The Role of FGF23 on Cardiac and Vascular Function in Experimental Chronic Kidney Disease

Verkaik, M.

2019

document version
Publisher's PDF, also known as Version of record

CHAPTER 1

General introduction and thesis outline

Chronic kidney disease

Chronic kidney disease (CKD) is defined as abnormalities of kidney structure or function, which include albuminuria (AER ≥30 mg/24 hours) and/or a glomerular filtration rate (GFR) of <60 mL/min/1.73 m^2, present for >3 months with implications for health[1]. To characterize the progression of CKD, a six-stage classification system based on the GFR and a three-stage classification system based on the degree of albuminuria have been internationally accepted[1]. The most prevalent causes of developing CKD are aging, hypertension, diabetes, obesity and smoking[2-5]. The median prevalence of CKD is 7.2% in persons aged 30 years or older and 23.4% to 35.8% in persons aged 64 years or older[6]. Patients with CKD are at high risk for cardiovascular diseases and progression to end stage renal disease (ESRD, CKD stage 5), a condition in which dialysis or kidney transplantation is needed.

Risk for cardiovascular diseases in CKD

Patients with chronic kidney disease usually die from cardiovascular diseases (CVD) before reaching ESRD[7]. In a Canadian cohort study life expectancy was found to be shortened due to cardiovascular disease by 1.3, 7.0, 12.5, and 16.7 years in patients aged 30 years with CKD stages 3A, 3B, 4, and 5 respectively, compared to individuals with normal kidney function[8]. Of those patients with CKD that eventually reach ESRD, 50% die from a cardiovascular cause. The age-adjusted cardiovascular mortality in ERSD is 15 to 30 times higher than in the general population and even more striking is that ESRD patients aged 25 to 34 years have a 500-fold higher cardiovascular mortality rate compared to age-matched individuals with normal kidney function[9]. Risk factors for CVD that are highly prevalent among CKD patients at all stages include hypertension, diabetes mellitus type II, dyslipidemia, activation of the renin-angiotensin system, endothelial dysfunction, oxidative stress and inflammation[10]. Interestingly, when correcting for these established cardiovascular risk factors, CKD itself remains an important independent cardiovascular risk factor[11]. Since hypertension and diabetes mellitus often cause renal failure, it has long been thought that the increased cardiovascular risk in CKD patient was mainly due to these underlying diseases in particular. In two meta-analyses however, the risk for cardiovascular mortality in CKD patients was indeed independent of hypertension and diabetes mellitus[12, 13]. This suggests that other CKD-specific factors contribute to the aforementioned cardiovascular risks.

Impaired kidney function is associated with a wide range of cardiovascular diseases. The risks for coronary heart disease, peripheral artery disease, stroke and atrial fibrillation are all increased in CKD patients and are largely independent from age, sex and ethnicity. Vascular calcification is a frequently encountered structural abnormality commonly seen in patients with advanced CKD[7, 9, 14, 15]. Vascular calcification is most prevalent among patients

on dialysis (stage 5 CKD) and not surprisingly strongly associates with its severity, which was shown by a coronary artery calcium score that is 2.5 to 5 times higher in dialysis patients compared to non-dialysis patients[16]. Even in young adults undergoing dialysis, coronary artery calcification (CAC) is highly prevalent[17].

A large population-based study showed that CKD is also strongly associated with an increased risk of incident MI and was independent from common cardiovascular risk factors such as hypertension, diabetes, BMI, and dyslipidemia[18], again pointing to CKD-specific factors being involved. In addition, the 1-year mortality after MI was higher for patients with moderate CKD compared with patients without renal dysfunction (66% versus 24%)[19].

Besides abnormalities in conduit arteries, like the coronary arteries, CKD patients suffer from other myocardial and vascular abnormalities. Myocardial capillary blood supply is lower in both uremic animals and CKD patients compared to controls[20, 21]. Remarkably, impaired coronary flow reserve is already present in early CKD and decreased by 27% in ESRD as compared to individuals with preserved kidney function[22]. Coronary flow reserve also appears to be a strong predictor of cardiovascular risk in both CKD and ESRD patients[23, 24]. This impairment of cardiac capillary blood supply can be explained by anatomical as well as functional changes. In both experimental models and human subject with renal impairment, capillary density in the myocardium is decreased[25, 26], a phenomenon known as rarefaction. Increased circulating inhibitors of angiogenesis and NO synthesis, and inhibition of proliferation and increased apoptosis of coronary endothelial cell could explain this capillary rarefaction[21]. In addition to anatomical causes, an explanation of this reduced cardiac capillary blood supply could be a disrupted functional regulation of perfusion. Indeed, reduced kidney function is associated with reduced coronary flow reserve in patients without obstructive coronary artery disease and points to coronary vascular dysfunction[27, 28]. To date, a clear explanation of this impaired cardiac microvascular function in CKD patients is still lacking.

Other cardiac morbidities are also often observed in CKD patients. In the general population, left ventricular hypertrophy (LVH) has a prevalence of 15-21%[29], while already at early stages of CKD, patients have a prevalence of 51% for LVH and by the time they reach ESRD, more than 70% of patients have LVH[30-33]. LVH in dialysis patients is not only predictive for adverse cardiovascular events[30] but also a predictor of renal disease progression to dialysis[34-36]. The mechanisms underlying LVH in CKD involve afterload-related factors including arterial hypertension and decreased aortic compliance, which result in myocardial cell thickening and concentric LV remodeling[37]. Preload-related factors involved in CKD-related LVH include expansion of total blood volume and decreased blood viscosity due to anemia (<10 g hemoglobin/dl), resulting in myocardial cell lengthening and cardiac remodeling. Cardiomyocyte hypertrophy and subsequent myocyte ischemia and fibrosis can lead to impaired contractility and a stiffening of the myocardial wall, leading to systolic

and diastolic dysfunction and ultimately to dilated cardiomyopathy and diastolic and/or systolic heart failure[37].

Indeed, heart failure (HF) is a frequently encountered cardiac abnormality observed in CKD patients. As defined by the 2016 ESC guidelines, HF is a clinical syndrome characterized by typical symptoms (e.g. shortness of breath, ankle edema and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, pulmonary crackles and peripheral edema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress[38]. The risk of HF is doubled in CKD patients compared to individuals with preserved kidney function[39] and is the predominant cardiovascular complication among patients with CKD[40]. In a large population-based study, the incidence of HF was 3-fold higher in individuals with an eGFR <60 mL/min/1.73 m^2 compared to non-CKD individuals[41] and likewise, more than 40% of HF patients have CKD[42]. HF can be subdivided in heart failure with preserved ejection fraction (≥50%, HFpEF) and with reduced ejection fraction (<50%, HFrEF). CKD-associated mortality risk is worse in diastolic HF (HFpEF) patients than in those with systolic HF (HFrEF)[43] and diastolic dysfunction is already present in early stages of CKD[44, 45]. This could in part explain the increased cardiovascular mortality among young CKD patients.

LVH and cardiac fibrosis also have been implicated in the increased risk for sustained ventricular arrhythmias and the predisposition to sudden cardiac death (SCD) associated with CKD[46]. SCD refers to an unexpected death from a cardiovascular cause with or without structural heart disease and is often caused by electrical instability and ventricular arrhythmias followed by hemodynamic failure. The risk for SCD is increased twofold in patients with mild kidney disease as compared to individuals with normal kidney function and approximately 20%–25% of all-cause mortality in ESRD is attributed to SCD[47]. Some of the underlying processes linked to the increased predisposition for SCD in people with CKD are electrophysiological and structural remodeling of the heart, e.g. LVH or fibrosis, vascular disease, and sympathetic activation.

The role of FGF23, Klotho and vitamin D in CKD

As CKD progresses, plasma phosphate increases, due to a reduction in phosphate glomerular ultrafiltration[48]. As a consequence plasma fibroblast growth factor 23 (FGF23), a hormone promoting renal wasting excretion, by reducing renal reabsorption of filtered phosphate and restoring the phosphate balance[49, 50]. However, FGF23 levels are frequently elevated in early CKD even before plasma phosphate levels are increased, and studies suggest that early CKD may be a state of primary FGF23 excess[51].

Klotho is a transmembrane protein expressed predominantly in the kidney and its most important feature is to function as a co-factor for FGF23 signaling. The close correlation between Klotho and FGF23 was discovered after the observation that FGF23-

deficient and Klotho-deficient mice show phenotypes with similar characteristics such as hyperphosphatemia, vascular calcification, osteopenia and elevated plasma vitamin D[52-55]. Decreased expression of Klotho is present already at early stages of CKD and is therefore considered an important factor in CKD and the associated cardiovascular disease. In the same endocrine system a third key player in CKD is vitamin D, as increased FGF23 levels in CKD reduce circulating active vitamin D levels[52, 56]. In mice, injections of FGF23 reduced renal mRNA for 25(OH)-vitamin D 1α-hydroxylase, the enzyme converting the inactive 25(OH)-vitamin D (25vitD) into the active form of vitamin D 1,25(OH)2-vitamin D (1,25vitD). Also, FGF23 injections increased renal mRNA for 24-hydroxylase, which is the enzyme converting 1,25vitD into the inactive 24,25 vitD[57]. These alterations in enzyme expression led to decreased levels of active vitamin D.

Vitamin D also has a suppressive effect on the renin-angiotensin-aldosteron system (RAAS) by inhibiting transcription of the renin gene[58]. The other way around, RAAS is also closely correlated to the FGF23/Klotho/vitamin D axis. Various studies have shown that angiotensin II downregulates renal expression of Klotho[59-61]. Animal studies showed that continuous administration of angiotensin II downregulated Klotho expression at both the mRNA and protein level, which was also observed in rat renal tubular epithelial cells[59, 61]. This effect is most probably regulated by the angiotensin II type 1 (AT1) receptor as treatment with losartan, an AT1 receptor blocker, reversed decreased Klotho expression in kidney of mice with renal injury[60].

Thus, CKD alters levels of FGF23, Klotho and vitamin D, resulting in a vicious circle that accelerates progression of CKD. in the next section, FGF23, Klotho and vitamin D will be discussed in more detail in their relation to renal and vascular function.

Klotho

Klotho is essential for FGF23 signaling

Klotho was discovered as a gene that is involved in the suppression of several ageing-like phenotypes and deletion led to a shortened life span[62]. In contrast, overexpression of Klotho extends life span and Klotho is therefore regarded as an aging-suppressor gene[63]. The Klotho gene encodes a long type I transmembrane protein, with a large ectodomain that can be cleaved resulting in soluble Klotho[64, 65]. Soluble Klotho might have remote systemic effects. The Klotho protein binds directly to multiple FGFRs, in which FGF23 binds with higher affinity to the Klotho-FGFR complex than to FGFR or Klotho alone[66]. With this binding of Klotho to FGFR, enhanced activation of FGF signaling by FGF23 was seen, which indicates that Klotho is essential for FGF23 signaling[53, 66].

Renal Klotho expression is mostly found in the distal tubule and is downregulated by long-term infusion of angiotensin II, production of reactive oxygen species, activation of NF-

κB and TNF-α[59, 67-69]. In contrast, upregulation of renal Klotho is induced by PPAR-gamma[2] and 1,25(OH)$_2$D$_3$[54, 67]. In early stage CKD, Klotho expression and shedding are already decreased[70, 71] and decreased plasma Klotho therefore might be an early biomarker for CKD.

Klotho and the vasculature

Klotho attenuates vascular calcification in chronic kidney disease

After the discovery that klotho mice showed acceleration of aging-related disorders like atherosclerosis, research focused on effects of Klotho on the cardiovascular system. Klotho has been shown to influence cardiovascular calcification (VC) in numerous studies[62, 72-74]. CKD mice overexpressing Klotho showed less VC compared to wild-type CKD mice [70]. Conversely, CKD mice deficient of Klotho showed more calcification compared to wild-type CKD mice. It is assumed that Klotho deficiency accelerates medial calcification, a hallmark of CKD-related atherosclerosis[75]. Soluble Klotho might interfere with vascular calcification as well, since intraperitoneal injections with soluble Klotho reduced this[76]. Klotho deficiency thus might not only be an early hallmark of CKD but also an important contributor to vascular calcification as commonly seen in CKD patients.

Klotho improves vascular function by increasing NO production

Another role of Klotho in vascular remodeling is by reducing atherosclerosis. It is believed that apoptosis and senescence of vascular endothelial cells are closely related to the development of atherosclerotic plaques[77, 78]. Klotho is an important factor that can attenuate these pathological processes, partly by reducing oxidative stress[79, 80]. Indeed, Klotho was able to protect HUVECs from both H_2O_2-induced apoptosis and oxidative stress[81]. In addition, Klotho protects also VSMCs from superoxide production and oxidative stress[82].

Besides the direct interference of Klotho with vascular cells, it also influences vascular function. Shortly after the discovery of Klotho, it was found that the vasodilator response of arterioles in heterozygous klotho-deficient mice was attenuated[83, 84]. Endothelium-dependent relaxation of aortic rings and arterioles induced by acetylcholine was higher in wild-type mice compared to heterozygous klotho mice[83, 84]. Further, NO metabolites in urine were decreased in heterozygous klotho mice[83, 84]. These results indicate a role for Klotho in vascular endothelial cell function, possibly by regulation of NO production.

Not only seems Klotho to influence larger vessels, also the smaller vessels might benefit from increased Klotho levels. In heterozygous *klotho* mice, impaired vasculogenesis and angiogenesis, accompanied by reduced endothelium-derived NO release, were observed[85]. A correlation between Klotho and vascular improvement was found in humans as well[86]. In CKD patients, serum Klotho levels was negatively associated with arterial stiffness, although no correlation was found for endothelial dysfunction, atherosclerosis or vascular

calcification. Since most data indicate that Klotho improves vascular function by increasing endothelial NO, several studies assessed a direct link with the endothelium and NO production by endothelial nitric oxide synthase (eNOS)[87, 88]. Six et al. showed that Klotho could directly increase NO production in aortic rings, which was explained by increased eNOS phosphorylation[89]. Thus, Klotho directly regulates NO production in endothelial cells by its effect on eNOS.

Of interest, a recent paper showed only low or absent Klotho transcript and protein expression in mouse arteries and these low levels did not mediate FGF23 signaling[90], suggesting that Klotho effects on blood vessels are mediated by its circulating form (soluble Klotho). The absence of vascular Klotho was confirmed by our group, where using several independent and validated methods did not detect full-length membrane-bound Klotho in both uremic and healthy human vascular tissue[91]. These findings are in contradiction with those of others, who have reported Klotho expression in human vascular tissue[92, 93]. So, whether the beneficial effects of Klotho on the vasculature are a consequence of local Klotho expression or are mediated by circulating soluble Klotho still has to be determined. The clinical implications of these findings is currently difficult to weigh. Currently available techniques to measure soluble Klotho are unreliable and it is unknown how to increase Klotho levels in patients. Therefore, although being a highly intriguing aspect of CKD, the exact clinical role of Klotho in cardiovascular disease in CKD patients is not yet clear.

Vitamin D

Vitamin D levels are regulated by enzymes and control calcium levels

Vitamin D is synthesized in the skin after exposure to UV rays of sunlight, or may be ingested from the diet[94]. Native vitamin D from the skin is transported to the liver where it is converted into the storage form 25-hydroxyvitamin D (25vitD). This main circulating metabolite of vitamin D can be converted into the circulating active 1,25-dihydroxyvitamin D (1,25vitD) by 1α-hydroxylase, mainly by the kidney and serves as an endocrine/humoral factor[95]. Degradation of vitamin D is accomplished by 24-hydroxylase, expressed in target cells containing the vitamin D receptor (VDR), including kidney, bone and many other organs[96].

Vitamin D is involved in maintaining the very constant plasma levels of calcium. Besides its roles in active uptake of calcium and phosphate by the intestines, and increased bone resorption of calcium and phosphate, vitamin D also regulates calcium reabsorption in the distal tubules and collecting ducts of the kidney[97-101].

Vitamin D levels are decreased in chronic kidney disease

An effect of increased FGF23 levels in CKD is the reduction of circulating 1,25vitD levels. Indeed, serum vitamin D levels decrease with CKD progression in which FGF23 is the strongest determinant of circulating 1,25vitD[102]. These decreased vitamin D levels were already observed in early stages of CKD, which might be explained by increased levels of FGF23 early in CKD. As mentioned above, FGF23 reduces vitamin D levels by a direct effect on 1α-hydroxylase[52, 56, 57].

Vitamin D and the vasculature

A dual role for vitamin D in vascular disease

Vitamin D plays a complex role in vascular disease. In a study among 1108 haemodialysis patients, severe vitamin D deficiency was strongly associated with sudden cardiac death, cardiovascular events and mortality[103]. On the other hand, increased 1,25vitD levels are associated with induction of vascular calcification[104-111]. It has to be noted that very high concentrations of active vitamin D were used in these studies and cannot directly be extrapolated to the human situation, since such high levels are hardly seen in the general population.

On the other hand, vitamin D shows to be a protective factor in atherosclerosis by inhibiting proliferation and calcification of VSMCs[112, 113] and protection of macrophages[114, 115]. Thus, most data point to a protective role of vitamin D in calcification and atherosclerosis, at least when pharmacological doses are avoided.

Vitamin D deficiency impairs vascular function

In healthy adults, research showed that vitamin D insufficiency is associated with increased arterial stiffness in the carotid artery and endothelial dysfunction in healthy subjects[116]. In this study, 25vitD concentrations were independently associated with FMD and microvascular function assessed by reactive hyperemia index (RHI). Importantly, a recent randomized controlled clinical trial showed that in 3 to 4 stage CKD patients 1,25vitD supplementation improved endothelium-dependent vasodilation[117]. Since both the vitamin D receptor and 1α-hydroxylase are found in vascular endothelial cells and show lower expression in vitamin D-deficient subjects, vitamin D may directly regulate vasoreactivity[118-120]. A direct effect of vitamin D on the vasculature was tested in a study set-up using a pressure myograph[121]. Resistance arteries of young rats with vitamin D deficiency showed decreased endothelium-dependent vasodilatation after acetylcholine addition compared to resistance arteries of control rats, and it was concluded that vitamin D deficiency resulted in a ±50 percent reduction of the contribution of NO to endothelium-dependent vasorelaxation[121]. Endothelium-independent vasodilatation in response to SNP was only reduced in resistance

arteries from vitamin D-deficient female rats, and not in male rats. This study suggests that vitamin D at least directly regulates endothelium-dependent vasodilatation. The role of NO within this mode of action of vitamin D was confirmed by a study on HUVECs[122], where it was shown that 1,25vitD increases NO production through activation of the VDR. Thus, vitamin D deficiency diminishes endothelium-dependent vascular function, through decreased NO synthesis.

Fibroblast growth factor 23

FGF23 is an endocrine factor, primarily produced by osteocytes

FGF23 belongs to the FGF ligand superfamily[123] and sequence analysis revealed most similarities with FGF19 and FGF21, members of the FGF19 subfamily, in which FGF15 is the mouse ortholog of human FGF19[124, 125]. The most striking feature of the FGF19 subfamily is that they function as endocrine factors and thereby having effects on distant tissues different from where it is secreted, and as such qualify as hormones[50, 126]. This feature might be due to its low affinity to heparin, which allows FGF23 to escape from heparin sulfate (HS)-rich extracellular matrices at its production site which allows it to reach the systemic circulation[127]. The majority of FGFs have a stable FGF-FGF receptor binding and dimerization, caused by the regulation of a conserved heparin-binding domain with a high affinity to heparin sulfate (HS)[128, 129]. In contrast, members the FGF19 subfamily, including FGF23, exhibit a poor HS-binding affinity, by preventing the formation of hydrogen binding between HS and amino acid residues in the HS-binding domain[127, 130]. This weak binding to HS also results in its reduced ability to bind FGF receptors (FGFR), as high affinity binding of FGF to its FGFR requires HS[131-133]. Since FGF23 exhibits low affinity to FGFR, it needs a cofactor for efficient receptor binding and signal transduction[134]. Tissue-specific expression of such a cofactor together with selective usage of FGFR subtypes determines the target organ of these endocrine-acting FGFs and creates tissue-specific effects[134]. The classic co-factor for FGF23 is Klotho, which induces high affinity binding to its receptor and is expressed predominantly in the kidney, and the parathyroid gland[53].

FGF23 is primarily produced by osteocytes and its production is directly regulated by vitamin D and dietary phosphate[135-139]. Previous research has shown that vitamin D administration increases serum FGF23 levels[137, 138]. In addition, in osteoblast cell cultures administration of 1,25(OH)2D3 stimulates FGF23 expression, most probably mediated by a vitamin D-responsive element in the FGF23 promoter[137]. Also a direct stimulating effect of parathyroid hormone (PTH) on FGF23 levels in osteoblast-like cells was observed, and in addition, long-term PTH infusion in mice increased serum FGF23 levels[140]. On the other hand, short-term PTH infusion in adult subjects decreased FGF23 levels[141]. Thus, the role of PTH in regulation of FGF23 levels remains controversial.

FGF23 regulates phosphate and vitamin D levels

The most important functions of FGF23 are inhibition of sodium-dependent phosphate reabsorption by internalization of its main transporter NaPi2a in proximal segments of the nephron and inhibition of 1α-hydroxylase activity in the proximal tubule of the kidney, leading to phosphaturia and decreased plasma levels of circulating $1,25(OH)_2D_3$, respectively[52, 56, 102, 142-144].

High FGF23 levels are associated with poor prognosis in CKD patients

FGF23 levels are closely associated with CKD progression[145]. Levels of FGF23 increase early in CKD, independently and long before serum phosphate levels are elevated[102]. This might be explained by the fact that patients with an early stage of CKD, the reduced ultrafiltration of phosphate is compensated by a diminution of tubular phosphate reabsorption, under the influence of FGF23. Many studies have shown that chronic elevation of FGF23 predicts a worse outcome of CKD patients[146-148]. In these studies, both incident and prevalent hemodialysis patients, FGF23 levels were found to be independently associated with all-cause mortality. Interestingly, this association was independent of serum phosphate levels.

FGF23 and the risk for cardiovascular disease

Besides all-cause mortality, there is a growing body of evidence from epidemiological studies for an independent association of increased FGF23 concentrations with cardiovascular diseases. In patients with coronary artery disease (CAD) a 2-fold higher risk for mortality and CV events was observed in patients with FGF23 concentrations in the highest tertile as compared to patients in the lowest tertile[149]. Comparable results were observed in patients from all stages of CKD[150-152]. Remarkably, also in the general population increased FGF23 concentrations are associated with an increased risk for cardiovascular mortality[153]. Among patients with heart failure, FGF23 is also a predictor of both all-cause and CV death[154, 155].

FGF23 and cardiac disease

Multiple epidemiological studies found a relationship between FGF23 concentrations and cardiac diseases. The incidence and prevalence of atrial fibrillation (AF) was associated with FGF23 concentrations in multiple studies[156-158] although another large cohort study with up to 20 years of follow-up, did not confirm this[159]. It thus remains debated whether FGF23 is linked to AF in CKD. Also, coronary heart disease is strongly associated with FGF23 concentrations. In a large prospective cohort, each 20 pg/ml higher FGF23 concentrations was associated with a 14% greater risk of coronary artery disease (CAD)[160, 161]. Next to a positive and independent associations of serum FGF23 with the presence of CAD, also the number of stenotic vessels was associated with serum FGF23[162].

CAD is the most common cause of heart failure and associates with FGF23 levels. In individuals with CKD and high FGF23 concentrations, incident heart failure was 2-fold higher as compared to individuals with low FGF23 concentrations[163]. Interestingly, this increased incidence remained significant in individuals without CKD, albeit less strong. Comparable results were observed in a large cohort study, wherein incident heart failure was 1.75-fold higher in individuals with high FGF23 concentrations and also remained significant in non-CKD individuals[161]. In addition, FGF23 was found to be associated with heart failure disease severity measured by NYHA class[164, 165]. These studies suggested a dose dependent worsening of cardiac function with increasing FGF23 concentrations and might therefore explain the high prevalence of heart failure in patients with ESRD, who typically have high FGF23 concentrations. That might also explain the higher incidence of heart failure in CKD patients as compared to non-CKD patients.

The increased incidence of heart failure among individuals with high FGF23 concentrations might in part be explained by its effects on left ventricular hypertrophy (LVH). Observational studies have shown that higher FGF23 concentrations are independently associated with greater left ventricular mass and higher prevalence of LVH and was observed in both CKD and non-CKD patients[166-168]. Experimental studies demonstrated that FGF23 can directly induce cardiomyocyte hypertrophy[166], which is likely mediated by the FGF receptor 4 (FGFR4) in a klotho-independent pathway[169]. Indeed, FGFR blockade reduced LV mass and improved cardiac function in CKD rats[170]. FGF23 thus seems to be strongly involved in LVH induction and could subsequently induce heart failure. Nonetheless, the molecular changes that may underlie the increased prevalence of heart failure and cardiac mortality in CKD are poorly understood.

FGF23 and the vasculature

FGF23 is independently associated with vessel calcification

Human vascular smooth muscle cells (VSMCs) with increasing phosphate concentrations show a dose-dependent increase in calcification, mediated by a sodium-dependent phosphate cotransporter[171, 172]. Since FGF23 is closely related to circulating phosphate levels, it is not surprising that serum FGF23 is independently associated with vascular calcification next to age[148, 173, 174]. In both prevalent and incident haemodialysis patients, FGF23 was independently associated with aortic and carotid artery calcification[173, 175]. This correlation has not only been observed in dialysis patients, but also CKD patients showed higher aortic and coronary calcification scores when FGF23 levels were elevated[176]. Moreover, a recent study demonstrated that FGF23 was present in calcified lesions of coronary arteries in patients undergoing heart transplantation[177]. However, several recent papers showed no direct effect of FGF23 on vascular calcification[90, 178, 179]. Whether FGF23 has an indirect or direct effect on the vessel wall has yet to be determined.

Direct actions of FGF23 on vascular function?

A direct effect of FGF23 on the vascular wall has been suggested to be involved in the cardiovascular complications of CKD patients. Several studies claimed to have found both FGFR and Klotho in the vasculature[92, 93, 180, 181], although a recent paper showed only low or absent Klotho transcript and protein expression in mouse arteries and these low levels did not mediate FGF23 signaling[90]. This was also confirmed by our own group in human vascular tissue[91]. It is therefore highly doubtful that effects of FGF23, if any, on the vasculature are the consequence of membrane-bound Klotho signal transduction via FGFR, but may rather be mediated by soluble klotho[182].

In a large cohort study, higher serum FGF23 levels, even within the normal range, were independently associated with impaired vasoreactivity and increased arterial stiffness[183]. In addition, FGF23 positively associates with arterial stiffness in patients with diminished renal function (eGFR < 60 mL/min/1.73 m^2) and FGF23 negatively associates with both endothelium-dependent and -independent vasodilatation. Another study showed that FGF23 was inversely related to endothelium-dependent vasodilation in patients with 3-4 CKD[184]. In contrast to these studies, a recent study showed no association of FGF23 with endothelial dysfunction and arterial stiffness in dialysis patients[185].

In addition to these observational studies, data describing the effects of FGF23 on vascular functions *ex vivo* are conflicting as well. Incubation of mouse aortic rings with FGF23 at supraphysiological concentrations diminishes their response to acetylcholine, which was explained by a decreased NO bioavailability[180]. Whether these effects on endothelial function are also present with more physiological levels of FGF23 remains unclear. Another recent study showed that FGF23 induces reactive oxygen species (ROS) production in endothelial cells[179], again pointing to the interference of FGF23 with NO bioavailability. In contrast, both acute and long term exposure, i.e. 30 minutes and 3 hours respectively, of FGF23 to mouse mesenteric arteries did not affect endothelium-dependent and independent dilatory contractile responses[90]. These contrasting observations of FGF23 on the microvasculature might be explained by differences of study setup, including different vascular beds, different FGF23 concentrations and exposure time of FGF23 to the vasculature. Whether FGF23 directly impairs endothelial function or via other mechanisms, is therefore under debate. Also, it is unknown if blocking FGF23 in CKD can prevent endothelial dysfunction and therefore could be a potential target to treat cardiovascular disease in CKD.

In conclusion, the high burden of cardiovascular disease in CKD patients is strongly associated with increased FGF23 levels and decreased klotho and vitamin D levels. Especially FGF23 is a strong predictor of cardiovascular events in CKD. A dose dependent worsening of cardiac function with increasing FGF23 concentrations is observed, which might be explained by direct effects of FGF23 on myocardial tissue. Whether

this only involves effects on cardiomyocyte hypertrophy resulting in LVH or also other pathophysiological mechanisms in unknown yet. Although the role of FGF23 in vascular calcification remains controversial, a clear correlation with endothelial dysfunction was observed in both clinical and preclinical studies. Most likely this vascular dysfunction is mediated trough impaired NO production in endothelial cells, but different experimental set-ups and variable FGF23 concentrations make it difficult to interpret these data. Since FGF23 is elevated already in early stages of CKD, treatment focus on lowering FGF23 levels to reduce the risk of cardiovascular events in CKD patients may be justifiable. However, it is unknown if the detrimental effects of FGF23 on the cardiovascular system are due to chronic exposure or also acute exposure of FGF23 to the vasculature. Although many associations with increased FGF23 levels and cardiovascular complications are shown, the molecular changes involved in these processes are poorly understood, and for many of these aspects proof of causality is still lacking.

Thesis outline

The main aim of this thesis is to explore the underlying physiological processes and molecular changes that could explain the increased cardiovascular risk in CKD patients with high FGF23 levels.

This thesis aims to contribute to bridging this knowledge gap by:

- Assessing the effect of FGF23 on endothelial function in a mouse model of renal insufficiency, with physiological or slightly increased FGF23 levels and different experimental setups.
- Describing the effect of FGF23 on different cardiovascular beds, like the myocardium.
- Evaluating if the effect of increased FGF23 levels on the vasculature is driven by acute or chronic exposure.
- Identifying the molecular effects of FGF23 on cardiomyocytes, besides cardiomyocyte hypertrophy.
- Assessing if cardiovascular complications in experimental CKD models can be prevented by lowering or blocking FGF23.

Coronary arterioles are the main regulators of flow through the myocardium and are able to increase myocardial blood flow in response to increased demand. Failure of the myocardial vasculature to respond to increased demand will reduce perfusion and will eventually lead to cardiac hypoxia and dysfunction. Therefore, research on myocardial perfusion derangements is essential in understanding cardiac ischemia and failure. In **Chapter 2** we highlight the technical aspects that determine myocardial perfusion results obtained using myocardial contrast echocardiography (MCE), and highlight factors that influence

cardiovascular hemodynamics that should be taken into account when using MCE in mice.

Impaired mineral homeostasis and inflammation are hallmarks of chronic kidney disease (CKD), yet the consequences of CKD-induced alterations in FGF23-αklotho-vitamin-D signaling on renal tubular electrolyte regulatory mechanisms are, however, still unclear. In **Chapter 3** we studied two different murine models, partial nephrectomy and adenine-enriched dietary intervention, to induce kidney failure and to investigate the subsequent impact on systemic and local renal factors involved in Ca^{2+} and P_i regulation.

FGF23 is independently associated with endothelial dysfunction and cardiovascular mortality. Whether FGF23 directly impairs endothelial function or indirectly via other mechanisms, is unclear. Also, mechanistic data are lacking on how FGF23 might affect cardiac function independent from LVH induction. In **Chapter 4** we tested the hypothesis that FGF23 directly impairs vascular function in CKD and whether this can be restored by blocking FGF23 effects with FGF23 antibodies. In addition, vascular function in the heart was studied by visualizing and quantifying myocardial perfusion with MCE.

In **Chapter 5** we used an approach that is more suitable for CKD patients to combat cardiovascular disease. Unfortunately, FGF23 antibodies can only be used in an experimental setup. Calcimimetics are used in CKD patients to treat hyperparathyroidism, but are also associated with a decrease in FGF23 concentrations and improved cardiovascular outcome proportionally to the decrease of FGF23 concentrations. Therefore, we tested the hypothesis that treatment with the calcimimetic R568 in experimental moderate CKD improves vascular function by lowering FGF23.

Heart failure is the predominant clinical cardiovascular presentation of patients with CKD. FGF23 has been linked directly to LVH, but the molecular changes that may underlie the increased prevalence of heart failure and cardiac mortality in CKD are poorly understood. In **Chapter 6** we hypothesized that CKD directly impairs cardiac function, besides established structural change, due to a direct effect of high FGF23 concentrations on cardiomyocyte contraction and relaxation, by modifying calcium fluxes in cardiomyocytes.

References

1. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. *Kidney inter, Suppl* 2013**:** 1–150.
2. Zhang H, Li Y, Fan Y, *et al.* Klotho is a target gene of PPAR-gamma. *Kidney Int* 2008; **74:** 732-739.
3. James MT, Hemmelgarn BR, Tonelli M. Early recognition and prevention of chronic kidney disease. *Lancet* 2010; **375:** 1296-1309.
4. Haroun MK, Jaar BG, Hoffman SC, *et al.* Risk factors for chronic kidney disease: a prospective study of 23,534 men and women in Washington County, Maryland. *J Am Soc Nephrol* 2003; **14:** 2934-2941.
5. Fox CS, Larson MG, Leip EP, *et al.* Predictors of new-onset kidney disease in a community-based population. *JAMA* 2004; **291:** 844-850.
6. Zhang QL, Rothenbacher D. Prevalence of chronic kidney disease in population-based studies: systematic review. *BMC Public Health* 2008; **8:** 117.
7. Schiffrin EL, Lipman ML, Mann JF. Chronic kidney disease: effects on the cardiovascular system. *Circulation* 2007; **116:** 85-97.
8. Hemmelgarn BR, Clement F, Manns BJ, *et al.* Overview of the Alberta Kidney Disease Network. *BMC Nephrol* 2009; **10:** 30.
9. Sarnak MJ, Levey AS, Schoolwerth AC, *et al.* Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Circulation* 2003; **108:** 2154-2169.
10. Sarnak MJ. Cardiovascular complications in chronic kidney disease. *Am J Kidney Dis* 2003; **41:** 11-17.
11. Go AS, Chertow GM, Fan D, *et al.* Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004; **351:** 1296-1305.
12. Fox CS, Matsushita K, Woodward M, *et al.* Associations of kidney disease measures with mortality and end-stage renal disease in individuals with and without diabetes: a meta-analysis. *Lancet* 2012; **380:** 1662-1673.
13. Mahmoodi BK, Matsushita K, Woodward M, *et al.* Associations of kidney disease measures with mortality and end-stage renal disease in individuals with and without hypertension: a meta-analysis. *Lancet* 2012; **380:** 1649-1661.
14. Covic A, Kanbay M, Voroneanu L, *et al.* Vascular calcification in chronic kidney disease. *Clin Sci (Lond)* 2010; **119:** 111-121.
15. Russo D, Palmiero G, De Blasio AP, *et al.* Coronary artery calcification in patients with CRF not undergoing dialysis. *Am J Kidney Dis* 2004; **44:** 1024-1030.
16. Braun J, Oldendorf M, Moshage W, *et al.* Electron beam computed tomography in the evaluation of cardiac calcification in chronic dialysis patients. *Am J Kidney Dis* 1996; **27:** 394-401.
17. Goodman WG, Goldin J, Kuizon BD, *et al.* Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. *N Engl J Med* 2000; **342:** 1478-1483.
18. Meisinger C, Doring A, Lowel H, *et al.* Chronic kidney disease and risk of incident myocardial infarction and all-cause and cardiovascular disease mortality in middle-aged men and women from the general population. *Eur Heart J* 2006; **27:** 1245-1250.
19. Shlipak MG, Heidenreich PA, Noguchi H, *et al.* Association of renal insufficiency with treatment and outcomes after myocardial infarction in elderly patients. *Ann Intern Med* 2002; **137:** 555-562.
20. Amann K, Wiest G, Zimmer G, *et al.* Reduced capillary density in the myocardium of uremic rats--a stereological study. *Kidney Int* 1992; **42:** 1079-1085.
21. Charytan DM, Padera R, Helfand AM, *et al.* Increased concentration of circulating angiogenesis and nitric oxide inhibitors induces endothelial to mesenchymal transition and myocardial fibrosis in patients with chronic kidney disease. *Int J Cardiol* 2014; **176:** 99-109.
22. Charytan DM, Skali H, Shah NR, *et al.* Coronary flow reserve is predictive of the risk of cardiovascular death regardless of chronic kidney disease stage. *Kidney Int* 2018; **93:** 501-509.
23. Murthy VL, Naya M, Foster CR, *et al.* Coronary vascular dysfunction and prognosis in patients with chronic kidney disease. *JACC Cardiovasc Imaging* 2012; **5:** 1025-1034.
24. Shah NR, Charytan DM, Murthy VL, *et al.* Prognostic Value of Coronary Flow Reserve in Patients with Dialysis-Dependent ESRD. *J Am Soc Nephrol* 2016; **27:** 1823-1829.
25. Amann K, Breitbach M, Ritz E, *et al.* Myocyte/capillary mismatch in the heart of uremic patients. *J Am Soc Nephrol* 1998; **9:** 1018-1022.
26. Amann K, Neimeier KA, Schwarz U, *et al.* Rats with moderate renal failure show capillary deficit in heart but not skeletal muscle. *Am J Kidney Dis* 1997; **30:** 382-388.
27. Chade AR, Brosh D, Higano ST, *et al.* Mild renal insufficiency is associated with reduced coronary flow in patients with non-obstructive coronary artery disease. *Kidney Int* 2006; **69:** 266-271.

28. Sakamoto N, Iwaya S, Owada T, *et al.* A reduction of coronary flow reserve is associated with chronic kidney disease and long-term cardio-cerebrovascular events in patients with non-obstructive coronary artery disease and vasospasm. *Fukushima J Med Sci* 2012; **58:** 136-143.
29. Levy D, Garrison RJ, Savage DD, *et al.* Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med* 1990; **322:** 1561-1566.
30. Zoccali C, Benedetto FA, Mallamaci F, *et al.* Prognostic value of echocardiographic indicators of left ventricular systolic function in asymptomatic dialysis patients. *J Am Soc Nephrol* 2004; **15:** 1029-1037.
31. Paoletti E, Bellino D, Cassottana P, *et al.* Left ventricular hypertrophy in nondiabetic predialysis CKD. *Am J Kidney Dis* 2005; **46:** 320-327.
32. Foley RN, Parfrey PS, Harnett JD, *et al.* Clinical and echocardiographic disease in patients starting end-stage renal disease therapy. *Kidney Int* 1995; **47:** 186-192.
33. London GM, Pannier B, Guerin AP, *et al.* Alterations of left ventricular hypertrophy in and survival of patients receiving hemodialysis: follow-up of an interventional study. *J Am Soc Nephrol* 2001; **12:** 2759-2767.
34. Paoletti E, Bellino D, Gallina AM, *et al.* Is left ventricular hypertrophy a powerful predictor of progression to dialysis in chronic kidney disease? *Nephrol Dial Transplant* 2011; **26:** 670-677.
35. Chen SC, Su HM, Hung CC, *et al.* Echocardiographic parameters are independently associated with rate of renal function decline and progression to dialysis in patients with chronic kidney disease. *Clin J Am Soc Nephrol* 2011; **6:** 2750-2758.
36. Tsioufis C, Kokkinos P, Macmanus C, *et al.* Left ventricular hypertrophy as a determinant of renal outcome in patients with high cardiovascular risk. *J Hypertens* 2010; **28:** 2299-2308.
37. Glassock RJ, Pecoits-Filho R, Barberato SH. Left ventricular mass in chronic kidney disease and ESRD. *Clin J Am Soc Nephrol* 2009; **4 Suppl 1:** S79-91.
38. Ponikowski P, Voors AA, Anker SD, *et al.* 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur Heart J* 2016; **37:** 2129-2200.
39. Gansevoort RT, Correa-Rotter R, Hemmelgarn BR, *et al.* Chronic kidney disease and cardiovascular risk: epidemiology, mechanisms, and prevention. *Lancet* 2013; **382:** 339-352.
40. Segall L, Nistor I, Covic A. Heart failure in patients with chronic kidney disease: a systematic integrative review. *Biomed Res Int* 2014; **2014:** 937398.
41. Kottgen A, Russell SD, Loehr LR, *et al.* Reduced kidney function as a risk factor for incident heart failure: the atherosclerosis risk in communities (ARIC) study. *J Am Soc Nephrol* 2007; **18:** 1307-1315.
42. Shiba N, Shimokawa H. Chronic kidney disease and heart failure--Bidirectional close link and common therapeutic goal. *J Cardiol* 2011; **57:** 8-17.
43. Ahmed A, Rich MW, Sanders PW, *et al.* Chronic kidney disease associated mortality in diastolic versus systolic heart failure: a propensity matched study. *Am J Cardiol* 2007; **99:** 393-398.
44. Otsuka T, Suzuki M, Yoshikawa H, *et al.* Left ventricular diastolic dysfunction in the early stage of chronic kidney disease. *J Cardiol* 2009; **54:** 199-204.
45. Hayashi SY, Rohani M, Lindholm B, *et al.* Left ventricular function in patients with chronic kidney disease evaluated by colour tissue Doppler velocity imaging. *Nephrol Dial Transplant* 2006; **21:** 125-132.
46. Haider AW, Larson MG, Benjamin EJ, *et al.* Increased left ventricular mass and hypertrophy are associated with increased risk for sudden death. *J Am Coll Cardiol* 1998; **32:** 1454-1459.
47. Whitman IR, Feldman HI, Deo R. CKD and sudden cardiac death: epidemiology, mechanisms, and therapeutic approaches. *J Am Soc Nephrol* 2012; **23:** 1929-1939.
48. Levin A, Bakris GL, Molitch M, *et al.* Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: results of the study to evaluate early kidney disease. *Kidney Int* 2007; **71:** 31-38.
49. Farrow EG, Davis SI, Summers LJ, *et al.* Initial FGF23-mediated signaling occurs in the distal convoluted tubule. *J Am Soc Nephrol* 2009; **20:** 955-960.
50. Hu MC, Shi M, Zhang J, *et al.* Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. *Kidney Int* 2010; **78:** 1240-1251.
51. Wolf M. Update on fibroblast growth factor 23 in chronic kidney disease. *Kidney Int* 2012; **82:** 737-747.
52. Shimada T, Kakitani M, Yamazaki Y, *et al.* Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest* 2004; **113:** 561-568.
53. Urakawa I, Yamazaki Y, Shimada T, *et al.* Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* 2006; **444:** 770-774.

54. Tsujikawa H, Kurotaki Y, Fujimori T, *et al.* Klotho, a gene related to a syndrome resembling human premature aging, functions in a negative regulatory circuit of vitamin D endocrine system. *Mol Endocrinol* 2003; **17:** 2393-2403.
55. Yoshida T, Fujimori T, Nabeshima Y. Mediation of unusually high concentrations of 1,25-dihydroxyvitamin D in homozygous klotho mutant mice by increased expression of renal 1alpha-hydroxylase gene. *Endocrinology* 2002; **143:** 683-689.
56. Perwad F, Zhang MY, Tenenhouse HS, *et al.* Fibroblast growth factor 23 impairs phosphorus and vitamin D metabolism in vivo and suppresses 25-hydroxyvitamin D-1alpha-hydroxylase expression in vitro. *Am J Physiol Renal Physiol* 2007; **293:** F1577-F1583.
57. Shimada T, Hasegawa H, Yamazaki Y, *et al.* FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J Bone Miner Res* 2004; **19:** 429-435.
58. de Borst MH, Vervloet MG, ter Wee PM, *et al.* Cross talk between the renin-angiotensin-aldosterone system and vitamin D-FGF-23-klotho in chronic kidney disease. *J Am Soc Nephrol* 2011; **22:** 1603-1609.
59. Mitani H, Ishizaka N, Aizawa T, *et al.* In vivo klotho gene transfer ameliorates angiotensin II-induced renal damage. *Hypertension* 2002; **39:** 838-843.
60. Yoon HE, Ghee JY, Piao S, *et al.* Angiotensin II blockade upregulates the expression of Klotho, the anti-ageing gene, in an experimental model of chronic cyclosporine nephropathy. *Nephrol Dial Transplant* 2011; **26:** 800-813.
61. Zhou Q, Lin S, Tang R, *et al.* Role of Fosinopril and Valsartan on Klotho Gene Expression Induced by Angiotensin II in Rat Renal Tubular Epithelial Cells. *Kidney Blood Press Res* 2010; **33:** 186-192.
62. M K-o, Matsumura Y, Aizawa H, *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390:** 45-51.
63. Kurosu H, Yamamoto M, Clark JD, *et al.* Suppression of aging in mice by the hormone Klotho. *Science* 2005; **309:** 1829-1833.
64. Chen CD, Podvin S, Gillespie E, *et al.* Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. *Proc Natl Acad Sci U S A* 2007; **104:** 19796-19801.
65. Matsumura Y, Aizawa H, Shiraki-Iida T, *et al.* Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein. *Biochem Biophys Res Commun* 1998; **242:** 626-630.
66. Kurosu H, Ogawa Y, Miyoshi M, *et al.* Regulation of fibroblast growth factor-23 signaling by klotho. *J Biol Chem* 2006; **281:** 6120-6123.
67. Forster RE, Jurutka PW, Hsieh JC, *et al.* Vitamin D receptor controls expression of the anti-aging klotho gene in mouse and human renal cells. *Biochem Biophys Res Commun* 2011; **414:** 557-562.
68. Shimizu H, Bolati D, Adijiang A, *et al.* Indoxyl sulfate downregulates renal expression of Klotho through production of ROS and activation of nuclear factor-kB. *Am J Nephrol* 2011; **33:** 319-324.
69. Thurston RD, Larmonier CB, Majewski PM, *et al.* Tumor necrosis factor and interferon-gamma down-regulate Klotho in mice with colitis. *Gastroenterology* 2010; **138:** 1384-1394, 1394.
70. Hu MC, Shi M, Zhang J, *et al.* Klotho deficiency causes vascular calcification in chronic kidney disease. *J Am Soc Nephrol* 2011; **22:** 124-136.
71. Koh N, Fujimori T, Nishiguchi S, *et al.* Severely reduced production of klotho in human chronic renal failure kidney. *Biochem Biophys Res Commun* 2001; **280:** 1015-1020.
72. Leibrock CB, Alesutan I, Voelkl J, *et al.* NH4Cl Treatment Prevents Tissue Calcification in Klotho Deficiency. *J Am Soc Nephrol* 2015; **26:** 2423-2433.
73. Voelkl J, Alesutan I, Leibrock CB, *et al.* Spironolactone ameliorates PIT1-dependent vascular osteoinduction in klotho-hypomorphic mice. *J Clin Invest* 2013; **123:** 812-822.
74. Zhao Y, Zhao MM, Cai Y, *et al.* Mammalian target of rapamycin signaling inhibition ameliorates vascular calcification via Klotho upregulation. *Kidney Int* 2015; **88:** 711-721.
75. Mencke R, Hillebrands JL, consortium N. The role of the anti-ageing protein Klotho in vascular physiology and pathophysiology. *Ageing Res Rev* 2017; **35:** 124-146.
76. Chen TH, Kuro OM, Chen CH, *et al.* The secreted Klotho protein restores phosphate retention and suppresses accelerated aging in Klotho mutant mice. *Eur J Pharmacol* 2013; **698:** 67-73.
77. Minamino T, Miyauchi H, Yoshida T, *et al.* Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation* 2002; **105:** 1541-1544.
78. Tricot O, Mallat Z, Heymes C, *et al.* Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques. *Circulation* 2000; **101:** 2450-2453.
79. Ikushima M, Rakugi H, Ishikawa K, *et al.* Anti-apoptotic and anti-senescence effects of Klotho on vascular endothelial cells. *Biochem Biophys Res Commun* 2006; **339:** 827-832.
80. de Oliveira RM. Klotho RNAi induces premature senescence of human cells via a p53/p21 dependent pathway. *FEBS Lett* 2006; **580:** 5753-5758.

81. Carracedo J, Buendia P, Merino A, *et al.* Klotho modulates the stress response in human senescent endothelial cells. *Mech Ageing Dev* 2012; **133:** 647-654.
82. Wang Y, M K-o, Sun Z. Klotho gene delivery suppresses Nox2 expression and attenuates oxidative stress in rat aortic smooth muscle cells via the cAMP-PKA pathway. *Aging Cell* 2012; **11:** 410-417.
83. Saito Y, Yamagishi T, Nakamura T, *et al.* Klotho protein protects against endothelial dysfunction. *Biochem Biophys Res Commun* 1998; **248:** 324-329.
84. Nakamura T, Saito Y, Ohyama Y, *et al.* Production of nitric oxide, but not prostacyclin, is reduced in klotho mice. *Jpn J Pharmacol* 2002; **89:** 149-156.
85. Shimada T, Takeshita Y, Murohara T, *et al.* Angiogenesis and vasculogenesis are impaired in the precocious-aging klotho mouse. *Circulation* 2004; **110:** 1148-1155.
86. Kitagawa M, Sugiyama H, Morinaga H, *et al.* A decreased level of serum soluble Klotho is an independent biomarker associated with arterial stiffness in patients with chronic kidney disease. *PLoS One* 2013; **8:** e56695.
87. Maekawa Y, Ishikawa K, Yasuda O, *et al.* Klotho suppresses TNF-alpha-induced expression of adhesion molecules in the endothelium and attenuates NF-kappaB activation. *Endocrine* 2009; **35:** 341-346.
88. Rakugi H, Matsukawa N, Ishikawa K, *et al.* Anti-oxidative effect of Klotho on endothelial cells through cAMP activation. *Endocrine* 2007; **31:** 82-87.
89. Six I, Okazaki H, Gross P, *et al.* Direct, acute effects of Klotho and FGF23 on vascular smooth muscle and endothelium. *PLoS One* 2014; **9:** e93423.
90. Lindberg K, Olauson H, Amin R, *et al.* Arterial klotho expression and FGF23 effects on vascular calcification and function. *PLoS One* 2013; **8:** e60658.
91. Mencke R, Harms G, Mirkovic K, *et al.* Membrane-bound Klotho is not expressed endogenously in healthy or uraemic human vascular tissue. *Cardiovasc Res* 2015; **108:** 220-231.
92. Donate-Correa J, Mora-Fernandez C, Martinez-Sanz R, *et al.* Expression of FGF23/KLOTHO system in human vascular tissue. *Int J Cardiol* 2011.
93. Lim K, Lu TS, Molostvov G, *et al.* Vascular Klotho deficiency potentiates the development of human artery calcification and mediates resistance to fibroblast growth factor 23. *Circulation* 2012; **125:** 2243-2255.
94. Dusso AS, Brown AJ, Slatopolsky E. Vitamin D. *Am J Physiol Renal Physiol* 2005; **289:** F8- 28.
95. Fraser DR, Kodicek E. Unique biosynthesis by kidney of a biological active vitamin D metabolite. *Nature* 1970; **228:** 764-766.
96. Jones G, Prosser DE, Kaufmann M. 25-Hydroxyvitamin D-24-hydroxylase (CYP24A1): Its important role in the degradation of vitamin D. *Arch Biochem Biophys* 2011.
97. SCHACHTER D, ROSEN SM. Active transport of Ca45 by the small intestine and its dependence on vitamin D. *Am J Physiol* 1959; **196:** 357-362.
98. CARLSSON A. Tracer experiments on the effect of vitamin D on the skeletal metabolism of calcium and phosphorus. *Acta Physiol Scand* 1952; **26:** 212-220.
99. Bouillon R, Carmeliet G, Verlinden L, *et al.* Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocr Rev* 2008; **29:** 726-776.
100. Yamamoto M, Kawanobe Y, Takahashi H, *et al.* Vitamin D deficiency and renal calcium transport in the rat. *J Clin Invest* 1984; **74:** 507-513.
101. Wang Y, Borchert ML, DeLuca HF. Identification of the vitamin D receptor in various cells of the mouse kidney. *Kidney Int* 2012.
102. Gutierrez O, Isakova T, Rhee E, *et al.* Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. *J Am Soc Nephrol* 2005; **16:** 2205-2215.
103. Drechsler C, Pilz S, Obermayer-Pietsch B, *et al.* Vitamin D deficiency is associated with sudden cardiac death, combined cardiovascular events, and mortality in haemodialysis patients. *Eur Heart J* 2010; **31:** 2253-2261.
104. Zittermann A, Schleithoff SS, Koerfer R. Vitamin D and vascular calcification. *Curr Opin Lipidol* 2007; **18:** 41-46.
105. Razzaque MS. The dualistic role of vitamin D in vascular calcifications. *Kidney Int* 2011; **79:** 708-714.
106. Rodriguez M, Martinez-Moreno JM, Rodriguez-Ortiz ME, *et al.* Vitamin D and vascular calcification in chronic kidney disease. *Kidney Blood Press Res* 2011; **34:** 261-268.
107. Bas A, Lopez I, Perez J, *et al.* Reversibility of calcitriol-induced medial artery calcification in rats with intact renal function. *J Bone Miner Res* 2006; **21:** 484-490.
108. Lopez I, guilera-Tejero E, Mendoza FJ, *et al.* Calcimimetic R-568 decreases extraosseous calcifications in uremic rats treated with calcitriol. *J Am Soc Nephrol* 2006; **17:** 795-804.
109. Henley C, Colloton M, Cattley RC, *et al.* 1,25-Dihydroxyvitamin D3 but not cinacalcet HCl (Sensipar/Mimpara) treatment mediates aortic calcification in a rat model of secondary hyperparathyroidism. *Nephrol Dial Transplant* 2005; **20:** 1370-1377.
110. Mohtai M, Yamamoto T. Smooth muscle cell proliferation in the rat coronary artery induced by vitamin D. *Atherosclerosis* 1987; **63:** 193-202.

111. Zebger-Gong H, Muller D, Diercke M, *et al.* 1,25-Dihydroxyvitamin D3-induced aortic calcifications in experimental uremia: up-regulation of osteoblast markers, calcium-transporting proteins and osterix. *J Hypertens* 2011; **29:** 339-348.

112. Leelahavanichkul A, Yan Q, Hu X, *et al.* Angiotensin II overcomes strain-dependent resistance of rapid CKD progression in a new remnant kidney mouse model. *Kidney Int* 2010; **78:** 1136-1153.

113. Aoshima Y, Mizobuchi M, Ogata H, *et al.* Vitamin D receptor activators inhibit vascular smooth muscle cell mineralization induced by phosphate and TNF-alpha. *Nephrol Dial Transplant* 2012.

114. Li X, Speer MY, Yang H, *et al.* Vitamin D receptor activators induce an anticalcific paracrine program in macrophages: requirement of osteopontin. *Arterioscler Thromb Vasc Biol* 2010; **30:** 321-326.

115. Oh J, Weng S, Felton SK, *et al.* 1,25(OH)2 vitamin d inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus. *Circulation* 2009; **120:** 687-698.

116. Al M, I, Patel R, Murrow J, *et al.* Vitamin D status is associated with arterial stiffness and vascular dysfunction in healthy humans. *J Am Coll Cardiol* 2011; **58:** 186-192.

117. Zoccali C, Curatola G, Panuccio V, *et al.* Paricalcitol and endothelial function in chronic kidney disease trial. *Hypertension* 2014; **64:** 1005-1011.

118. Zehnder D, Bland R, Chana RS, *et al.* Synthesis of 1,25-dihydroxyvitamin D(3) by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. *J Am Soc Nephrol* 2002; **13:** 621-629.

119. Wong MS, Delansorne R, Man RY, *et al.* Vitamin D derivatives acutely reduce endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat. *Am J Physiol Heart Circ Physiol* 2008; **295:** H289-H296.

120. Jablonski KL, Chonchol M, Pierce GL, *et al.* 25-Hydroxyvitamin D deficiency is associated with inflammation-linked vascular endothelial dysfunction in middle-aged and older adults. *Hypertension* 2011; **57:** 63-69.

121. Tare M, Emmett SJ, Coleman HA, *et al.* Vitamin D insufficiency is associated with impaired vascular endothelial and smooth muscle function and hypertension in young rats. *J Physiol* 2011; **589:** 4777-4786.

122. Molinari C, Uberti F, Grossini E, *et al.* 1alpha,25-dihydroxycholecalciferol induces nitric oxide production in cultured endothelial cells. *Cell Physiol Biochem* 2011; **27:** 661-668.

123. Yamashita T, Yoshioka M, Itoh N. Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. *Biochem Biophys Res Commun* 2000; **277:** 494-498.

124. Itoh N, Ornitz DM. Evolution of the Fgf and Fgfr gene families. *Trends Genet* 2004; **20:** 563-569.

125. Itoh N, Ornitz DM. Functional evolutionary history of the mouse Fgf gene family. *Dev Dyn* 2008; **237:** 18-27.

126. Kusaba T, Okigaki M, Matui A, *et al.* Klotho is associated with VEGF receptor-2 and the transient receptor potential canonical-1 Ca2+ channel to maintain endothelial integrity. *Proc Natl Acad Sci U S A* 2010; **107:** 19308-19313.

127. Goetz R, Beenken A, Ibrahimi OA, *et al.* Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members. *Mol Cell Biol* 2007; **27:** 3417-3428.

128. Mohammadi M, Olsen SK, Goetz R. A protein canyon in the FGF-FGF receptor dimer selects from an a la carte menu of heparan sulfate motifs. *Curr Opin Struct Biol* 2005; **15:** 506-516.

129. Mohammadi M, Olsen SK, Ibrahimi OA. Structural basis for fibroblast growth factor receptor activation. *Cytokine Growth Factor Rev* 2005; **16:** 107-137.

130. Harmer NJ, Pellegrini L, Chirgadze D, *et al.* The crystal structure of fibroblast growth factor (FGF) 19 reveals novel features of the FGF family and offers a structural basis for its unusual receptor affinity. *Biochemistry* 2004; **43:** 629-640.

131. Schlessinger J, Plotnikov AN, Ibrahimi OA, *et al.* Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol Cell* 2000; **6:** 743-750.

132. Rapraeger AC, Krufka A, Olwin BB. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* 1991; **252:** 1705-1708.

133. Yayon A, Klagsbrun M, Esko JD, *et al.* Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 1991; **64:** 841-848.

134. Fukumoto S. Actions and mode of actions of FGF19 subfamily members. *Endocr J* 2008; **55:** 23-31.

135. Liu S, Guo R, Simpson LG, *et al.* Regulation of fibroblastic growth factor 23 expression but not degradation by PHEX. *J Biol Chem* 2003; **278:** 37419-37426.

136. Saji F, Shigematsu T, Sakaguchi T, *et al.* Fibroblast growth factor 23 production in bone is directly regulated by 1{alpha},25-dihydroxyvitamin D, but not PTH. *Am J Physiol Renal Physiol* 2010; **299:** F1212-F1217.

137. Liu S, Tang W, Zhou J, *et al.* Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. *J Am Soc Nephrol* 2006; **17:** 1305-1315.

138. Saito H, Maeda A, Ohtomo S, *et al.* Circulating FGF-23 is regulated by 1alpha,25-dihydroxyvitamin D3 and phosphorus in vivo. *J Biol Chem* 2005; **280:** 2543-2549.

139. Martin A, David V, Quarles LD. Regulation and Function of the FGF23/Klotho Endocrine Pathways. *Physiol Rev* 2012; **92:** 131-155.

140. Lavi-Moshayoff V, Wasserman G, Meir T, *et al.* PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: a bone parathyroid feedback loop. *Am J Physiol Renal Physiol* 2010; **299:** F882-F889.

141. Gutierrez OM, Smith KT, Barchi-Chung A, *et al.* (1-34) Parathyroid hormone infusion acutely lowers fibroblast growth factor 23 concentrations in adult volunteers. *Clin J Am Soc Nephrol* 2012; **7:** 139-145.

142. Liu S, Quarles LD. How fibroblast growth factor 23 works. *J Am Soc Nephrol* 2007; **18:** 1637-1647.

143. Shimada T, Urakawa I, Yamazaki Y, *et al.* FGF-23 transgenic mice demonstrate hypophosphatemic rickets with reduced expression of sodium phosphate cotransporter type IIa. *Biochem Biophys Res Commun* 2004; **314:** 409-414.

144. Segawa H, Kawakami E, Kaneko I, *et al.* Effect of hydrolysis-resistant FGF23-R179Q on dietary phosphate regulation of the renal type-II Na/Pi transporter. *Pflugers Arch* 2003; **446:** 585-592.

145. Fliser D, Kollerits B, Neyer U, *et al.* Fibroblast growth factor 23 (FGF23) predicts progression of chronic kidney disease: the Mild to Moderate Kidney Disease (MMKD) Study. *J Am Soc Nephrol* 2007; **18:** 2600-2608.

146. Olauson H, Qureshi AR, Miyamoto T, *et al.* Relation between serum fibroblast growth factor-23 level and mortality in incident dialysis patients: are gender and cardiovascular disease confounding the relationship? *Nephrol Dial Transplant* 2010; **25:** 3033-3038.

147. Gutierrez OM, Mannstadt M, Isakova T, *et al.* Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. *N Engl J Med* 2008; **359:** 584-592.

148. Jean G, Terrat JC, Vanel T, *et al.* High levels of serum fibroblast growth factor (FGF)-23 are associated with increased mortality in long haemodialysis patients. *Nephrol Dial Transplant* 2009; **24:** 2792-2796.

149. Parker BD, Schurgers LJ, Brandenburg VM, *et al.* The associations of fibroblast growth factor 23 and uncarboxylated matrix Gla protein with mortality in coronary artery disease: the Heart and Soul Study. *Ann Intern Med* 2010; **152:** 640-648.

150. Kendrick J, Cheung AK, Kaufman JS, *et al.* FGF-23 associates with death, cardiovascular events, and initiation of chronic dialysis. *J Am Soc Nephrol* 2011; **22:** 1913-1922.

151. Seiler S, Reichart B, Roth D, *et al.* FGF-23 and future cardiovascular events in patients with chronic kidney disease before initiation of dialysis treatment. *Nephrol Dial Transplant* 2010; **25:** 3983-3989.

152. Scialla JJ, Xie H, Rahman M, *et al.* Fibroblast growth factor-23 and cardiovascular events in CKD. *J Am Soc Nephrol* 2014; **25:** 349-360.

153. Arnlov J, Carlsson AC, Sundstrom J, *et al.* Higher fibroblast growth factor-23 increases the risk of all-cause and cardiovascular mortality in the community. *Kidney Int* 2013; **83:** 160-166.

154. Plischke M, Neuhold S, Adlbrecht C, *et al.* Inorganic phosphate and FGF-23 predict outcome in stable systolic heart failure. *Eur J Clin Invest* 2012; **42:** 649-656.

155. Gruson D, Lepoutre T, Ketelslegers JM, *et al.* C-terminal FGF23 is a strong predictor of survival in systolic heart failure. *Peptides* 2012; **37:** 258-262.

156. Mathew JS, Sachs MC, Katz R, *et al.* Fibroblast growth factor-23 and incident atrial fibrillation: the Multi-Ethnic Study of Atherosclerosis (MESA) and the Cardiovascular Health Study (CHS). *Circulation* 2014; **130:** 298-307.

157. Mehta R, Cai X, Lee J, *et al.* Association of Fibroblast Growth Factor 23 With Atrial Fibrillation in Chronic Kidney Disease, From the Chronic Renal Insufficiency Cohort Study. *JAMA Cardiol* 2016; **1:** 548-556.

158. Seiler S, Cremers B, Rebling NM, *et al.* The phosphatonin fibroblast growth factor 23 links calcium-phosphate metabolism with left-ventricular dysfunction and atrial fibrillation. *Eur Heart J* 2011; **32:** 2688-2696.

159. Alonso A, Misialek JR, Eckfeldt JH, *et al.* Circulating fibroblast growth factor-23 and the incidence of atrial fibrillation: the Atherosclerosis Risk in Communities study. *J Am Heart Assoc* 2014; **3:** e001082.

160. Kestenbaum B, Sachs MC, Hoofnagle AN, *et al.* Fibroblast growth factor-23 and cardiovascular disease in the general population: the Multi-Ethnic Study of Atherosclerosis. *Circ Heart Fail* 2014; **7:** 409-417.

161. Lutsey PL, Alonso A, Selvin E, *et al.* Fibroblast growth factor-23 and incident coronary heart disease, heart failure, and cardiovascular mortality: the Atherosclerosis Risk in Communities study. *J Am Heart Assoc* 2014; **3:** e000936.

162. Hu X, Ma X, Pan X, *et al.* Fibroblast growth factor 23 is associated with the presence of coronary artery disease and the number of stenotic vessels. *Clin Exp Pharmacol Physiol* 2015; **42:** 1152-1157.

163. Ix JH, Katz R, Kestenbaum BR, *et al.* Fibroblast growth factor-23 and death, heart failure, and cardiovascular events in community-living individuals: CHS (Cardiovascular Health Study). *J Am Coll Cardiol* 2012; **60:** 200-207.

164. Poelzl G, Trenkler C, Kliebhan J, *et al.* FGF23 is associated with disease severity and prognosis in chronic heart failure. *Eur J Clin Invest* 2014; **44:** 1150-1158.

165. Imazu M, Takahama H, Asanuma H, *et al.* Pathophysiological impact of serum fibroblast growth factor 23 in patients with nonischemic cardiac disease and early chronic kidney disease. *Am J Physiol Heart Circ Physiol* 2014; **307:** H1504-1511.

166. Faul C, Amaral AP, Oskouei B, *et al.* FGF23 induces left ventricular hypertrophy. *J Clin Invest* 2011; **121:** 4393-4408.

167. Mirza MA, Larsson A, Melhus H, *et al.* Serum intact FGF23 associate with left ventricular mass, hypertrophy and geometry in an elderly population. *Atherosclerosis* 2009; **207:** 546-551.

168. Gutierrez OM, Januzzi JL, Isakova T, *et al.* Fibroblast growth factor 23 and left ventricular hypertrophy in chronic kidney disease. *Circulation* 2009; **119:** 2545-2552.

169. Grabner A, Amaral AP, Schramm K, *et al.* Activation of Cardiac Fibroblast Growth Factor Receptor 4 Causes Left Ventricular Hypertrophy. *Cell Metab* 2015; **22:** 1020-1032.

170. Di Marco GS, Reuter S, Kentrup D, *et al.* Treatment of established left ventricular hypertrophy with fibroblast growth factor receptor blockade in an animal model of CKD. *Nephrol Dial Transplant* 2014; **29:** 2028-2035.

171. Reynolds JL, Joannides AJ, Skepper JN, *et al.* Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. *J Am Soc Nephrol* 2004; **15:** 2857-2867.

172. Jono S, McKee MD, Murry CE, *et al.* Phosphate regulation of vascular smooth muscle cell calcification. *Circ Res* 2000; **87:** E10-E17.

173. Nasrallah MM, El-Shehaby AR, Salem MM, *et al.* Fibroblast growth factor-23 (FGF-23) is independently correlated to aortic calcification in haemodialysis patients. *Nephrol Dial Transplant* 2010; **25:** 2679-2685.

174. Mirza MA, Hansen T, Johansson L, *et al.* Relationship between circulating FGF23 and total body atherosclerosis in the community. *Nephrol Dial Transplant* 2009; **24:** 3125-3131.

175. Balci M, Kirkpantur A, Gulbay M, *et al.* Plasma fibroblast growth factor-23 levels are independently associated with carotid artery atherosclerosis in maintenance hemodialysis patients. *Hemodial Int* 2010; **14:** 425-432.

176. Desjardins L, Liabeuf S, Renard C, *et al.* FGF23 is independently associated with vascular calcification but not bone mineral density in patients at various CKD stages. *Osteoporos Int* 2011.

177. van Venrooij NA, Pereira RC, Tintut Y, *et al.* FGF23 protein expression in coronary arteries is associated with impaired kidney function. *Nephrol Dial Transplant* 2014.

178. Scialla JJ, Lau WL, Reilly MP, *et al.* Fibroblast growth factor 23 is not associated with and does not induce arterial calcification. *Kidney Int* 2013; **83:** 1159-1168.

179. Richter B, Haller J, Haffner D, *et al.* Klotho modulates FGF23-mediated NO synthesis and oxidative stress in human coronary artery endothelial cells. *Pflugers Arch* 2016; **468:** 1621-1635.

180. Silswal N, Touchberry CD, Daniel DR, *et al.* FGF23 directly impairs endothelium-dependent vasorelaxation by increasing superoxide levels and reducing nitric oxide bioavailability. *Am J Physiol Endocrinol Metab* 2014.

181. Jimbo R, Kawakami-Mori F, Mu S, *et al.* Fibroblast growth factor 23 accelerates phosphate-induced vascular calcification in the absence of Klotho deficiency. *Kidney Int* 2014; **85:** 1103-1111.

182. Chen G, Liu Y, Goetz R, *et al.* alpha-Klotho is a non-enzymatic molecular scaffold for FGF23 hormone signalling. *Nature* 2018; **553:** 461-466.

183. Mirza MA, Larsson A, Lind L, *et al.* Circulating fibroblast growth factor-23 is associated with vascular dysfunction in the community. *Atherosclerosis* 2009; **205:** 385-390.

184. Yilmaz MI, Sonmez A, Saglam M, *et al.* FGF-23 and vascular dysfunction in patients with stage 3 and 4 chronic kidney disease. *Kidney Int* 2010; **78:** 679-685.

185. Sarmento-Dias M, Santos-Araujo C, Poinhos R, *et al.* Fibroblast growth factor 23 is associated with left ventricular hypertrophy, not with uremic vasculopathy in peritoneal dialysis patients. *Clin Nephrol* 2016; **85:** 135-141.