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

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CHAPTER 7

General summary and overall conclusions

Patients with CKD are at high risk for cardiovascular diseases and usually die from cardiovascular diseases (CVD) before reaching ESRD. There is a growing body of evidence supporting a causal role for increased FGF23 concentrations in cardiovascular diseases.[1] The exact molecular changes that underlie cardiac dysfunction are incompletely understood and whether the effect of FGF23 on the vasculature is direct or indirect is under debate. The main aim of this thesis was to elucidate the underlying physiological processes and molecular changes that could explain the increased cardiovascular risk in CKD patients with high FGF23 levels.

Experimental models of CKD

The primary experimental mouse model we used to reflect renal disease throughout this thesis is the 5/6 nephrectomy (5/6Nx) surgical ablation model, which mimics advanced renal failure after loss of renal mass in humans. This model is the most used animal model in both mice and rats to determine cardiovascular function in CKD.[2, 3] In **Chapter 3** we used the 5/6Nx mouse model to assess effects of kidney failure on calcium and phosphate homeostasis. Three weeks after 5/6Nx uremic parameters and FGF23 concentrations were measured. Compared to sham operated mice, serum creatinine was increased 1.6 times, serum urea was increased 2.1 times and serum FGF23 increased 2.3 times. Although mice have an average life span of only two years, measuring outcome three weeks after an intervention, e.g. 5/6Nx surgery, is relatively short, especially when the research questions concern chronic kidney disease. Therefore, to better reflect chronic renal failure, we increased the time after 5/6Nx surgery to six weeks for **Chapter 4, 5 and 6**. Serum creatinine, urea and FGF23 did not further increase after three weeks. This was also shown in chapter 6, were we measured serum creatinine, urea and FGF23 both three and six weeks after 5/6Nx surgery. Although we increased time after surgery from three to six weeks, one can still question whether this mouse model truly reflects chronic kidney disease or is still an model of early renal failure. Since we did not detect hypertension or LVH in our models, which are both typical features of CKD, we believe the mouse models used in this thesis indeed still reflect an early model of renal failure. This early phase, which features early but not late pathogenic processes, is particularly interesting to be able to distinguish between causes and effect in cardiovascular diseases in CKD. Also, by detecting early alterations in the cardiovascular system during CKD we might identify pathways suitable for early intervention, in the development of cardiovascular diseases and thereby even prevent cardiovascular pathophysiology in patients with CKD. For example, LVH might be induced by increased FGF23 levels in prolonged CKD, but cardiomyocyte hypertrophy could also be caused by functional alterations within cardiomyocytes that precede development or are independent of LVH. This can only be distinguished if using experimental models of early

CKD. By interfering in cardiomyocyte function in early stages of CKD one might thereby prevent or postpone the development of LVH in these patients, if these early changes are involved in the pathogenesis. Also, if FGF23 directly induces cardiovascular pathophysiology and is increased in early stages of CKD, therapeutic interventions might focus on lowering FGF23 levels in CKD patients as soon as FGF23 levels above normal are detected. We therefore believe this model of early renal failure is not a limitation of our experimental setup in, but rather a strength to detect early cardiovascular changes in CKD. In addition, this experimental model can be used to test interventions in early CKD, in order to reduce CKD progression and/or associated cardiovascular disease, since structural cardiovascular and other organ damage have not developed yet.

On the other hand, the 5/6Nx mouse model has some disadvantages. First, due to the small animal size, both the surgery and assessment of cardiovascular parameters are challenging and somewhat variable. For example, measuring vascular function in resistance arteries of mice, i.e. the gracilis artery, requires experience in order to obtain qualitative well-functioning arteries and consistent results. To solve this issue, one could consider using a rat model if genetic alterations are not necessary to study the research question. Also, larger blood volume samples and more tissues samples can be collected from rats allowing for multiple measurements within one animal. Furthermore, the 5/6Nx mouse model does not reflect all CKD features observed in humans. For example, calcification, atherosclerosis and hypertension are rarely present in normal mice. Hypertension is also dependent on the mouse strain used.[4] Indeed, C57BL/6 mice, which we used throughout this thesis, appear to be resistant against development of several renal disease features like glomerulosclerosis, proteinuria and hypertension.[5] Lastly, the induction of renal disease by 5/6Nx surgery is abrupt and as CKD in human patients usually progresses gradually, 5/6Nx surgery induces acute injury of the kidney and therefore might not be representative of the clinical situation.

Another mouse model we used in chapter 3 to assess calcium and phosphate homeostasis in CKD is the adenine-enriched dietary (ADE) treatment. In this experimental model renal disease is induced by the tubular toxicity of adenine metabolites, i.e. crystal are formed in the proximal tubular epithelia leading to inflammation and subsequent tubulointerstitial fibrosis.[6] To circumvent the problem that mice avoid eating adenine, the compound usually is mixed with a casein-containing diet that blunts the unattractive smell and taste.[7] The adenine-induced CKD animal model has gained attention due to its relative ease of use since surgery is not needed. Four weeks after the adenine diet serum FGF23 was highly increased compared to control and thus this mouse model can be used to assess FGF23-induced alterations *in vivo*. On the other hand, this model primary reflects a tubulointerstitial disease and thus tubular specific alterations might not be caused by CKD itself but rather by local toxic effects of adenine metabolites.

Unilateral ureteral obstruction (UUO) in mice is characterized by severe renal fibrosis in the obstructed kidney while an unaffected contralateral kidney exists. Within two weeks the obstructed kidney reaches end stage.[8] The advantage of this model is good reproducibility, short time-course, easy performance and the presence of the contralateral kidney as a control, while disadvantages include lack of functional readouts, i.e. increased serum creatinine and proteinuria are absent because the injured kidney is completely obstructed and has no urine output.[9] Also serum FGF23 levels are only mildly increased 3 and 7 days after UUO.[10]

Another mouse model of spontaneous CKD is the Col4a3 null mouse model of human autosomal-recessive Alport syndrome. This is an inherited genetic disease, caused by mutations in the type IV collagen genes, e.g. *COL4A3*, and patients with mutations in these genes develop ESRD early in life.[11] Col4a3 null mice exhibit progressive glomerulonephritis, declining 1,25(OH)2D levels, increments in PTH, late onset hypocalcemia and hyperphosphatemia, high-turnover bone disease, increased mortality and at week 12 of age even show a 23 times increase of serum FGF23 levels as compared to wild-type mice.[12] Increased mortality already begins around 10-weeks-of-age, pointing to a rather severe mouse model of CKD. Interestingly, when a different genetic background is used, i.e. C57Bl6/J instead of 129X1/SvJ, a slower progression of CKD and longer survival is observed, despite having the same gene mutated.[13] At 10 weeks of age C57Bl6/J-Col4a3 null mice have modest FGF23 elevations without other alterations of mineral metabolism, LVH or hypertension, and would therefore qualify as model to assess the role of specifically FGF23 on cardiovascular function *in vivo*.

Method to assess myocardial vascular function in mice

FGF23 has been linked to impaired cardiac function, even in the absence of LVH.[14] One hypothesis could be that FGF23 alters myocardial perfusion leading to heart failure, the second most prevalent cardiovascular disease among CKD patients after atherosclerotic heart disease.[15] Indeed, failure of the myocardial vasculature to respond to increased tissue oxygen demand will eventually lead to cardiac hypoxia and dysfunction.[16] Myocardial perfusion derangements can therefore explain part of the increased cardiac mortality in CKD patients, possibly caused by increased circulating FGF23 levels. In **Chapter 2** we describe different techniques to measure myocardial perfusion in mice.

A suitable technique to measure myocardial perfusion in detail *in vivo* in mice is myocardial contrast echocardiography (MCE). The MCE technique uses contrast agents (microbubbles) enabling visualization of the vasculature, including the microcirculation. To test myocardial vascular function, vasoactive substances can be used while performing MCE. Moreover, previous studies have shown that not basal myocardial blood flow but rather the ability to increase flow, for instance after stimulation by specific agonists, are most

strongly impaired in heart failure.[16, 17] In chapter 4 and 5 we used this technique to assess myocardial microvascular function in mice with CKD and increased FGF23 levels. While we observed increased microvascular blood volume after acetylcholine infusion, we could not detect significant differences between control mice and mice with increased FGF23 levels, either by injection or after 5/6Nx. Therefore, FGF23 might not play a major regulatory role in myocardial perfusion. Also, the small size of mice, their physiological responses to vasodilators and the anesthetics regimen can differ between mice and thereby result in variable outcomes. It is therefore important that future studies take this into account by choosing a sample size that is large enough and by minimizing factors that interfere with cardiovascular physiology, or by varying the degree of uremia.

Electrolyte homeostasis in early CKD

A hallmark of CKD is disturbed mineral homeostasis, i.e. calcium and phosphate deregulation, which can contribute to the development of cardiovascular disease.[18] A complex interplay between PTH, vitamin D, klotho and FGF23 regulates blood calcium and phosphate levels. In CKD both PTH and FGF23 are increased, and vitamin D is decreased, and subsequently disturb calcium and phosphate homeostasis. However, the consequences of CKD-induced alterations in FGF23-klotho-vitamin-D signaling on renal tubular electrolyte regulatory mechanisms are still unclear. In **Chapter 3** we assessed the impact of renal failure on FGF23-klotho-vitamin-D signaling and calcium and phosphate transport regulation using two different mouse models. Both the 5/6Nx and the ADE induced model of renal failure show disturbed levels of FGF23, klotho and vitamin D, and deregulated calcium and phosphate homeostasis. As in the clinical setting, PTH was increased in both models. Unlike the clinical setting however hypercalcemia in our models developed, possibly due to increased vitamin D levels. Gene expression of Ca^{2+} transporters TRPV5 and calbindin-D_{28k} was elevated upon CKD and can therefore provide another explanation for hypercalcemia as observed in both models.

Phosphate metabolism was different between the two mouse models. While ADE mice presented a rise in plasma phosphate, 5/6Nx did not increase plasma phosphate levels. In the latter model, increased PTH and FGF23 concentrations might be sufficient to maintain physiological plasma phosphate levels in this early model of CKD by promoting phosphate excretion. Indeed, in CKD increases of FGF23 levels precede increases in plasma phosphate levels.[19] Another explanation for the discrepancy between these two experimental models could be the intervention used to induce renal damage. While with 5/6 nephrectomy renal tissue is removed and a small amount of otherwise normally functioning renal tissue is maintained, with ADE induced renal damage oxidized adenine forms precipitates and crystals in all tubules causing tubular injury, inflammation, obstruction and fibrosis. ADE mice thus lack normal functioning nephrons to act upon PTH and FGF23, possibly by downregulation of membrane-bound klotho inducing FGF23 resistance.

On the other hand, expression of the renal phosphate transporters NaP_i2a and PIT2 was decreased in both mouse models and correlated with increased urinary phosphate excretion. Vitamin D was also dysregulated in both mouse models. Unexpectedly, circulating vitamin D was increased in both models, probably by the upregulation of the enzyme converting inactive vitamin D to active vitamin D, and might be a consequence of increased PTH levels seen in both mouse models. Why PTH was not lowered by the observed increased plasma calcium, FGF23 and vitamin D levels remains to be determined.

Both models reflect renal failure, but also display different effects on the expression of calcium and phosphate transporters and blood levels. Also not all parameters reflect the clinical features of CKD, e.g. hypercalcemia and elevated vitamin D levels, which makes interpretation of the model phenotype and clinical translation difficult. This might partly be due to the short duration of both CKD-induced mouse models, but also by the different methods used to induce renal damage. Calcium and phosphate homeostasis resulting from the interplay of the klotho-FGF23-vitamin D axis remains complex and correcting these factors in CKD patients is challenging.

Vascular effects of FGF23

Several studies showed that increased FGF23 levels are associated with increased arterial stiffness and impaired vasoreactivity, both in experimental models of renal failure and CKD patients.[20-23] Whether FGF23 is a confounder or causes vascular dysfunction, and if vascular dysfunction can be prevented in CKD by lowering FGF23 is described in **Chapter 4**. We observed endothelial dysfunction both in mice with CKD and mice receiving exogenous FGF23 *in vivo*, while vascular smooth muscle cell (VSMC) function was not changed. This effect in CKD mice is caused by FGF23, since FGF23 blockade prevents this pathological effect of FGF23 on endothelial cell function.

Others have also examined the effects of FGF23 on vascular function, yet the results of these studies are conflicting. *Col4a3*$^{-/-}$ mice, known for their high FGF23 levels, showed endothelial dysfunction of aortic rings *ex vivo* and no effect on VSMC function was observed,[22] which is in accordance with our study. In contrast, endothelium-dependent and independent vasodilatory and contractile responses of mouse mesenteric arteries were not affected by both acute and long-term exposure to FGF23 (6ng/ml), although long-term exposure in this study was only three hours.[24] These negative findings were confirmed by others, who incubated mouse aortic rings with FGF23 (10 ng/ml) and also did not observe an attenuated response to acetylcholine.[25] These contradictory findings on effects of FGF23 on the vasculature, even in the same type of vessels, i.e. aorta, might be explained by the use of different exposure times of the vasculature to FGF23. We observed endothelial dysfunction after long-term, i.e. days or weeks, exposure to FGF23, and not in acute *ex vivo* exposure where resistance arteries, i.e. gracilis arteries, were incubated with FGF23 for

one hour. This is in accordance with Lindberg et al.[24] who also used resistance arteries, i.e. mesenteric arteries, and did not observe vascular dysfunction after short-term exposure, i.e. 3 hours, to FGF23 *ex vivo*. Six et al.[25] used aortic rings and incubated these vessels with FGF23 to study vasodilator function (time of incubation was not reported). Endothelium-dependent vasodilation was not altered, but again, we consider this incubation with FGF23 as short-term exposure. Silswal et al.[22] used aortic rings from *Col4a3*$^{-/-}$ mice and observed endothelial dysfunction which can be explained by the long-term exposure of vessels to high FGF23 levels *in vivo*. These studies, together with our results, thus suggest that long-term, but not short-term, exposure of the vasculature to high FGF23 concentrations induces endothelial dysfunction.

As an explanation for the FGF23-induced endothelial dysfunction, we observed increased levels of the endogenous competitive NO synthase inhibitor asymmetrical dimethyl arginine (ADMA) in our mouse models with increased FGF23 levels, which was negatively correlated with the maximum response of resistance arteries to acetylcholine. Previously, an association between decreased NO levels and increased FGF23 levels was also shown in CKD patients.[21, 26] If however FGF23 does not have acute effects on endothelial cells, it remains speculative how it induces endothelial dysfunction after long-term exposure. One explanation could lie in the presence of dimethylarginine dimethylaminohydrolases (DDAHs) in endothelial cells. ADMA is released by protein hydrolysis and mainly eliminated by DDAH.[27] Inhibition of DDAH is post-transcriptionally regulated by reactive oxygen species (ROS) and oxidative stress in endothelial cells can therefore increase ADMA concentrations. Two recent studies showed that FGF23 induces ROS production in endothelial cells,[22, 28] which could link the increased ADMA levels observed with high FGF23 concentrations (Figure 1). On the other hand, both studies assessed short-term effects of FGF23 *ex vivo* i.e. maximum 30 minutes of incubation, and as explained above, it is questionable if short-term exposure of increased FGF23 concentrations to endothelial cells induces endothelial cell dysfunction. Also, our results indicate that ROS is not involved in endothelial dysfunction in our study. Overproduction of ROS leads to oxidation of tetrahydrobiopterin (BH4) and thereby promotes eNOS uncoupling. We did not find eNOS uncoupling, which indicates that ROS production in endothelial cells was minimal, at least did not induce eNOS uncoupling. Alternatively, chronic exposure of endothelial cells to FGF23, as in CKD, might only generate small amounts of ROS, too low to induce eNOS uncoupling, but high and long enough to decrease DDAH function. To test this hypothesis, simultaneous measures of ROS production, DDAH function, eNOS uncoupling and endothelial function after acute and chronic exposure to increased FGF23 levels should be performed in both *ex vivo* and *in vivo* models.

Alternatively, ADMA is generated in the presence of protein arginine methyltransferase (PRMT) and its increased expression in endothelial cells can induce increased ADMA levels.

Indeed, in a 5/6Nx rat model expression of PRMT was increased,[29] which might be explained by increased circulating FGF23 levels in this experimental model (Figure 1) although this was not formally tested in that study. To our knowledge, no studies addressed the interplay between increased FGF23 levels in CKD and PRMT expression and we suggest to further focus on the effect of FGF23 on both PRMT and DDAH expression and function in future research.

Lastly, in chapter 3 we showed that inflammatory stimuli, i.e. Concanavalin A (ConA) and Tumor Necrosis Factor (TNF), increase FGF23 expression. Vice versa, FGF23 has also shown to increase inflammatory factors like TNF.[30] In turn, TNF also is involved in endothelial dysfunction[31] and could thereby explain impaired endothelial function with increased FGF23 levels (Figure 1). We have however not measured inflammatory markers in our mice with endothelial dysfunction. Future research should therefore explore if increased FGF23 levels in CKD increase inflammatory cytokines and thereby induce endothelial dysfunction, or if these processes are independent of each other.

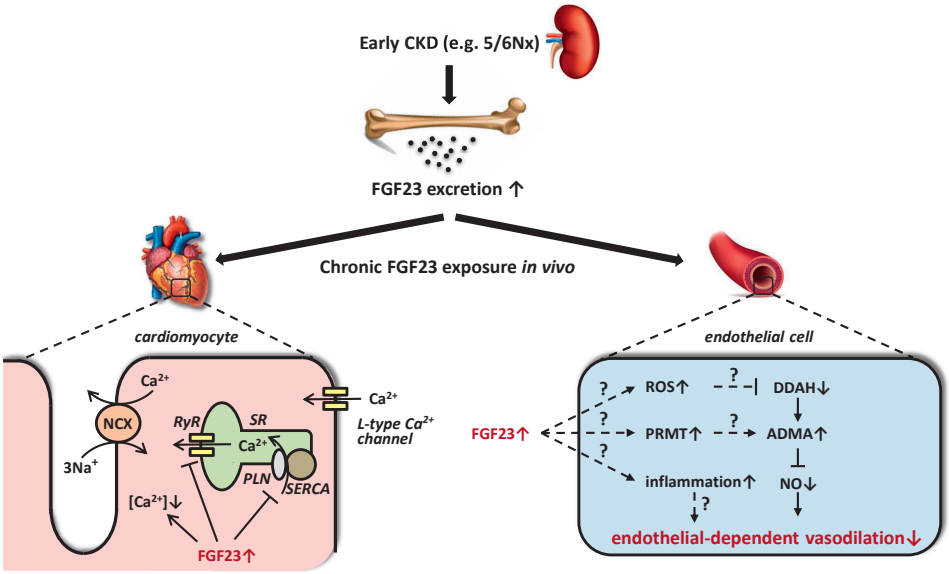

Figure 1. Increased FGF23 levels in CKD disturb calcium handling in cardiomyocytes and impairs endothelial function.

In an early model of experimental CKD (e.g. 5/6Nx) circulating FGF23 concentrations increase due to increased excretion of FGF23 from bone. Chronic exposure of cardiomyocytes to high FGF23 levels *in vivo* lowers systolic calcium content in the cytosol, decreases calcium influx velocities from the SR into the cytosol and decreases calcium efflux velocities out of the cytosol. Chronic exposure of resistance arteries to high FGF23 levels *in vivo* impairs endothelial-dependent vasodilation. ADMA levels are increased with increased FGF23 levels and might explain this endothelial dysfunction. Possible explanations for increased ADMA levels could be increased ROS production or increased PRMT expression. Alternatively, increased inflammation might explain endothelial dysfunction caused by high FGF23 concentrations. Changes in both cardiac and vascular function after exposure of high FGF23 levels might explain the increased cardiovascular morbidity in CKD patients.

Solid line represents proven pathway; dotted line with question mark represents hypothetical pathway. 5/6Nx: 5/6 nephrectomy; ADMA: asymmetrical dimethyl arginine; CKD: chronic kidney disease; DDAH: dimethylarginine dimethylaminohydrolase; FGF23: fibroblast growth factor 23; NCX: Na^{+}- Ca^{2+} exchange channel; NO: nitric oxide; PLN: phospholamban; PRMT: protein arginine methyltransferase; ROS: reactive oxygen species; RyR: Ryanodine receptor; SERCA: sarcoplasmic reticulum Ca^{2+}-ATPase; SR: sarcoplasmic reticulum.

In disease, e.g. heart failure, vasodilator reserve in resistance arteries in the myocardium is decreased, consequently limiting perfusion reserve of the coronary microcirculation, which is associated with heart failure.[16, 17, 32] FGF23 is suggested to impair cardiac function, even in the absence of LVH.[14] Since we observed peripheral vascular dysfunction with increased FGF23 levels as discussed above, this might also apply to the coronary circulation. In our experimental CKD mouse model we did observe indeed a trend for myocardial endothelial dysfunction, independent of LVH, but this was not explained by increased FGF23 levels, but most likely by other metabolic features of CKD. Different properties of microvascular beds, e.g. variation in receptor expression, release of neurotransmitters and signal transduction pathways, but also different experimental setups or different DDAH activity in endothelial cells could explain this discrepancy between peripheral and myocardial vascular responses upon increased FGF23 concentrations.

Lowering FGF23 in CKD patients might qualify as a new target to combat cardiovascular disease. In experimental studies FGF23 antibodies are used to lower circulating FGF23, but unfortunately this cannot be used as a drug in CKD patients, since the consequent increase in plasma phosphate concentrations did increase mortality, as was shown before in animal models.[33] Alternatively, lower FGF23 levels in CKD patients could be achieved by lowering phosphate levels with phosphate binders. This was observed in dialysis patients treated with the phosphate binder sevelamer and sucroferric oxyhydroxide.[34, 35] Moreover, in hyperphosphatemic patients with stage 4 CKD treatment with sevelamer leads to improved FMD, proportional to the magnitude of FGF23 level changes and was independent of phosphate levels.[23] This was confirmed in another study that also showed an improved FMD upon sevelamer treatment.[36] Sevelamer could thus be a therapeutic option to improve or maybe even prevent vascular dysfunction in CKD patients, but only in the presence of high phosphate levels, and hence in more advanced CKD. Another alternative to lower FGF23 levels in CKD patients would be the use of calcimimetics.[37] Calcimimetics have originally been developed to treat secondary hyperparathyroidism in dialysis-dependent CKD by increasing the sensitivity of the calcium-sensing receptor on tissues, including the parathyroid gland.

In **Chapter 5** we used the calcimimetic R568 to lower FGF23 concentrations in an experimental CKD mouse model and assessed if this would improve vascular function. As expected, R568 decreased circulating FGF23 concentrations in CKD mice, although only modestly, but this did not improve peripheral and myocardial vascular function. These results are not in line with what others have found in a clinical study using the calcimimetic cinacalcet. In a posthoc analysis of the EVOLVE trial[38] treatment-induced reductions in serum FGF23 were associated with lower rates of cardiovascular death and major cardiovascular events. Several factors can explain why we did not observe improved vascular function with calcimimetic treatment. First, the observed endothelial dysfunction in CKD mice in this study was less pronounced compared to our CKD mice described in chapter 4. Amelioration of this small decrease in endothelial function by the calcimimentic is hard to detect. We do not have a clear explanation why we observed different degree of disturbed vascular responses between mice from chapter 4 and 5, since we used mice with the same genetic background, the same surgery intervention, equal time after surgery to assess vascular function, similar experimental set-ups and similar read-outs. Repeating these experiments might confirm the observed results to rule out a time issue.

Another explanation for the lack of improved vascular function with calcimimetic treatment might be ascribed to structural vascular changes. Indeed, calcimimetics treatment in both experimental models and in hemodialysis patients reduced vascular calcification.[39, 40] Since wild-type C57Bl/6 mice do not develop vascular calcification, it is possible that the lack of improvements on vascular function upon R568 treatment is due to a lack of vascular calcification in our mouse model. Future research should rule out or confirm calcification as an explanation for the assumed improved vascular outcome when calcimimetics are used in an experimental CKD model with high FGF23 levels. The role of phosphate, calcium, FGF23 and vascular dysfunction in both larger and smaller vessels herein should be further explored.

Cardiac effects of FGF23

Molecular changes within the myocardium that may underlie the increased prevalence of heart failure and cardiac mortality in CKD are poorly understood. Therefore, we assessed whether disturbed calcium fluxes in cardiomyocytes could explain the increased cardiac mortality in CKD patients (**chapter 6**). We found disturbances of calcium kinetics in both mice with CKD and in mice with increased FGF23 levels in isolation. During the cardiac action potential L-type Ca^{2+} channels are activated and Ca^{2+} enters the cell via Ca^{2+} current and via the Na^{+}- Ca^{2+} exchange (NCX) channel.[41] Ca^{2+} influx triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) through the Ryanodine receptor (RyR) and thereby raises cytosolic free Ca^{2+}. When Ca^{2+} binds to the thin-filament protein troponin C (TnC) it switches on the myofilaments in a cooperative manner activating contraction. We showed that CKD

mice and mice with increased FGF23 levels in isolation had decreased calcium influx velocities and lower systolic calcium content (Figure 1). This was in the absence of altered sarcomere length and contractility. For relaxation, free cytosolic Ca^{2+} declines and dissociates from TnC. This removal of Ca^{2+} is mainly performed by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and sarcolemmal NCX, facilitating reuptake into the sarcoplasmic reticulum. Again, in both CKD mice and mice with increased FGF23 levels in isolation calcium efflux velocities out of the cytosol were decreased (Figure 1). This could however not be explained by altered protein expression of SERCA or phosphorylation status of phospholamban (PLN), which regulates SERCA activity. A possible explanation could be posttranslational modification of these calcium channels by increased reactive oxygen species (ROS) production by FGF23. Indeed, a recent study showed that FGF23 increases ROS formation in endothelial cells.[28] Others have also found that ROS induce disturbed calcium handling in cardiomyocytes.[28, 42, 43] So whether FGF23 has a direct effect on calcium channels in cardiomyocytes or via increased ROS production remains to be determined. Nonetheless, our data support that FGF23 directly disturbs cardiomyocyte function, independently from LVH possibly by increased sensitivity or an increased maximal force generating capacity. In contrast to our study, Touchberry et al. found increased intracellular calcium and improved contractility with acute FGF23 treatment, possibly by binding of FGF23 to calcium channels on the cellular membrane of cardiomyocytes.[44] One could speculate that FGF23 thus improves cardiac function, but long-term treatment with increased FGF23 concentrations of these cardiomyocytes could eventually be detrimental, by unexplored mechanisms. Importantly, this acute exposure of FGF23 *ex vivo* might be different from the clinical setting, in which cardiac tissue is exposed to high FGF23 concentrations *in vivo* for extended periods of time. In our study we tried to mimic this clinical situation by exposing mice to high FGF23 levels *in vivo* for a longer period, i.e. days to weeks, by using the 5/6Nx mouse model and chronic FGF23 injections.

The question arises if these disturbances are caused by anatomical changes, i.e. cardiomyocyte hypertrophy, or if altered calcium handling might be an early step in LVH development. We assessed LVH both by heart weight over tibia length ratio and cardiac MRI. Both CKD mice and mice receiving FGF23 injections did not show LVH and this was confirmed by the lack of increased mRNA expression of LVH markers. This is contradiction with others, who demonstrated that FGF23 can directly induce cardiomyocyte hypertrophy,[1] which is likely meditated by the FGF receptor 4 (FGFR4) in a klotho-independent pathway.[45, 46] Indeed, FGFR blockade reduced LV mass and improved cardiac function in CKD rats.[47] A reason for this contradiction with our results might be explained by different experimental set-ups, i.e. *ex vivo* vs. *in vivo*, different animal use, i.e. mice vs. rats, or diet use, i.e. high vs. normal phosphate diet. Since we did not observe cardiac dysfunction as assessed by MRI, but only disturbed calcium handling by cardiomyocytes, it is unclear if these alterations

also result in impaired cardiac function and LVH in CKD over time. Future research should therefore explore if this disturbed calcium handling by cardiomyocytes precedes cardiac dysfunction and/or LVH and if this can be ascribed to increased FGF23 concentrations. Also, since congestive heart failure, in part induced by diastolic dysfunction, is the most common cardiovascular complication among CKD patients,[15] it would be interesting to explore if lowering FGF23 in CKD can potentially prevent or at least delay development of cardiac dysfunction.

Future perspectives

The results described in this thesis and many other studies have shown that FGF23 is deeply involved in cardiovascular diseases in CKD patients. Still questions remain on what molecular mechanism cause this increased cardiovascular risk by high FGF23 levels. Our results show that chronic exposure of the vasculature to FGF23 causes endothelial dysfunction, but question about the exact mechanism remain. Possibly ROS are involved in this process, but data are conflicting. Richter et al.[28] observed increased ROS in endothelial cells after FGF23 exposure, but the lack of eNOS uncoupling in our experiments argues against this hypothesis. On the other hand, we observed increased ADMA levels in mice with increased FGF23 levels, which might point to involvement of ROS via DDAH in our mouse models. Future research thus should address the interplay between chronic and acute exposure to FGF23 on vessels, ROS and ADMA production, and vascular function. PRMT and DDAH play an important role in ADMA concentrations and might explain the interplay between increased FGF23 levels and endothelial dysfunction. Alternatively, inflammation plays a role in the pathophysiological effect of FGF23 on endothelial function. Since data point to an interplay between FGF23 and inflammation[48], this might also explain endothelial dysfunction with increased FGF23 levels. Future research should thus explore if FGF23 increases inflammatory cytokines, or other inflammatory processes and thereby disturbs endothelial function.

Simultaneously, interventions to lower FGF23 in CKD should be explored to search for proof of causality and subsequently to optimize care for patients. Possible interventions to lower FGF23 in CKD that have been proven to be effective are reducing dietary phosphate intake[49] and use of phosphate binders.[35, 50-55] Also calcimimetics are a pharmacological option to lower FGF23 in hemodialysis patients.[37, 38, 56] A posthoc analysis of the EVOLVE trial showed that reductions in serum FGF23 after cinacalcet treatment were associated with lower rates of cardiovascular death and major cardiovascular events, strongly suggesting a beneficial effect of targeting FGF23. We also showed that the calcimimetic R568 lowers FGF23 concentrations in an experimental CKD mouse model, but this did not improve peripheral and myocardial vascular function. Future research on experimental models with

and without calcification and increased FGF23 levels should shed some light on the role of calcimimetics on cardiovascular function and how this improves outcome, when its use is titrated to FGF23 reduction, instead of PTH control.

Lastly, FGF23 antibodies, or selective inhibition of FGF receptors might be a treatment option to lower FGF23 levels in CKD patients. Burosumab, a monoclonal antibody against FGF23, has been used in patients with X-linked hypophosphatemia, a condition with increased secretion of FGF23.[57] Although this is a different condition than CKD, the use of FGF23 antibodies in the clinical setting are an interesting innovative treatment option. We have also used FGF23 antibodies in our experimental CKD mouse models, although this was in the presence of a low phosphate diet. Future research should determine if FGF23 antibodies can be used in patients with CKD and how serum phosphate levels should be managed while under this treatment.

Conclusions

This thesis increases our understanding about the role of FGF23 on vascular and cardiac function in CKD. Specifically, we showed that FGF23 impairs endothelial function already early in the development of CKD and that this pathological effect of FGF23 can be prevented by lowering circulating FGF23. Next to the effects of FGF23 on vascular function, we also showed that FGF23 induces disturbances in calcium kinetics of cardiomyocytes. Previous research already showed that FGF23 induces structural changes in the cardiovascular system, e.g. LVH, while the studies in this thesis show the role of FGF23 on functional parameters of the cardiovascular system. Thus, FGF23 induces pathophysiological mechanisms in both vascular and cardiac function and support the concept of FGF23 as a therapeutic target to prevent cardiovascular morbidity and mortality in CKD patients.

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