Summary and future perspectives

Summary

Patients with CKD have a high burden of cardiovascular disease. The main goal in the treatment of patients with CKD is to lower this increased risk of cardiovascular comorbidity and mortality, besides limiting CKD progression. To a large extent the strategy to manage this increased risk in CKD is currently based on cardiovascular risk management strategies used in the general population. This implies that CKD-specific contributors to overall risk, other than proteinuria, are generally left untreated. Accumulating data suggest that a deranged FGF23-Klotho-vitamin D axis contributes to this high cardiovascular risk. Therefore, this thesis focused specifically on different clinical interventions that reduce serum FGF23 concentrations and increase α-Klotho concentrations in patients with CKD.

In the first part of this thesis, the current knowledge on dietary and pharmacological interventions to modulate serum FGF23 concentrations is described (chapter 2). Emerging data have indicated that in addition to the amount of dietary phosphorus intake, also the protein type (organic vs. inorganic) and source (animal vs. plant derived), as well as the protein-to-phosphorus ratio are very important in the bioavailability of phosphorus from food. This knowledge should be better implemented in daily practice. The combined use of a phosphate-restricted diet and phosphate binders is a well-established intervention to control serum phosphate concentrations in end-stage renal disease. Currently this therapy is not applied in patients with earlier stages of CKD where serum phosphate levels are still normal. Generally in these patients, however, FGF23 is already increased. Importantly, this early rise in FGF23 is associated with increased cardiovascular disease risk, suggesting that lowering FGF23 in early CKD could be beneficial. However, literature on the capability of dietary phosphate restriction and/or phosphate binders to lower FGF23 concentrations is inconsistent. Even more, its effects on cardiovascular comorbidity and mortality are unknown. Based on the literature, we concluded that FGF23 is best decreased by a combination of phosphate-restricted diet and a phosphate binder, as described in chapter 2. Whereas calcium-containing binders appear to increase serum FGF23 concentrations, the greatest FGF23-lowering effect seems to be achieved by sevelamer. Therefore, we designed the study protocol of the SoFT trial in which patients with CKD are treated with a forced uptitration regimen of phosphate restricted diet and sevelamer in order to evaluate the efficacy, predictability and sustainability of the decline in serum FGF23 concentration (Chapter 3). Currently this study is ongoing, and expected to be completed by the end of 2017.

FGF23 is closely related to Klotho, mainly through synergistic effects on phosphaturia. In CKD FGF23 generally rises, while Klotho declines. Both changes are independently associated with dismal outcome. Therefore, besides declining FGF23 in CKD, exploring in parallel options to increase Klotho is conceptionally appealing. In the second part of this thesis our research focused on α-Klotho. In chapter 4 we reviewed the literature on the role of α-Klotho in vascular...
calcification and endothelial dysfunction in chronic kidney disease. A key function of Klotho in vascular protection is mediated by inhibiting phosphate entrance into vascular smooth muscle cells. Maintaining endothelial integrity by Klotho is accomplished by limiting calcium entrance into these cells, as such protecting them from apoptosis and detachment. In addition, decreased concentrations of α-Klotho result in endothelial dysfunction due to its effects on NO-availability and oxidative stress. Endothelial dysfunction emerged as an important link between CKD and the increased CV risk. Because of these deleterious effects on the cardiovascular system, we focused on possible interventions that upregulate endogenous α-Klotho production. However, studying α-Klotho needs reliable assays to measure α-Klotho in both serum and urine accurately. There has been a lot of discussion about accuracy of the current available α-Klotho assays in serum.

Research from our institute showed that the IBL-assay performs best among the commercially available tests (1). In addition, we wanted to measure α-Klotho in urine with the IBL-assay as well. This method to determine its concentration in urine has already been used and reported in previous studies. However, these studies did not address the reliability of the method. Therefore we determined the stability of α-Klotho in both fresh catheter, fresh voided urine, and after several storage conditions. The results are described in chapter 5. We noted that α-Klotho is very unstable in urine. Storage of the urine for a few hours in the bladder decreases α-Klotho concentrations already with >80%. Storage in the freezer and additional freeze-thaw cycles decreased α-Klotho concentrations even further. The addition of a protease inhibitor or 0.1% albumin to fresh voided urine seems to overcome this decrease in α-Klotho. However, this effect is completely nullified after freezing, and can not overcome the issue of α-Klotho instability during residence in the bladder. These limitations prevent reliable determination of α-Klotho in individual specimens. Therefore, we refrained from urinary α-Klotho measurements in our subsequent studies.

Because animal studies suggested that oxidative stress downregulates α-Klotho, and CKD is associated with increased oxidative stress, we hypothesized that reversal of oxidative stress could restore α-Klotho. In chapter 6, we assessed the effect of anti-oxidative therapy on serum α-Klotho concentrations. A post-hoc analysis was performed in a prospective cohort of 62 patients with mild to moderate CKD who had been treated with the combination of pravastatin and vitamin E or placebo during background treatment with renin-angiotensin system (RAS-) inhibition. α-Klotho concentrations were measured at baseline and after 12 months of treatment. Unexpectedly, we were not able to show a significant increase in α-Klotho concentrations in this study population despite an effective decrease of oxidized LDL (oxLDL) as parameter for oxidative stress reduction. However, we cannot rule out the possibility that a more pronounced reduction of oxidative stress might have been effective to increase α-Klotho concentrations.

Moreover, most of the previous in vivo and in vitro animal studies quantified membrane bound Klotho instead of circulating α-Klotho concentrations. A final explanation for the lack of efficacy of anti-oxidant therapy is that all participants in this study used RAS-inhibition, which is known to increase α-Klotho concentrations and possibly precluding a further increase in α-Klotho concentrations during additional anti-oxidative therapy.
Another possible therapeutic option to increase α-Klotho concentrations was tested in chapter 7, based on observations that patients with acromegaly have very high concentrations of α-Klotho. Here we conducted a prospective, single-center open case-control pilot study in 16 subjects to evaluate the effect of subcutaneous injections of growth hormone for 7 consecutive days on serum α-Klotho concentrations. We measured α-Klotho concentrations at start of the study, after 7 days of treatment and 1 week after discontinuation of the therapy. α-Klotho concentrations increased significantly in these subjects. Our findings are in line with other studies that showed increased α-Klotho concentrations after growth hormone replacement therapy.

**Future perspectives**

Prevalent CKD is accompanied by an excessive risk of cardiovascular (CV) disease and mortality (2). Disordered phosphate homeostasis indicated by elevated circulating fibroblast growth factor 23 (FGF23) concentrations arises already in the early stages of CKD (3). Epidemiologic studies have demonstrated that higher serum phosphate and FGF23 concentrations are associated with progression of renal disease, CV disease and mortality (4-16). Regarding FGF23 as a possible explanation for these findings, it could be that FGF23 is an indicator of increased cardiovascular risk, but not causally related to it. The recent study of Udell et al. for instance showed that in subjects with high FGF23 levels at baseline, the risk of CV death or heart failure was much higher when compared to subjects with low FGF23 levels at baseline. Striking was that risk reduction by allocation to the angiotensin-converting enzyme inhibitor group as compared to the placebo-group, was only accomplished if baseline FGF23 was high (17). Alternatively, FGF23 could be a uremic toxin itself, directly inducing pathological changes to tissues and organs. In line with this perspective are experimental data implicating high phosphate in the pathogenesis of vascular calcification and endothelial dysfunction whereas high FGF23 appears to be a potential mechanistic contributor to the development of left ventricular hypertrophy in CKD (18-23). Therefore, designing studies that test the assumption that lowering serum phosphate and FGF23 concentrations are important. The current KDIGO guideline on CKD-MBD recommends phosphate-lowering treatments only for CKD patients with overt hyperphosphatemia. Unfortunately, this recommendation is supported by a low level of evidence (24). To date, serum phosphate concentrations can be effectively lowered with a phosphate restricted diet and/or phosphate binders, but there is no evidence that any of the currently available phosphate binders improve mortality when compared to placebo (25). However, as the increased CV risk is already shown in early CKD, when serum phosphate concentrations are still normal, and previous studies are mostly performed in hemodialysis patients with overt hyperphosphatemia, it is plausible that treatment strategies have to be applied at early CKD stages. The SoFT study was designed to test the FGF23- and phosphate-lowering capacity of the combination of a phosphate-restricted diet and phosphate binder therapy in non-dialysis CKD patients with normal serum phosphate levels. Current data show conflicting results on the effect of restricting dietary phosphate intake on
FGF23 concentrations. And although all dietary phosphate binders have proven capacity to decrease serum phosphate levels, their capability to lower FGF23 concentrations also differs widely (26-42). This can partially be explained by the use of varying dosages across the studies, or a suboptimal use because of poor adherence or side effects. The sample sizes of these studies were usually small with a short follow-up. In addition the calcium content from calcium-based binders might directly upregulate FGF23 (43). The physiological background of FGF23 upregulation following calcium loading is, most likely, the inhibitory effect of FGF23 on vitamin D activation, as such preventing the development of hypercalcemia. Furthermore, previous data showed that in a situation of dietary phosphate depletion there is a low luminal phosphate concentration gradient that limits passive paracellular diffusion through tight junctions across the intestinal mucosa. As adaptive response an increased active transcellular transport of phosphate via sodium phosphate (NPT2b) co-transporters occurs (44). Therefore, the addition of NPT2b blockade to low dietary phosphate intake and luminal binding of dietary phosphate could yield maximal reduction of phosphate absorption leading to maximal serum phosphate and FGF23 reduction. This is currently being studied in the COMBINE study (45).

Nowadays it is becoming clearer that serum phosphate concentration is not the sole trigger for FGF23 production and secretion as FGF23 levels are increased at the early stages of CKD where serum phosphate concentrations might even be relatively low (46). Therefore, other factors must play a role and could be possible additional targets for intervention that aim to decline FGF23. Recently, inflammation and iron deficiency emerged as FGF23 inducing factors (47). In addition to a direct effect, an increase in expression of hypoxia inducible factor 1α (HIF-1α) in osteoblasts and osteocytes is another mechanism through which inflammation and iron deficiency lead to increased syntheses of FGF23 (48). Interventions to target iron deficiency are widely established in clinical practice and are indeed associated with reductions of FGF23 concentrations in hemodialysis patients (49, 50). An alternative approach could be to target FGF23 actions downstream, however this may worsen hyperphosphatemia and has been demonstrated to promote CV disease and mortality in the preclinical setting (51). Conceptually, this issue could be overcome using more selective therapies instead of global inhibiting FGF23 effects, because the latter also blocks adaptive FGF23 increases as homeostatic defense against hyperphosphatemia. As recently demonstrated, FGF23 can directly promote the development of left ventricular hypertrophy and production of C-reactive protein in the liver by activating the Fibroblast Growth Factor Receptor 4 (FGFR4), thereby establishing FGFR4 as a pharmacological target for reducing cardiovascular risk in CKD (52, 53). A selective FGFR4-inhibitor is already developed and tested in vitro in order to treat hepatocellular carcinoma (54). This selective FGFR4-inhibitor holds promise for nephrology as well, because this approach could block FGF23 actions selectively, but without comprising the beneficial phosphaturic effects of FGF23.

In conclusion, it is still unknown which intervention performs best aiming at a safe and sustainable lowering of FGF23 concentrations with a good long-term tolerability. Therefore, lowering FGF23 as a useful strategy to improve cardiovascular outcome in CKD has not been
validated in the clinical setting yet. Besides methods described in this thesis, interventions aiming at other inducers of FGF23 besides phosphate require further exploration. Subsequently, studies need to be performed to test the best intervention on intermediate endpoints, e.g. kidney function, LVH, endothelial function and vascular calcification, and hard endpoints like reduction of CV events and mortality. It is conceivable that a combination of therapies to modulate FGF23 is necessary in order to achieve these goals.

In addition to increased serum FGF23 concentrations, accumulating evidence suggests that loss of α-Klotho also plays an important role in the increased risk of CKD patients.

Loss of α-Klotho may be one of the factors connecting impaired kidney function with the increasing CV risk (see figure 1) (55). Even more, it is also plausible that in the future, α-Klotho can be used in addition to serum creatinine to predict the renal-driven CV risk of an individual patient. For example, subjects with an eGFR of 60 ml/min/m² and normal α-Klotho concentrations have a better CV prognosis than the subjects with an eGFR of 60 ml/min/m² and reduced α-Klotho concentrations.

However, before we can use α-Klotho as a risk predictor, a reliable method to measure α-Klotho concentrations is required first. In addition, in the future, α-Klotho may also play a role in the treatment of CKD and its CV complications, as administration of exogenous α-Klotho to mice with CKD showed improvement of kidney injury, attenuation of vascular calcifications and restrained development of LVH (56-61). However, as described in the introduction of this thesis, long-term exogenous supplementation of the relatively large α-Klotho-protein (130kDa) will not be easy to accomplish and therefore upregulation of the
production of endogenous α-Klotho might be more feasible, at least in the predialysis phase, as the kidney is the primary production site of α-Klotho. Previous studies in humans already showed upregulation of α-Klotho concentrations by angiotensin-receptor blockers (62, 63). However, despite the widespread use of renin-angiotensin-system blockers, as they are proven to be cardio- and renoprotective, the frequency of CV events and mortality in patients with CKD remains high. Vitamin D analogs increase α-Klotho concentrations (64), in line with the presence of a vitamin D-responsive element in the promoter region of the Klotho gene (65). Indeed, Donate-Correa et al. demonstrated that the administration of paricalcitol to kidney transplant recipients resulted in declined methylation of the Klotho promoter gene and increased Klotho gene expression in peripheral blood mononuclear cells (66). Administration of active vitamin D analogs is a mainstay in the treatment of dialysis-dependent patients, but evidence that this improves risk for these patients is still lacking (67). On the contrary, current data do demonstrate an increased risk of hypercalcemia, hyperphosphatemia and increased serum FGF23 concentrations in hemodialysis patients treated with vitamin D analogs (68, 69). Moreover, since the kidney is the main production site of α-Klotho, ESRD patients are unlikely to benefit from vitamin D's virtue to increase α-Klotho. Previous conflicting data on the clinical benefit of the application of active vitamin D might be explained by differences among individuals in terms of induced increments of Klotho versus the development of hypercalcemia or hyperphosphatemia. Based on these concepts future studies could focus on tailored treatment in using vitamin D. Currently however, the 2009 KDIGO guideline and its very recently published update suggest restrictive use of calcitriol or its analogs to unselected patients with CKD stages 3-5 and moderate hyperparathyroidism because current data failed to demonstrate improvements in clinically relevant outcomes (24, 70, 71). α-Klotho concentrations were not measured in these studies and there is a possibility that the α-Klotho concentrations of these patients were already low-normal to normal. Thus, an improvement in these endpoints were not to be expected and, according to this theory, administration of vitamin D analogs will only be useful in the early stages of CKD if the subject has also low α-Klotho concentrations. However, this still needs to be tested in clinical studies. Therefore, a search for other Klotho-upregulating therapies seems justified. Administration of the protein-bound uremic retention solutes indoxyl sulfate (IS) and p-cresyl sulfate (PCS) to uremic mice induced downregulation of renal Klotho by hypermethylation of the Klotho promoter gene. Specific inhibition of DNA methyltransferase 1 his with 5-aza-2′-deoxycytidine caused demethylation of the Klotho gene and increased Klotho expression in vitro (61, 72). As IS and PCS originate from bacterial protein fermentation in the large intestines, bacterial metabolism may be an important therapeutic target in CKD (73). Preliminary data demonstrated that the use of dietary therapy, prebiotics, probiotics, synbiotics and/or adsorbent therapies that bind precursors of the uremic retention solutes in the intestine leads to reduced serum concentrations of p-cresol, PCS and/or IS (74-77). It would be very interesting to investigate the effect of these therapies on α-Klotho concentrations in CKD patients. Two randomized, relatively large-scale studies with AST-120, a gastrointestinal binder of these
uremic toxins, showed no beneficial effect on progression of CKD. Unfortunately, CV effects were not addressed (78, 79). More direct intervening in epigenetic regulation of Klotho gene expression might also be an attractive future approach. Haruna et al. already showed that increased Klotho expression through genetic manipulation of the Klotho gene ameliorated progressive renal injury in a mouse model of glomerulonephritis (58). Likewise, the group of Abraham et al. conducted a high-throughput screen, using the Klotho promoter to drive expression of luciferase to identify compounds that activate Klotho transcription. Compounds that elevated luciferase expression with at least 30% were further evaluated in vitro by incubation with kidney as well as choroid plexus cells of rats, which express Klotho endogenously. All animals showed increased Klotho protein compared to controls (80). These techniques need further optimization and establishment of its safety before being suitable for use in humans. Furthermore, α-Klotho concentrations can be elevated by increased shedding of α-Klotho into the circulation. As described in the introduction of this thesis, α-Klotho is proteolytically cleaved from the cell surface by a disintegrin and metalloproteinase 10 and 17 (ADAM 10 and 17) and shed into the circulation (81, 82). Chen et al. revealed in an in vitro model with Klotho-transfected COS-7 cells that insulin increases shedding of α-Klotho through increased proteolytic activity of ADAM 10 and 17 (81). However, administration of insulin to patients without diabetes carries the risk of inducing hypoglycemia. Similar to our results in chapter 7, the very recent paper of Rubinek et al. also demonstrated increased α-Klotho concentrations after treatment with growth hormone; an in vitro model showed mammalian target of rapamycin (mTOR)-dependent induction of Klotho-shedding mediated by IGF-1. Inhibition of mTORC1 using rapamycin, an mTOR-inhibitor, partially reduced shedding of Klotho (83). This contradicts the earlier findings of the report of Zhao et al., who showed in vitro and in vivo that rapamycin increases membrane and secreted Klotho (84). Further research on this subject is warranted and could have major implications for kidney transplant practice as these patients are regularly treated with mTOR-inhibitors. However, shedding of α-Klotho is probably an acute phase reaction (85) but is not inexhaustible and non-functional membrane-bound Klotho remains in the already injured kidney, possibly precluding this approach as a feasible long-term therapy.

In summary, although there is still a long way to go to find safe and applicable FGF23-lowering and Klotho-increasing therapies to treat patients with CKD, many data support the concept that these are major goals in the treatment of CKD. Interventions that are both safe and effective in improving these features of CKD may translate in an urgently needed improvement of the cardio-renal outcome of patients with CKD.
References


