the sample had no vascular abnormalities, one third had stenosis of <50%, and the remainder had stenosis ≥50% or occlusions [196]. The intra-observer reproducibility was good (kappa value=0.73) with intra-observer agreement in 94% of the segments. Inter-observer reproducibility was excellent (kappa value=0.83) with inter-observer agreement in 77% of the segments [196]. The mean length of time between the basic PIVUS investigation and WBMRA was 16 months (range 3–24 months). Exclusion criteria for the WBMRA examination were pacemaker, valvular prostheses, intracranial clips and claustrophobia. No significant differences in the basic characteristics and major cardiovascular risk factors were found between the total PIVUS sample, the WBMRA subsample, and those not attending the basic investigation [189, 196].

Echocardiography and Doppler

A comprehensive two-dimensional and Doppler echocardiography was performed with an Acuson XP124 cardiac ultrasound unit (Acuson, California, USA). A 2.5 MHz transducer was used for the majority of the examinations. The echocardiograms were performed approximately 1 week after the main examination. The echocardiographer (Dr. Lars Lind) was blinded to the clinical data. Presence of stenosis or regurgitations in the mitral and aortic valves was recorded by use of colour and continuous Doppler. LV dimensions were measured with M-mode on-line from parasternal projections, using a leading-edge to leading-edge convention. Measurements included left atrial diameter (LA), interventricular septal thickness (IVS), LV posterior wall thickness (PW), LV diameter in end diastole and end systole (LVEDD, LVESD). LV wall thickness was calculated as IVS+PW; relative wall thickness (RWT) as (IVS+PW)/LVEDD; and LVM as 0.8x(1.04x([IVS+LVEDD+PW]3–

LVEDD3)+0.6 g [197]. LVMI was obtained by indexing LVM to height$^{2.7}$ [198]. LV geometry was also divided into four categories according to Ganau et al. [199]. A normal LV geometry was considered to be present if LVMI was normal (<51 g/m$^{2.7}$) and RWT <0.45. Concentric LVH was defined as LVMI above the threshold for LVH together with RWT >0.44, but if RWT was below this cutoff for RWT, eccentric LVH was present. If LVMI was normal but RWT >0.45, the LV geometry was denoted concentric remodeling.

The MrOS population

Study subjects

Osteoporotic fractures in Men (MrOS) is a multi-center prospective, longitudinal, observational study of risk factors for vertebral and all non-vertebral fractures in older men, and of the sequelae of fractures in men. The specific aims of the study include: (1) to define the skeletal determinants of fracture risk in older men, (2) to define lifestyle and medical factors related to fracture risk, (3) to establish the contribution of fall frequency to fracture risk in older men, (4) to determine to what extent androgen and estrogen concentrations influence fracture risk, (5) to examine the effects of fractures on quality of life, (6) to identify sex differences in the predictors and outcomes of fracture, (7) to collect and store serum, urine and DNA for future analyses as directed by emerging evidence in the fields of aging and skeletal health, and (8) define the extent to which bone mass/fracture risk and prostate diseases are linked.

The MrOS study population consists of community dwelling, ambulatory men aged 65 years or older. Inclusion criteria were designed to provide a study cohort that is representative of the broad population of older men. The inclusion criteria were: (1) ability to walk without the assistance of another, (2) absence of bilateral hip replacements, (3) ability to provide self-reported data, (4) residence near a clinical site for the duration of the study, (5) absence of a medical condition that (in the judgment of the investigator) would result in imminent death, and (6) ability to understand and sign an informed consent.

The Swedish MrOS cohort (n = 3014) consists of three subcohorts from three different Swedish cities (n = 1005 in Malmö, n = 1010 in Göteborg, and n = 999 in Uppsala), and the study subjects (men 69–80 yr of age) were randomly identified using national population registers. A total of 45% of the subjects who were contacted participated in the study. At the clinic visit, participants completed questionnaires about medical history, current medication used, and lifestyle characteristics. Height and weight were

measured, and blood samples were collected in EDTA treated tubes followed by subsequential centrifugation for collection of serum and finally stored in a -70°C freezer. Informed consent was obtained for all subjects and the study was approved by the local ethics committees at Uppsala (ethical approval number Ups 01-057), Malmö (LU-693-00) and Göteborg Universities (Gbg M 014-01) and conducted in accordance with the guidelines in The Declaration of Helsinki. The main features of the cohort investigated are presented in Table 4.

Table 4. MrOS study cohort data displaying basic characteristics of participants including biochemical and BMD measurements.

Variables	Reference Range	Study Subjects
Age (years)		75.4±3.1
Height (mm)		1747±65
Weight (kg)		80.7±12
Biochemistry		
FGF23(pg/mL)		49±40.8
phosphate (mmol/L)	0.75-1.4	1.08±0.20
calcium (mmol/L)	2.15-2.50	2.36±0.16
GFR (mL/min/1.73 m²)	>100	72.5±24.9
PTH (pmol/mL)	1.3-7.6	4.8±3.1
25(OH)D (nmol/mL)	15-100	69.7±23.6
Bone Mineral Density		
Lumbar Spine (mg/cm^2)		1142±201
Total Hip (mg/cm^2)		945±146
Femoral Neck (mg/cm^2)		837±133
Femoral Trochanter (mg/cm^2)		796±141

Values are mean ± SD.

Serum biochemistries

PTH levels were analyzed using the Immulite 2000 Intact PTH Assay (Diagnostic Products Corporation, CA, USA). 25-hydroxyvitamin D (25(OH)D) was measured on the Nichols Advantage automated assay system (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). Biochemical analyses of Pi and cystatin C were measured according to standard procedures at the Clinical Chemistry Laboratory at Uppsala University Hospital as previously described [179]. Estimated glomerular filtration rate (eGFR) was calculated from cystatin C using the estimate: GFR=(79.901(cystatin C)$^{-1.4389}$). The assay has very low total imprecision, good linearity and strong correlation with iohexol clearance (R^2=0.956) [190].

Serum lipids

Serum lipid analyses were performed on a Konelab 20 autoanalyzer (Thermo Electron Corporation, Vantaa, Finland). Total cholesterol and triglyceride levels were determined by fully enzymatic techniques. High-density lipoprotein (HDL) was determined after precipitation of apolipoprotein B (apoB)-containing lipoproteins with magnesium sulfate and dextran sulfate. Low-density lipoprotein (LDL) was calculated using Friedewald´s formula. ApoB and apolipoprotein A1 (apoA1) were determined by immunoprecipitation enhanced by polyethylene glycol at 340nm. Interassay CVs were below 5% for all Konelab analyses.

Serum levels of insulin and glucose

Fasting plasma glucose was quantitated by an enzymatic method on a Modular (Roche, Stockholm, Sweden) with an interassay CV of less than 4%. Homeostasis model assessment (HOMA) index was calculated as the product of fasting serum insulin level (micro-units per milliliter) and fasting plasma glucose level (millimoles per liter) divided by 22.5 [191].

Dual X-ray absorptiometry

BMD of the total hip and hip sub regions defined as femoral trochanter and femoral neck, and lumbar spine (vertebrae 1-4) were measured using dual energy x-ray absorptiometry (DXA) in all participants. A Lunar Prodigy DXA (GE Lunar Corp., Madison, WI USA,) was used for the Uppsala and Malmö parts of the MrOS groups and Hologic QDR 4500/A-Delphi (Hologic, Waltham, MA, USA) was used for the Göteborg sub group. BMD values for the left hip was used in calculations if present, otherwise BMD values for the right hip were used. The CVs for the area BMD (g/cm^2) measurements ranged from 0.5% to 3%, depending on the application. To be able to use DXA measurements performed with equipment from two different manufacturers, standardized BMD was calculated in the MrOS Study, as previously described [200-202].

Assessment of fractures

Participants were followed for a median of 3.35 years after the baseline examination. The follow-up time was recorded from the date of the baseline visit to the date of the first fracture or the date of death. Central registers covering all Swedish citizens were used to identify the subjects who experienced fractures and the time of death for those subjects who died during the study. X-ray archives in Malmö, Gothenburg and Uppsala were searched for new fractures occurring after the baseline visit, using the unique

personal citizenship registration number of the subjects [203]. All fractures that were reported by the study subjects after the baseline visit were confirmed by physician review of radiology reports. Fractures reported by the study subjects, which were not possible to confirm by the X-ray analyses, were not included in this study. All validated fractures were included in the main analyses, followed by exploratory subanalyses of fracture type. In the latter, we studied the associations between serum intact FGF23 and validated fractures, divided into three main groups: (1) X-ray verified clinical vertebral fractures, (2) non-vertebral osteoporosis fractures at the major osteoporosis-related locations (defined as hip, distal radius, proximal humerus, and pelvis) and (3) other fractures (radius/ulna, hand, fingers, humerus, elbow, skull, cervical vertebrae, clavicle, scapula, rib, femoral shaft, patella, upper tibia, ankle, foot and toes) [203].

Serum FGF23 measurements

Intact FGF23 was measured using an ELISA according to the manufacturer's protocol (Kainos Laboratories International; Tokyo, Japan) [12]. This second-generation, two-site, monoclonal antibody ELISA has previously been shown to recognize the biologically active, intact FGF23 [12]. The Kainos Intact FGF23 assay has a lower limit of detection of 3 pg/mL and intraassay and interassay coefficients of variation of less than 5%. The Kainos Intact assay was the most sensitive among three different two-site enzyme-linked immunosorbent assays for FGF23 measurements [37].

Statistical analyses

Paper I

In paper I, we employed the community-based PIVUS cohort to investigate the relation between serum FGF23, endothelium function and arterial stiffness.

Of the 1016 subjects in the PIVUS population, 967 subjects had valid measurements of FGF23, biochemical variables, established cardiovascular risk factors, and endothelium function and were included in our primary analyses. Secondary analyses were also performed in the following pre-specified subgroups: participants without previous cardiovascular disease (n=817), participants with an age-adjusted normal renal function (eGFR ≥60 mL/min/1.73 m^2, n=759) and participants with diminished renal function (eGFR <60 mL/min/1.73 m^2, n=208).

First, we modeled FGF23 as a linear continuous variable (expressed as 1-standard deviation (1-SD) increase). Second, we used multi-category and threshold models, based on the FGF23 distribution in our sample, comparing the association in FGF23 tertile 2 and 3 to that of tertile 1 (lowest) and the association in tertile 3 compared with tertile 1-2. The relation between FGF23 and endothelium function was evaluated using three models:

I Crude model.

II Adjusted for gender and biochemical factors (phosphate, calcium, PTH, 25(OH)D and eGFR).

III Adjusted for the Framingham risk score. The Framingham score was calculated as a gender-specific score to predict coronary heart disease risk according to age, diabetes, smoking, joint national committee-V (JNC-V) blood pressure categories, and national cholesterol education programme (NCEP) total cholesterol and LDL cholesterol categories [204].

Logarithmic transformation was performed to achieve normal distribution for skewed variables (FGF23, calcium, PTH, EDV-Ach and EIDV-SNP). P-values<0.05 from two-sided tests were considered statistically significant. The statistic software package STATA 10.1 (Stata Corporation, College Station, USA) was used for all calculations.

Paper II

In paper II, we further investigated the relation between FGF23 and cardiovascular disease by assessing the relation between FGF23 and atherosclerosis assessed by WBMRA in a clinical 1.5-T scanner. Analogous to the aortic calcification score or coronary artery calcification score, this method generates a 'total-body' atherosclerosis score (AS). Three hundred and six subjects (145 women, 161 men) from the PIVUS population underwent WBMRA and were included in our primary analyses. Secondary analyses were also performed in participants without previous cardiovascular disease (n=261). Since the AS variable did not follow the *Poisson* distribution, which count variables often do, it was further divided into a categorical variable used in all subsequent analyses:

1. No stenosis (AS = 0): 100 subjects.
2. Low AS (AS < 7): 99 subjects.
3. High AS (AS ≥ 7): 107 subjects.

In our primary analysis, we modeled FGF23 as a linear continuous variable (expressed as 1-standard deviation (1-SD) increase). We also used multi-category and threshold models comparing the odds in FGF23 tertile 2 and 3

to that of tertile 1 (lowest) and the odds in tertile 3 compared with tertile 1-2. Thresholds were based on the FGF23 distribution in our sample.
We addressed the following questions: are increased FGF23 levels associated with higher odds of having arterial stenosis (AS > 0 compared to AS = 0); and are increased FGF23 levels associated with increased odds of a high AS compared to low AS in subjects with AS > 0 (*n*=206). Three logistic regression models were used for all analyses:

I Crude model.
II Adjusted for gender and biochemical factors (phosphate, calcium, PTH, 25(OH)D and eGFR).
III Adjusted for the Framingham risk score (a compound cardiovascular risk score) [204].

Finally, we investigated whether the known interaction between FGF23 and renal function [11, 117, 179, 182] alters the association between FGF23 and total body AS, by invoking a multiplication term between FGF23 tertiles and the presence of impaired renal function (eGFR<60 mL/min/1.73 m^2) in the regression models.

Logarithmic transformation was performed to achieve normal distribution of skewed variables (FGF23, calcium and PTH). P-values<0.05 from two-sided tests were considered significant. The statistic software package STATA 10.1 (Stata Corporation, College Station, USA) was used for all calculations.

Paper III

In paper III, we evaluated the relation between FGF23 and LVMI, LVH, and LV geometry, employing the community-based PIVUS cohort. For the present study, we excluded participants with a self-reported history of myocardial infarction (n=72) or heart failure (n=22) since these conditions *per see* influence LV remodeling and could therefore distort the relationship between biomarkers and LVH. These subjects were however included in a secondary analysis. In some individuals, high quality echocardiographic measurements could not be obtained (n=84) and some did not have complete biochemical measurements due to lack of serum (n=43). These subjects did not differ in gender or any other demographic measurements compared to the whole cohort. Finally, 795 participants (52.3% women) were eligible for the present study. All analyses were performed on the total sample and separately in participants with eGFR <60 mL/min/1.73 m^2, n=164.

Non-normally distributed variables (FGF23, PTH and LVMI) were log-transformed before used in subsequent analysis. All analyses were defined *a priori* and performed on the total sample (n=795) and separately in participants with eGFR <60 mL/min/1.73 m^2, n=164. Relation between FGF23 and LVMI was evaluated in linear regression models using

continuous variables (per 1-SD increase) and presented as standardized beta-coefficients with 95% CIs. FGF23 relation to LVH was evaluated using logistic regression. Finally, the relation between FGF23 and LV geometry group was evaluated using multinomial logistic regression (LV geometric group as dependent variable coded as 0: normal geometry 1: eccentric hypertrophy 2: concentric hypertrophy 3: concentric remodeling).

Three models were used for all analyses:

I Model A: crude model.

II Model B: adjusting for gender, systolic blood pressure, diastolic blood pressure, BMI, diabetes and hypertension.

III Model C: adjusting for gender, serum biochemistries (phosphate, calcium, $25(OH)D_3$ and PTH), and eGFR.

Two-tailed significance values were given with $p<0.05$ regarded as significant. The statistic software package STATA 10.1 (Stata Corporation, College Station, USA) was used for all calculations.

Paper IV

In paper IV, we investigated the relation between FGF23 and bone mineral density (BMD) in the MrOS community-based population of elderly men.

BMD was assessed in femoral neck, femoral trochanter, hip and lumbar spine. The relation between FGF23 and BMD in the four regions of interest was analyzed in crude and models adjusted for serum biochemistries (phosphate, calcium, PTH, $25(OH)D_3$, and eGFR) and traditional confounding variables including age, height, weight, and smoking.

Non-normally distributed variables, such as FGF23, PTH and 25(OH)D were log-transformed before used in subsequent analysis. Pearson's correlation coefficient was used for univariate correlations. Analysis of covariance (ANCOVA) was used for all multivariate regression analysis. Confounding variables were adjusted for by including them as independent variables in the ANCOVA model. To minimize the possible confounding effect of using different DXA equipment and diverging laboratory procedures including sample handling at different study sites, study site (Göteborg, Malmö or Uppsala) was included as a covariate in all linear multivariate regression analyses. $p<0.05$ was regarded as statistically significant. SAS version 9.1 (SAS Institute Inc., Cary, N.C., USA) was used for all calculations.

Paper V

In paper V, we explored the association between a single serum FGF23 measurement and fracture risk, again employing the prospective population-

based MrOS cohort of elderly Swedish men. For the current study, 2868 subjects with complete measurements of FGF23, fractures and other parameters were included.

Initially, the distributional properties of all baseline variables were examined. Data are presented as mean ± SD, except for the non-normally distributed variables, which are presented as median (10^{th} – 90^{th} percentiles). Fracture rates were expressed as the number of subjects with a validated first fracture per 1000-person-years and 95% CIs.

Cox proportional hazards regression models with age during follow-up as underlying time scale [205] were used to study the associations between serum intact FGF23 and fracture outcomes. Proportional hazard assumptions were confirmed by inspecting Schoenfeld residuals and linearity assumptions by inspecting Martingale residuals. All validated fractures were included in the main analyses, followed by exploratory subanalyses of fracture type. Age and BMI were included as covariates in the first multivariate model followed by a model in which BMD was also included, and finally a model adjusted for age, BMI, BMD, GFR, vitamin D, and PTH. Additional potential confounders where then added and retained in the model if addition changed the HR for FGF23 by more than 10%. Confounders were serum biochemistries, grip strength, time to complete a "narrow walk", the ability to rise from a chair without aid, smoking, occurrence of fracture after age 50, previous 12 months fall occurrence, overall health rate and body composition. All models were stratified by the study center.

Three methods were used to evaluate the association between FGF23 and time to first fracture. FGF23 was first modeled as a continuous independent variable (per SD change) and also as quartiles based on the FGF23 distribution in the cohort. Because subjects in the first, second and third FGF23 quartile had similar risks of fracture, we created a dichotomous variable where the highest FGF23 quartile was compared to the other three quartiles. Second, cubic splines with three knots placed at the 5^{th}, 50^{th} and 95^{th}-percentile were used to allow for non-linear effects of FGF23. The splines are restricted to be linear below the first knot point and above the last knot point [206]. Third, we performed exploratory cut-point analysis. We dichotomized FGF23 at various quantiles using log likelihoods of Cox proportional hazard models. The cut-point at which FGF23 was dichotomized to produce the highest profile log likelihood was considered the best value for further dichotomizing. The cut-point analyses supported the use of the highest quartile as a cut-point.

All analyses were performed using SAS® Version 9.2 for Windows™ (SAS Institute, Cary, NC).

Paper VI

In paper VI, we investigated the relation between FGF23 and traditional cardiovascular risk factors. We studied the relation between FGF23 and anthropomorphic measurements of obesity, fat mass, serum lipids and the metabolic syndrome in the Gothenburg subpopulation of MrOS and the PIVUS population. After exclusion of participants with missing data for any variable, 964 and 946 subjects were included in subsequent analyses in MrOS and PIVUS respectively.

Initially, the distributional properties of all baseline variables were examined and non-normally distributed variables (triglycerides, HDL, leptin, calcium, PTH and FGF23) were log-transformed before used in subsequent analyses. Linear relations were investigated using linear regression models and standardized ß-values and 95% CIs are given for all analyses. All models were defined *a priori* and the variables included were chosen based on known associations with the outcome variable or FGF23.

Relations between FGF23 and clinical markers of general obesity (weight and BMI), central obesity (waist circumference and waist-to-hip ratio) and body composition were evaluated in two models:

I Adjusted for age and gender.

II Adjusted for age, gender and known FGF23-regulatory variables and factors of mineral metabolism (serum phosphate, albumin, calcium, 25(OH)D, PTH and eGFR).

Relations between FGF23 and serum lipids (total cholesterol, triglycerides, HDL, LDL, apoA1, apoB and leptin) were examined in three sets of models:

I Crude model.

II Adjusted for age and gender and BMI.

III Adjusted for age, gender, BMI, serum phosphate, albumin, calcium, 25(OH)D, PTH and eGFR.

Finally, we investigated whether FGF23 levels could predict the fulfillment of the NCEP metabolic syndrome criteria [192] using logistic regression.

P-values <0.05 from two-sided tests were considered statistically significant. SAS 9.2 (SAS Institute Inc, USA) was used for all calculations.

Results and Discussion

FGF23 and cardiovascular status

Paper I results

In paper I, we investigated the relation between FGF23 and endothelium function and arterial stiffness employing the community-based PIVUS cohort. The endothelium function was assessed by the *invasive forearm technique* (EDV-Ach and EIDV-SNP) and arterial stiffness by *pulse wave analysis* [change in reflection index (RI)]. Of the 1016 investigated subjects, 967 subjects had valid measurements of FGF23 and the vascular variables. The majority of these subjects were in CKD stage II with a mean eGFR of 77±19.5 mL/min/1.73 m^2, thus representing a valuable model of healthy individuals and early CKD. Median serum FGF23 was 42.1 pg/mL, ranging from 3.5 to 316 pg/mL, with an interquartile range from 33.2 to 53.6 pg/mL (Figure 6).

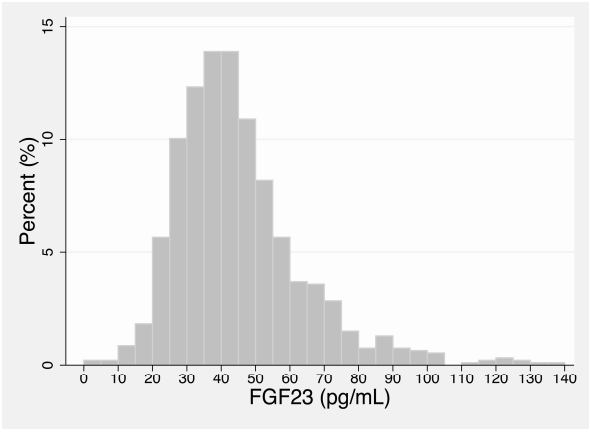

Figure 6. The distribution of serum FGF23 in the PIVUS cohort.
For the entire cohort (n = 967), the median FGF23 level was 42.1 pg/mL with the 10th and 90th percentile between 26.1 and 70.2 pg/mL, ranging between 3.5 and 316 pg/mL.

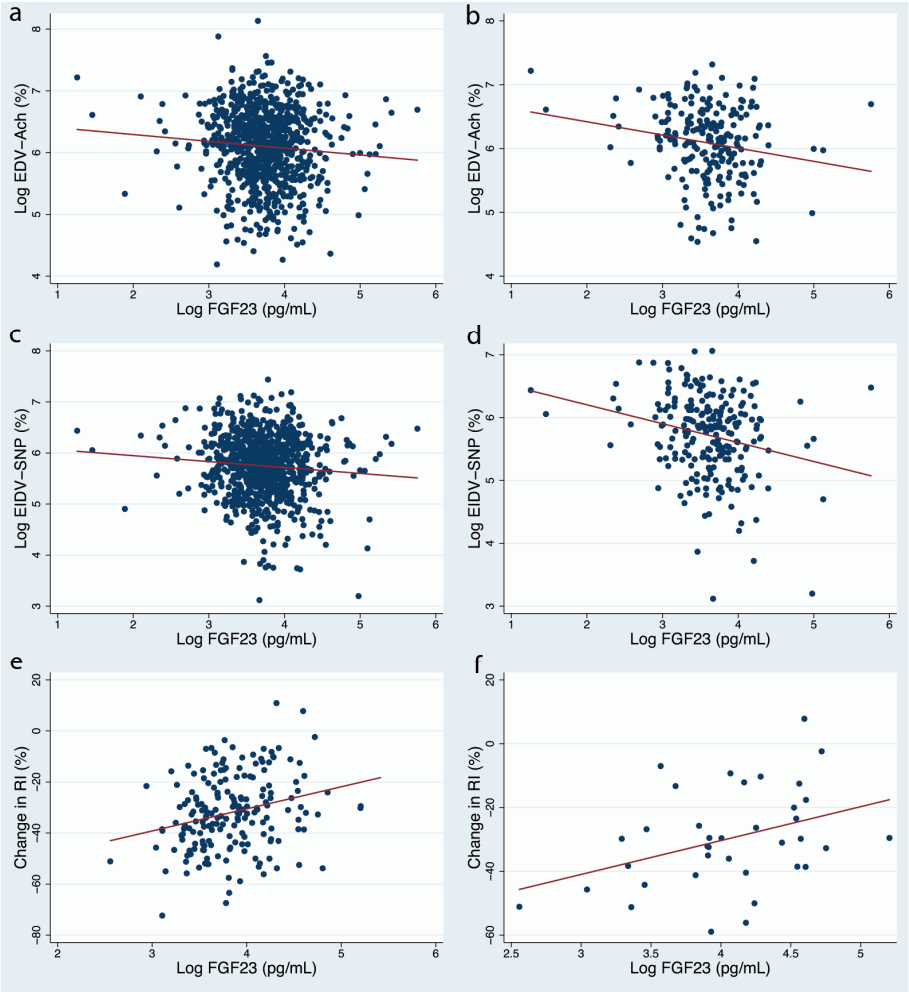

Figure 7. Relations between serum FGF23 and endothelium function, and arterial stiffness.
Endothelium function (EDV-Ach and EIDV-SNP) was assessed by the invasive forearm technique and arterial stiffness was assessed by pulse wave analysis (ΔRI). (a) FGF23 was negatively associated with EDV-Ach ($\beta = -0.08$, $p < 0.05$, CI -0.15 to -0.02, n=967). (b) The association was even stronger in subjects with eGFR ≥ 90 mL/min/1.73 m^2 ($\beta = -0.19$, $p < 0.01$, CI -0.32 to -0.06, n=235). (c) Similarly, a negative association was found between FGF23 and EIDV-SNP ($\beta = -0.08$, $p < 0.01$, CI -0.15 to -0.02, n=967). (d) This association was also stronger in subjects with eGFR ≥ 90 mL/min/1.73 m^2 ($\beta = -0.22$, $p < 0.001$, CI -0.35 to -0.09, n=235). (e) Finally, FGF23 was positively associated with ΔRI in subjects with eGFR < 60 mL/min/1.73 m^2 ($\beta = 0.26$, $p < 0.001$, CI 0.12 – 0.41, n=208), (f) and this association was further pronounced in subjects with eGFR < 50 mL/min/1.73 m^2 ($\beta = 0.38$, $p = 0.01$, CI 0.08 – 0.68, n=41).

FGF23 was negatively associated with both endothelium-dependent vasodilation and endothelium-independent vasodilation. The association was more pronounced in subjects with eGFR above 90 mL/min/1.73 m^2 (Figure 7). Thus, our data support an association between higher FGF23 and impaired endothelium function in subjects with normal renal function.

Furthermore, FGF23 was positively associated with arterial stiffness. This association was however only found in subjects with diminished renal function (<60 mL/min/1.73 m^2) and was stronger in subjects with eGFR below 50 mL/min/1.73 m^2 (Figure 7).

Collectively, we provide evidence for a continuous association between FGF23 and vascular dysfunction; higher FGF23 are associated with impaired vasoreactivity in subjects with normal renal function, and with arterial stiffness in subjects with clinically overt renal failure when eGFR falls below 60 mL/min/1.73 m^2.

Paper II results

In paper II, we studied the relation between FGF23 and atherosclerosis assessed by WBMRA. The result of the analysis was summarized in the total body atherosclerosis score (AS).

Out of 306 subjects included in this study:

- 100 subjects had a total body AS equal to 0 (FGF23 median 41.3 pg/mL and IQR 34.4 – 49.2 pg/mL)
- 206 subjects had total body AS ranging from 1 to 27.5 and a median of 7. We used the median value of AS as the cutoff between:
    - Subjects with a low AS (99 subjects; FGF23 median 40.4 pg/mL and IQR 31.5 – 52.5 pg/mL)
    - Subjects with a high AS (107 subjects; FGF23 median 48.7 pg/mL and IQR 38.1 – 59.0 pg/mL).

  FGF23 levels was significantly increased in the high AS group compared to the low AS group ($p<0.001$).

1-SD increase of FGF23 was associated with a 43-49% increased odds of having a high total body AS versus a low total body AS. In multicategory models, subjects in the highest FGF23 tertile (>50.2 pg/mL) had nearly a 3-fold increase risk of a high AS compared to the lowest tertile (Table 5). The odds ratios for FGF23 predicting degree of AS were significantly higher in subjects with eGFR<60 mL/min/1.73 m^2, supporting a nearly 6-fold increased risk for a high AS in the highest FGF23 tertile (Table 5).

Table 5. Interaction between FGF23 and renal function on the impact on severity of atherosclerosis score (AS). Note the increased risk for a high AS with increasing FGF23 levels in subjects with eGFR<60 mL/min/1.73 m^2 compared to those with a normal renal function.

	All subjects with AS > 0	**Subjects AS>0 and normal renal function**	**Subjects AS>0 and diminished renal function**
FGF23 tertile 1 (<37.4 pg/mL)	Referent	Referent	Referent
FGF23 tertile 2 (37.4-50.2 pg/mL)	2.15 (1.06-4.37)*	1.79 (0.86-3.71)	2.42 (1.02-5.73)**
FGF23 tertile 3 (>50.2 pg/mL)	3.01 (1.52-5.99)**	2.35 (1.56-4.77)*	5.64 (2.78 – 11.5)**

Values are odds ratios (95% CI) adjusted for the interaction between FGF23 and renal function. * p<0.05 ** p<0.01.

Finally, we found evidence for a non-linear relation between FGF23 and the presence of stenosis, with a 73% increased risk for a positive AS in the highest FGF23 tertile. However, this relationship was blunted when adjusting for serum biochemistries.

Paper III results

In paper III, we evaluated the relation between serum FGF23, left ventricular mass index (LVMI), left ventricular hypertrophy (LVH), and left ventricular geometry in the population-based PIVUS cohort.

FGF23 was positively associated with LVMI (Figure 8), with increased odds for LVH (Table 6) and specifically increased odds for concentric hypertrophy (Figure 9). All associations were stronger in subjects with eGFR <60 mL/min/1.73 m^2 and elevated serum FGF23 was associated with increased odds for both concentric and eccentric hypertrophy.

Participants with LVH had a small but significant increase in FGF23 compared to those without LVH (median FGF23 43.7 pg/mL vs. 40.8 pg/mL, p=0.003). A larger increase was observed in subjects with eGFR <60 mL/min/1.73 m^2 (median FGF23 54.3 pg/mL vs. 40.1 pg/mL, p=0.0003).

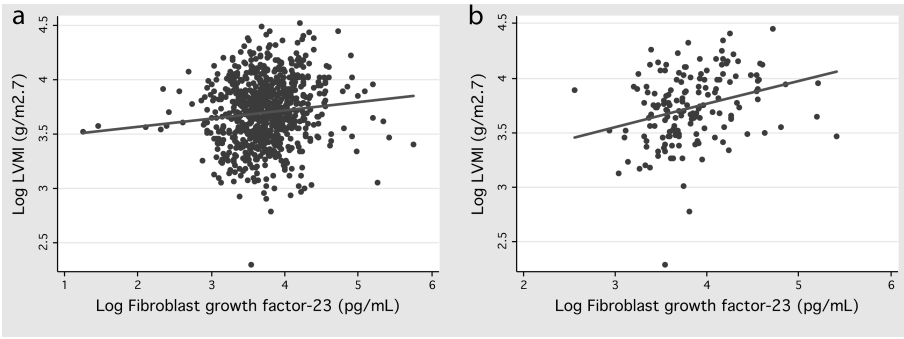

Figure 8. Relation between FGF23 and LVMI.
(a) Association between FGF23 and LVMI in all subjects (n = 795, β = 0.11, CI 0.04–018). (b) The association was more pronounced in subjects with eGFR < 60 mL/min/1.73 m^2 (n = 164, β = 0.31, CI 0.15–0.46).

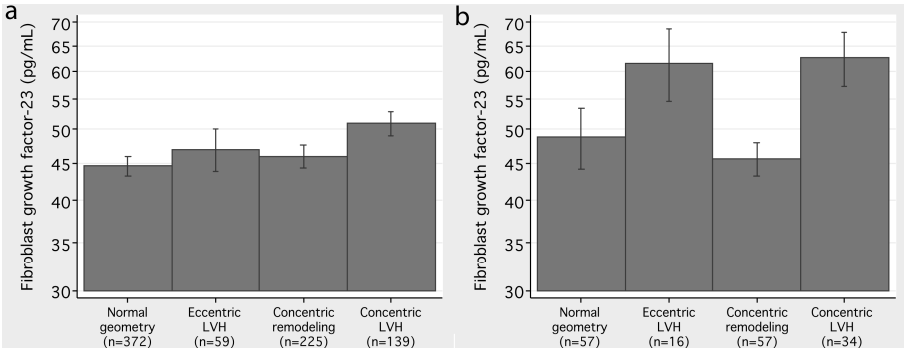

Figure 9. Differences in FGF23 levels over four groups according to left ventricular geometry.
(a) Analysis of variance (ANOVA) showed a significant difference in FGF23 levels between the groups (p = 0.003) and between the concentric LVH and normal geometry groups (p = 0.001) in the total population. (b) Similarly, ANOVA revealed a significant difference in FGF23 levels between groups (p = 0.002) and between concentric LVH and normal geometry (p = 0.01) in subjects with eGFR <60 mL/min/1.73 m^2. Y-axis is drawn on the log-scale.

Table 6. Relation between FGF23 and left ventricular hypertrophy (LVH).

	Crude model	**Adjusted for gender, systolic blood pressure, diastolic blood pressure, BMI, diabetes and hypertension**	**Adjusted for gender, serum phosphate, calcium, 25(OH)D3, PTH and eGFR.**
Whole PIVUS cohort (n=795)			
Continuous models			
1-SD increase	1.28** (1.09-1.51)	1.28* (1.05-1.55)	1.30** (1.09-1.54)
Multicategory models			
Tertile 1 (<36 pg/mL)	Referent	Referent	Referent
Tertile 2 (36-48 pg/mL)	1.14 (0.76-1.72)	1.00 (0.62-1.63)	1.13 (0.74-1.71)
Tertile 3 (>48 pg/mL)	1.71** (1.15-2.54)	1.63* (1.03-2.58)	1.70* (1.13-2.56)
Subjects with eGFR <60 mL/min/1.73 m^2 (n=164)			
Continuous models			
1-SD increase	1.86*** (1.30-2.67)	2.10*** (1.38-3.22)	1.78** (1.22-2.61)
Multicategory models			
Tertile 1 (<36 pg/mL)	Referent	Referent	Referent
Tertile 2 (36-48 pg/mL)	1.05 (0.38-2.92)	0.84 (0.26-2.73)	1.09 (0.37-3.18)
Tertile 3 (>48 pg/mL)	4.15** (1.74-9.92)	4.61** (1.61-13.17)	3.64** (1.42-9.34)

Values are odds ratios (95% confidence interval).
* p <0.05
** p <0.01
*** p <0.001

Discussion

In the first three papers, we show that FGF23 is linked to several dynamic measurements of vascular function, including endothelial dysfunction measured by an invasive forearm technique and arterial stiffness measured by pulse-wave velocity in both crude and multivariate-adjusted models [207]. This supports that FGF23 is linked to early changes in vascular function predisposing to an increased cardiovascular risk. Furthermore, in subjects that underwent the whole-body magnetic resonance angiography (WBMRA), which provides information about the degree of arterial stenosis as a surrogate marker of atherosclerosis in all major vascular territories, we show that a higher FGF23 level is associated with a higher atherosclerosis score, accentuating the role of FGF23 as a marker of early cardiovascular changes [208]. Finally, Serum FGF23 is positively associated with left ventricular mass index and increased risk of having left ventricular hypertrophy [209]. In particular, these associations were found in the highest FGF23 tertile (>48 pg/mL) and were strengthened when restricted to subjects with eGFR <60 mL/min/1.73 m^2 [209]. Interestingly FGF23 showed the strongest association with concentric hypertrophy, which represents the most malignant form of left ventricular hypertrophy from a cardiovascular standpoint.

Recently, other groups have also reported on associations between FGF23 and left ventricular hypertrophy in hemodialysis patients [181, 210], between FGF23 and vascular dysfunction expressed as forearm blood flow response to ischemia (flow-mediated vasodilatation, FMD) in patients with CKD stage 3 and 4 [211], and between FGF23 and mortality in hemodialysis patients [212, 213]. Increased FGF23 levels have also been shown to predict an increased risk for future cardiovascular events in CKD patients not yet on dialysis [214] and increased risk for mortality and cardiovascular events in patients with stable coronary artery disease with normal to moderate CKD [215]. It should be noted that FGF23 in some studies has been linked to peripheral vascular calcification and/or coronary artery calcification score, whereas other reports failed to show such an association [181, 212, 216-218]. This may, at least in part, be explained by imprecision and difficulties in standardizing the quantification of vascular calcification.

The correlation of FGF23 levels with serum phosphate in CKD patients [117, 178, 186, 219], and the association of hyperphosphatemia with adverse outcome in these patients [108, 110, 118, 119, 123, 126, 184, 185, 220-225] may partly explain why subjects with high FGF23 levels are at increased risk for prevalent and incident cardiovascular events. Likewise, calcitriol deficiency is a nontraditional cardiovascular risk factor in CKD [226-228]. Thus, FGF23 may also indirectly cause cardiovascular harm through suppressing calcitriol synthesis.

Although disordered phosphorous metabolism appears to be the obvious therapeutic target, serum phosphate levels in pre-dialysis CKD are within the normal reference range [180] and the magnitude of effect associated with changes in serum phosphate within the normal range is so small [108, 110, 126, 224, 225]. FGF23 holds promise of being a better biomarker of phosphate-related toxicity than serum phosphate. FGF23 screening could thus identify more patients with normal serum phosphate levels that would benefit from phosphorous related therapies. Among dialysis patients the level of FGF23 may also identify patients at risk for future therapy-resistant sHPT [187], and may help to predict the efficacy of calcitriol treatment in these patients [229].

There is also the possibility that FGF23 could have effects other than its regulation of serum phosphate and vitamin D levels. In support for this hypothesis is the fact that the association between FGF23 and multiple cardiovascular adverse events remained significant even after adjustment for serum biochemistries including phosphate, calcium, PTH and vitamin D [186, 188, 207]. Furthermore, FGF23 antagonizes some effects of vitamin D *in vitro*; vitamin D induces apoptosis in a cell culture model, whereas FGF23 and Klotho induce cell proliferation [230]. Finally, the supra-physiological, or rather pharmacological, concentrations of FGF23 present in many dialysis patients may induce unspecific, Klotho-independent, FGF receptor signaling, for example in endothelial cells [231]. In this regard, other members of the FGF-family, such as FGF1 and FGF2, have been linked to growth and repair of the cardiovascular system through their binding to FGFR1, which is expressed in the heart [232-235].

Preliminary data show that FGF23 reduces calcium deposition by vascular smooth muscle cells exposed to high calcium and phosphate concentrations [236]. The reduction was further enhanced in an inflamed setting similar to what may be observed in CKD patients [236]. Thus, yet another hypothesis is that if FGF23 is expressed locally in vascular walls, it could act as a local mineralization inhibitor of vascular calcification. However, whether FGF23, like many other bone-related proteins, is expressed in the vascular wall during atherosclerotic plaque formation is currently unknown.

To date there exist no drugs that target and directly reduce FGF23 levels, however, serum FGF23 levels may indirectly be lowered by use of phosphate binders [219, 237, 238]. Importantly, treatment with phosphate binders is associated with a reduction in 1-year mortality among incident hemodialysis patients [239]. Surprisingly, phosphate lowering did not only benefit those patients with overt hyperphosphatemia, but also those with physiological phosphate levels. In addition, the survival benefit was only marginally attenuated when adjusting for follow-up serum phosphate levels, which suggests benefits of phosphate binders beyond the reduction in serum phosphate. It will be of important to investigate whether lowering serum

FGF23 levels by phosphate binders can explain the benefits from this therapy and whether the outcome can be predicted by pre-treatment FGF23 levels.

Finally, there is still a lot of work to be done and many questions needing for an answer before FGF23 can be integrated into CKD practice. Specifically, the safety and efficacy of lowering serum FGF23 levels in pre-dialysis CKD subjects must be evaluated in the setting of controlled clinical trials [240].

FGF23 and bone

Paper IV results

In paper IV, we investigated the relation between circulating FGF23 and bone mineral density (BMD) in the MrOS population.

Univariate analysis revealed a weak but significant correlation between FGF23 and BMD in femoral neck, femoral trochanter, total hip and lumbar spine. Although the association remained significant when adjusting for biochemical confounders, it was abolished when adjusted for traditional confounding variables including age, height, weight and smoking. When we further explored the relation between FGF23 and these variables, only weight was significantly associated with FGF23 (β=0.15, p<0.0001).

Paper V results

In paper V, we studied the relation between FGF23 and future fracture risk in the MrOS population. 2868 subjects were included and followed prospectively for an average of 3.3±1.0 years (median 3.35 years, ranging from 6 days to 6.15 years) after the baseline examination.

1-SD increase in the baseline serum FGF23 level was associated with a 20-26% increased risk for any type of fracture and a 33-56% increased risk for clinical vertebral fractures (Table 7). Subjects in the highest FGF23 quartile (>57.4 pg/mL) were at a 62%-increased risk for all fractures compared to those in the lowest three quartiles. These subjects were also at an increased risk for clinical vertebral fractures, non-vertebral osteoporosis fractures and hip fractures (Table 7).

Importantly, the association between increased FGF23 and an increased fracture risk was not attributed to a diminished renal function as the association between FGF23 and increased fracture risk was significant exclusively in subjects with a normal renal function.

Table 7. Fibroblast growth factor-23 and the risk for fractures.

	Adjusted for age	Adjusted for age and BMI	Adjusted for age, BMI and BMD	Adjusted for age, BMI, BMD, GFR, vitamin D and PTH
Relation between fibroblast growth factor-23 and all fractures				
1-SD increase	1.20* (1.03-1.40)	1.23** (1.06-1.43)	1.26** (1.08-1.47)	1.24* (1.05-1.47)
Q4 vs. Q1-Q3 (>57.4 pg/mL)	1.54** (1.14-2.09)	1.62** (1.19-2.20)	1.64** (1.20-2.25)	1.56* (1.11-2.20)
Relation between fibroblast growth factor-23 and clinical vertebral fractures				
1-SD increase	1.33* (1.02-1.75)	1.39* (1.08-1.80)	1.44** (1.10-1.88)	1.56** (1.18-2.06)
Q4 vs. Q1-Q3 (>57.4 pg/mL)	2.02** (1.21-3.38)	2.26** (1.35-3.80)	2.30** (1.36-3.90)	2.72*** (1.54-4.79)
Relation between fibroblast growth factor-23 and non-vertebral osteoporosis fractures				
1-SD increase	1.09 (0.86-1.38)	1.11 (0.87-1.40)	1.16 (0.91-1.47)	1.06 (0.82-1.38)
Q4 vs. Q1-Q3 (>57.4 pg/mL)	1.63* (1.01-2.63)	1.68* (1.04-2.72)	1.73* (1.06-2.83)	1.50 (0.87-2.59)
Relation between fibroblast growth factor-23 and hip fractures				
1-SD increase	1.18 (0.83-1.38)	1.18 (0.82-1.69)	1.29 (0.89-1.85)	1.16 (0.78-1.72)
Q4 vs. Q1-Q3 (>57.4 pg/mL)	2.30* (1.16-4.58)	2.30* (1.15-4.60)	2.47* (1.22-5.01)	2.18* (1.00-4.73)

Values are Hazard Ratios (95% confidence interval). Q1-Q4: FGF23 quartiles 1 to 4.
* p <0.05 ** p<0.01 *** p<0.001

Discussion

FGF23 is candidate for hormonal regulation of BMD as overexpression of FGF23 in human disorders and transgenic animals indisputably cause mineralization defects and a reduction in BMD. That said, we did not find significant evidence for an association between FGF23 and BMD. Similar results have also been shown in patients with CKD on hemodialysis [241, 242]. We did however show that a higher serum FGF23 level is a novel independent predictor of overall fracture risk as well as vertebral fracture risk in elderly men (Figure 10). As evidenced by spline models and loss of significance when modeling FGF23 as a continuous variable, the relation between FGF23 and fracture risk was non-linear and stronger in individuals with FGF23 above the cut-point 56 pg/mL. Indeed, FGF23 levels above this cut-point were also associated with an increased risk for hip and non-vertebral fractures (Figure 10).

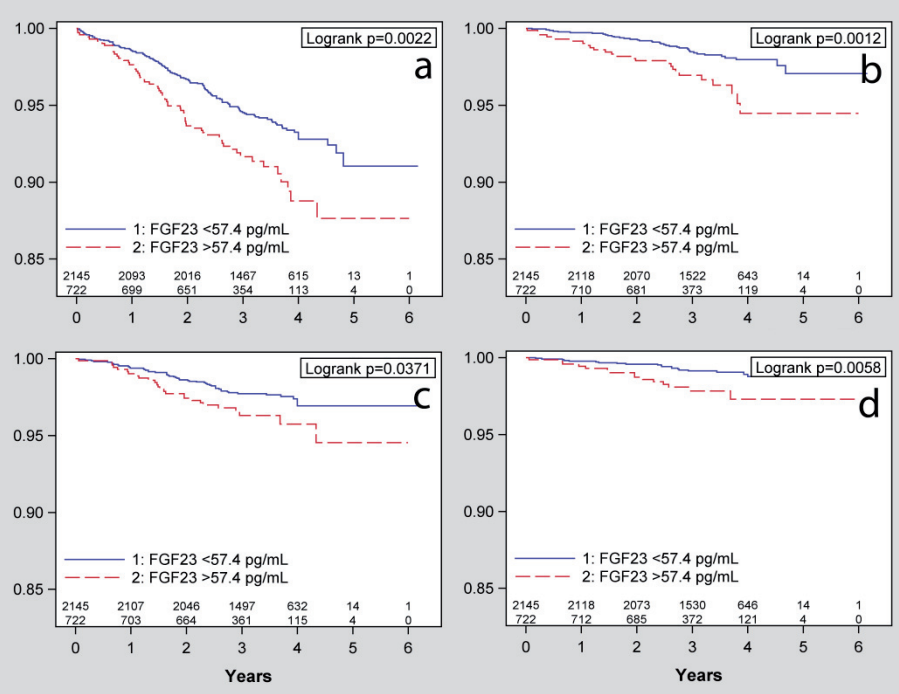

Figure 10. Fibroblast growth factor-23 and the risk for fractures.
Kaplan-Meier survival curves according to the concentration of FGF23 above and below the cut-off value (57.4 pg/mL) and the outcome of all fractures (a), clinical vertebral fractures (b), non-vertebral osteoporosis fractures (c) and hip fractures (d).

The skeletal phenotypic anomalies of several phosphate wasting disorders do not seem to be a consequence of hypophosphatemia alone, since hypophosphatemic *NaPi IIa null* mice do not develop rickets/osteomalacia [3]. Interestingly, an abnormal bone phenotype is observed both in hypophosphatemic *Fgf23* transgenic mice, which early develop severe osteomalacia [52] and hyperphosphatemic *Fgf23 null* mice, which have a reduced bone mineralization [243]. A genetic study on *Fgf23*$^{-/-}$/*NaPi IIa*$^{-/-}$ double-mutant mice recently demonstrated that despite reversal of the hyperphosphatemia of *Fgf23 null* mice into hypophosphatemia by *NaPi IIa* ablation, the skeletal phenotype remained unchanged [243]. This suggests that either an unknown factor, the temporal aspect of phosphate disturbance, the irreversibility of a bone phenotype, or the aberrant levels of FGF23 itself, rather than the phosphate levels *per see*, may be the actual cause of the bone phenotypes observed in phosphate disorders.

Although available data are conflicting [243-245], since osteocytes and osteoblasts are the predominant source of FGF23 [36, 44], FGF23 could at least have an 'intrinsic' function in bone, i.e. its expressional changes cause downstream effects in the same cell, which could affect other bone-produced

factors of relevance for bone health [14, 82, 246, 247]. FGF23 might also directly interfere with bone mineralization independently of its hypophosphatemic effect and its negative regulation of $1,25(OH)_2D$ levels. Expression of the FGF23 receptors in osteoblasts and osteoclasts supports this possibility [245, 246]. In addition, administration of FGF23 or transgenic expression of FGF23 results in a rapid and severe bone demineralization [243, 246]. However, direct endocrine effects *in vivo* are less likely given the complete absence of Klotho in bone. On the other hand, because of the mechanosensory role of the osteocyte, we cannot rule out the possibility that mechanical loading modulates FGF23 expression. Thus, it is possible that increased mechanical loading (due to a larger body weight) could stimulate FGF23 expression. Alternatively, it is also possible that yet undetermined factor(s) may cause both high FGF23 and fragility of bone.

FGF23 and traditional cardiovascular risk factors

Paper VI results

In paper VI, we studied the relation between FGF23 and anthropomorphic measurements of obesity, fat mass, serum lipids and the metabolic syndrome in the Gothenburg subpopulation of MrOS and the PIVUS population.

In both cohorts, 1-SD increase in log FGF23 was associated with 7-20% higher body weight and 7-17% higher BMI. In PIVUS, 1-SD increase in log FGF23 was associated with 9-10% higher waist circumference and 6-7% higher waist-to-hip ratio. Furthermore, in both cohorts individuals in the highest FGF23 tertile (>53.7 pg/mL) were at a significantly higher risk for overweight (BMI ≥26 as defined by the WHO criteria) than those in the lowest tertile (Figure 11). As supported by anthropomorphic measurements, 1-SD increase in log FGF23 was also associated with 7-16% higher total body fat mass and 9-18% higher trunk fat mass. We also analyzed the relation between FGF23 and traditional biochemical risk factors for cardiovascular disease. In this regard, 1-SD increase in log FGF23 was associated with 7-22% and 7-19% lower HDL and apoA1, respectively. In contrast, a corresponding 11-14% increase in triglycerides was observed. We found no significant evidence for an association between FGF23 and LDL or apoB. Finally, 1-SD increase in log FGF23 was associated with 9-12% higher leptin, however this relation was not significant in multivariate-adjusted models. Importantly, the association between FGF23 and fat mass remained significant but were attenuated when adjusting for leptin.

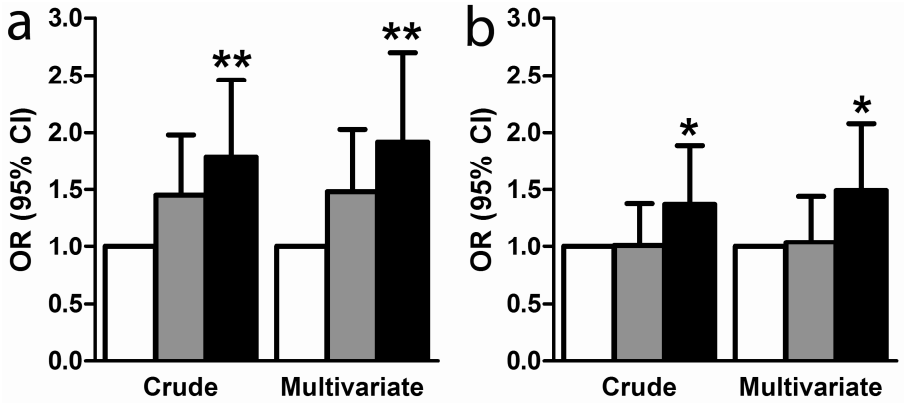

Figure 11. FGF23 and the risk for over-weight (BMI ≥ 26 as defined by the WHO criteria) in (a) MrOS and (b) PIVUS study, respectively.
The association between FGF23 tertiles (tertile 1 as referent in white) and the risk for over-weight was analyzed using logistic regression models. (Left) crude models (age-adjusted in MrOS and gender-adjusted in PIVUS); (Right) additional adjustments for phosphate, albumin, calcium, 25(OH)D, PTH and eGFR.
FGF23 Tertile 1 ≤ 33.1 pg/mL; FGF23 Tertile 2 33.1 - 53.7 pg/mL; FGF23 Tertile 3 > 53.7 pg/mL. * $p < 0.05$ ** $p < 0.01$

We also evaluated a potential role of FGF23 in the metabolic syndrome due to its link to dyslipidemia and increased fat mass. On the basis of the updated NCEP criteria, the metabolic syndrome was diagnosed in 23.3% (n=220) of all PIVUS subjects. These participants had significantly higher FGF23 levels compared to subjects without the metabolic syndrome (median FGF23 46.4 vs 41.2 pg/mL; $p<0.05$). 1-SD increase in log FGF23 was associated with a 21% (95% CI, 4-41%) increased risk of having the metabolic syndrome, although borderline significant in multivariate adjusted models. In multi-category models, subjects within the highest FGF23 tertile were at a nearly 2-fold increased risk of having the metabolic syndrome (Figure 12). FGF23 was also associated with increased risk for fulfilling the triglyceride and HDL criteria of the metabolic syndrome. Finally, we did not find any significant evidence for the relation between FGF23 levels and the number of metabolic syndrome criteria met. Hence, FGF23 may represent the burden of the metabolic syndrome risk factors rather than the clustering of these factors.

Discussion

We report on novel associations between serum FGF23 levels and higher BMI, larger waist circumference, elevated triglycerides, lower HDL cholesterol and apoA1 and increased total and truncal fat mass. These associations are important from the viewpoint that they may represent a novel pathway linking high FGF23 to an increased cardiovascular risk,

which to date is thought to be dependent on the role of FGF23 in mineral metabolism.

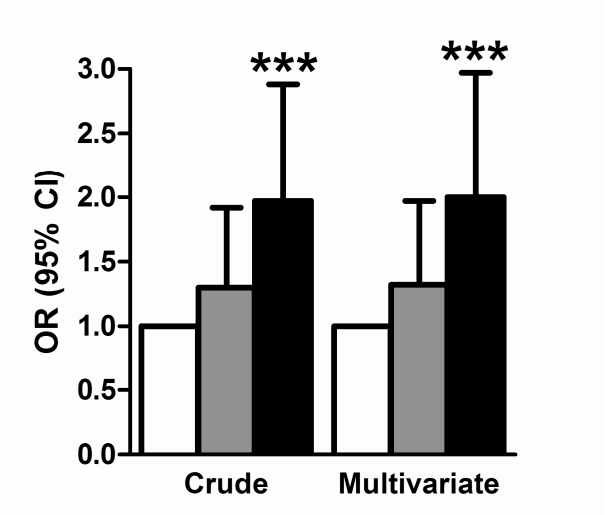

Figure 12. The relation between FGF23 and the metabolic syndrome in the PIVUS study.
Subjects in the highest FGF23 tertile (tertile 1 as referent in white) had an increased risk for the presence of the metabolic syndrome. (Left) crude model; (Right) multivariate model adjusted for age, gender, BMI, phosphate, albumin, calcium, 25(OH)D, PTH and eGFR.
FGF23 Tertile 1 ≤ 33.1 pg/mL; FGF23 Tertile 2 33.1 - 53.7 pg/mL; FGF23 Tertile 3 > 53.7 pg/mL. *** $p < 0.001$

There could be several explanatory factors for the observed association between FGF23, fat mass and dyslipidemia. FGF23 could exert indirect metabolic effects due to variations in mineral metabolism, or alternatively indicate, yet unidentified, end-organ effects of FGF23 beyond kidneys and parathyroid.

In support for the first hypothesis, elevated PTH levels and vitamin D insufficiency are associated with increased fat mass and the metabolic syndrome [248, 249]. On the other hand, it is tempting to infer parallels to other bone-derived factors such as osteocalcin that exerts profound effect on glucose homeostasis, insulin sensitivity and fat metabolism [250, 251]. In this regard, the FGF23-related growth factors, FGF19 and FGF21, exert hormonal control of fat mass and glucose metabolism and have anti-obesity properties [43, 252-255]. Our findings should prompt mechanistic studies addressing potential similar functions of FGF23.

Finally, a recent report showed that leptin directly stimulates FGF23 expression in bone [256], further accentuating the functional and biological relevance of this report.

Conclusions

Paper I

Higher serum FGF23 levels, even within the normal range, are independently associated with impaired vasoreactivity and increased arterial stiffness in the community.

Paper II

Circulating FGF23 is a novel marker of total body atherosclerosis *in vivo*.

Paper III

Higher serum FGF23 levels, even within the normal range, are associated with increased left ventricular mass index and increased risk for the presence of left ventricular hypertrophy in elderly subjects.

Paper IV

Despite the important role of FGF23 in maintaining a normal bone metabolism, serum FGF23 is only weakly related to bone mineral density. This association is largely driven by an independent relation between FGF23 and body weight.

Paper V

Circulating FGF23 is a novel, independent predictor of fracture risk in elderly men.

Paper VI

Higher serum FGF23 levels are associated with fat mass and dyslipidemia in humans, potentially representing novel pathway(s) linking high FGF23 to an increased cardiovascular risk.

Future perspectives - Clinical implications of FGF23 in CKD-MBD

FGF23 is likely one of the most important regulators of mineral metabolism and a novel key player in CKD-MBD. However, many questions remain unanswered, and the exact role of FGF23 in the development of the many complications in CKD-MBD is far from being completely understood.

First, it is of great importance to define 'normal' FGF23 ranges and cut-points, which will allow clinicians to recognize those patients with elevated FGF23 levels. Although many studies, including the ones presented in this thesis, have reported what could be regarded as normal FGF23 levels, defining normal FGF23 levels in elderly Caucasian individuals, establishing a reference range for serum FGF23 across various ages, genders, and races and in health, CKD and other relevant diseases is a necessity.

Another question is to understand the mechanisms underlying FGF23 regulation. In CKD, is it the increased phosphate load that leads to increased FGF23 levels, or is the kidney itself directly contributing to increased FGF23 levels, or could other factors associated with CKD lead to the increased FGF23 levels? In fact, kidney disease may be regarded as a state of Klotho deficiency [183], which is not only associated with hyperphosphatemia and elevated FGF23 levels [257, 258], but also with vascular calcification, accelerated aging, and premature death. Thus, CKD may represent a state of progressive renal resistance to FGF23, and the association between elevated FGF23 levels and adverse outcomes in CKD could be mediated by Klotho deficiency [259].

Finally, it is important to elucidate whether FGF23 is an innocent bystander (read biomarker) of cardiovascular status and phosphate toxicity, whether it exerts undesirable effects indirectly through calcitirol deficiency, or whether it has adverse effects independent of its role in mineral regulation. The answer to this question will have important implications on how FGF23 should be targeted in the treatment of CKD-MBD. If elevated FGF23 levels are shown to have adverse effects on for example the cardiovascular system, it is of great interest to design drugs that block excess FGF23 activity in CKD. However, if FGF23 is merely a biomarker of the adverse effects related to phosphate, blocking FGF23 action in CKD may have opposite effects potentially aggravating vascular calcification. In this

case, dietary phosphorous restriction and phosphorous binders could be used in order to weaken the source of FGF23 elevation [237, 238, 260].

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