General introduction and outline of this thesis

Introduction

Chronic kidney disease (CKD) is a well-established risk factor for cardiovascular comorbidity and mortality and this increased risk is already present in the early stages of CKD, as shown in figure 1 (1). Even when adjusted for the higher prevalence of traditional risk factors like diabetes, hypertension and dyslipidemia, there is still a dramatically increased risk. Therefore, other CKD-specific factors must be involved. To date, several factors are identified like proteinuria (1), hyperphosphatemia, disturbances in vitamin D metabolism (2), inflammation (3), oxidative stress and other less well characterized factors like retention of protein-bound uremic molecules (e.g. p-cresyl sulphate and indoxyl sulphate) (4, 5) and anemia (6, 7). The last two decades, an increasing role is attributed to fibroblast growth factor 23 (FGF23), a protein that most importantly regulates serum phosphate concentrations and Klotho, an anti-aging protein. These factors were found to co-act closely, together with vitamin D, also known as the FGF23-Klotho-vitamin D axis. This thesis focuses on the two most recently discovered components of this axis: FGF23 and Klotho.

Fibroblast growth factor-23

FGF23 is a bone-derived hormone that contributes to serum phosphate control by increasing urinary phosphate excretion through direct downregulation of sodium phosphate cotransporters in the renal proximal tubular epithelial cells. This enhances phosphaturia. In addition, FGF23 reduces plasma vitamin D levels through inhibition of 1α-hydroxylase activity and stimulation of 24-hydroxylase in the proximal tubules (8). FGF23 also reduces the release of parathyroid hormone (PTH) from the parathyroid gland (9). In physiology, binding of FGF23 to target cells requires co-expression and co-localization of the FGF1c-receptor and α-Klotho (10). Plasma FGF23 concentrations increase already in the early stages of CKD and rise further as CKD progresses to levels as high as 1000-fold above normal (11-13). Although increased FGF23 concentrations help to maintain serum phosphate levels in the normal range in the early stages of CKD, multiple observational studies have consistently shown that increased FGF23 concentrations are independently and linearly associated with progressive renal function decline (14-17) and an increased prevalence of cardiovascular disease and mortality (15, 16, 18-21). The
plausibility of a role of FGF23 in the incidence of these clinical pathologies is suggested by additional observations that increased plasma FGF23 concentrations are also associated with cardiovascular risk factors such as endothelial dysfunction (22, 23), total body atherosclerosis (24) and left ventricular hypertrophy (LVH) (25-27). However, the question whether FGF23 is just a sensitive biomarker reflecting the toxicity of underlying pathological processes, or is a direct cardiovascular toxin itself needs to be elucidated. There are studies that point to a direct toxic effect of FGF23. First, some observed a direct pathological effect on components of the cardiovascular system, like impaired vasoreactivity and increased arterial stiffness (22, 23, 28). Faul et al. showed that left ventricular hypertrophy was induced directly by FGF23 in a set of in vitro and in vivo experiments (29). In another study they showed that FGF23 can bind to FGFR4 in cardiac myocytes, independent of Klotho, resulting in stimulation of the phospholipase Cgamma/calcineurin/nuclear factor of activated T cells signaling (30). Activation of this pathway is an established potent inducer of cardiac hypertrophy (31). The development of LVH in rats with CKD was mitigated by a specific FGFR4-blocking antibody. Moreover, mice lacking FGFR4 did not develop LVH at all in response to elevated FGF23 levels (30). Another possible mechanism for FGF23 causing LVH was shown in the study of Andrukhova et al. This animal study showed that FGF23 causes increased sodium uptake in the distal tubular cells leading to volume expansion, hypertension and LVH (32). This is in line with the observation in humans where elevated FGF23 levels are more strongly associated with congestive heart failure than with atherosclerotic events (19).

In conclusion, the current perspective is that increased FGF23 concentrations have direct and indirect effects on the cardiovascular system contributing to increased cardiovascular morbidity and mortality in patients with CKD. However, it is still unclear if FGF23 is a modifiable risk factor. Several studies have attempted to decrease serum FGF23 concentrations using phosphate restricted diets and/or phosphate binding therapy (28, 33-47). These studies yielded varying results; most striking was that calcium-containing binders even increased FGF23 and the largest FGF23-lowering effect can be expected with a combination of phosphate restricted diet and a phosphate binder, in particular sevelamer. Second step is to determine if lowering of FGF23 leads to improved clinical outcome. Only thereupon FGF23 can be stated as an important target for therapy, like hypertension and proteinuria.

Klotho

The gene encoding the protein that was called Klotho was discovered in 1997 by Kuro-O and coworkers (48). Klotho is a 1,012-amino-acid long, single-pass transmembrane protein with a large extracellular domain (49). Mice defective in Klotho gene expression age prematurely, whereas transgenic mice overexpressing Klotho live longer than 'wild-type' mice (48, 50). Klotho is predominantly synthesized in the distal tubular epithelial cells of the kidneys and in lower levels in the proximal tubule (51). The parathyroid gland also expresses Klotho constitutively. The extracellular domain of tubular Klotho is cleaved by the membrane-anchored proteases A
Desintegrin and Metalloproteinase 10 and 17 (ADAM10 and ADAM17) and released into extracellular fluid, including blood, urine and cerebrospinal fluid (51-54). The membrane-bound and secreted domains of Klotho have different functions. The membrane bound form acts as a cofactor for FGF23 signal transduction by transforming the FGF receptor type 1c into a high-affinity FGF23 receptor, inducing the above-described classical actions of FGF23 on the proximal tubular segment and the parathyroid gland. The circulating form of Klotho functions as a humoral factor that regulates the activity of several ion channels and transporters including: 1) epithelial transient receptor potential calcium channels, specifically TRPV5 and TRPV6, to increase calcium reabsorption in the kidney and calcium absorption in the intestines respectively (55, 56); 2) renal outer medullary K⁺-channels (ROMK1) to promote renal potassium excretion in distal nephrons (57), and 3) NaPi-2a receptors to promote phosphaturia independently of FGF23 by deglycosylation of the NaPi-2a protein in the proximal tubules, which results in a decrease in number and activity of NaPi-2a (51). Circulating Klotho is also involved in the modulation of Wnt signal transduction (58), anti-oxidation (59), the regulation of nitric oxide production in the endothelium (60, 61), maintenance of endothelial integrity (62), and inhibition of intracellular insulin and insulin-like growth factor-1 signalling (50).

Loss of transmembrane and secreted Klotho occurs as early as CKD stages 1-2 and progresses with further renal function loss (63-65). On the one hand, this is caused by loss of renal tissue; on the other hand active downregulation appears in a uremic milieu with increased oxidative stress and inflammation (63, 66, 67). Lower Klotho levels are associated with progressive CKD (68), higher prevalence of cardiovascular disease (69), arterial stiffness (70) and vascular calcification (71, 72), and higher mortality (73). Moreover, accumulating data suggest that loss of Klotho is not just an early biomarker for CKD, but also a pathogenic intermediate factor for progression of renal function loss and development of complications. Therefore, restoring Klotho levels could have clinical benefit. Indeed, delivery of exogenous Klotho resulted in an impressive amelioration of kidney injury in different CKD mouse-models evidenced by enhanced phosphaturia, preserved glomerular filtration and direct inhibition of phosphate uptake by vascular smooth muscle cells, as such inhibiting initiation of vascular calcification (71). On top of that, recent data showed that supplementation of exogenous Klotho is still therapeutically effective even when kidney lesions are already established (74-76). However, long-term exogenous supplementation of the relatively large Klotho-protein will not be easy to accomplish. Therefore, upregulation of the production of endogenous Klotho might be more feasible not only to minimize complications of CKD but also preserve remnant kidney function. A previous study already demonstrated that α-Klotho concentrations increased with the administration of an angiotensin receptor blocker (ARB) (77). However, this finding has little clinical implications as blockade of the renin-angiotensin-system is already undisputed in CKD and therefore this insight won’t lead to important changes in clinical practice.
Outline of this thesis

One of the main goals in the treatment of CKD patients is to reduce the substantially increased risk of cardiovascular comorbidity and mortality accompanied with CKD, thereby improving both quality and length of life. As discussed above, the recently recognized FGF23-Klotho-vitamin D axis holds promise of so far unexplored opportunities to accomplish this to some extent. However, hard endpoint trials will be needed to substantiate the clinical benefits of targeting the FGF23-Klotho-vitamin D axis. The main challenge at this point is to generate clinical data that will allow the design of valid hard endpoint trials with an optimal chance to demonstrate clinical benefit. Therefore, the main studies described in this thesis explored specific clinical interventions to reduce serum FGF23 concentrations or to increase serum α-Klotho concentrations in patients with CKD.

Part 1: Interventions to reduce serum FGF23 concentrations in patients with CKD

The assumption that FGF23 plays a causal role in the development of cardiovascular morbidity is conceivable on the basis of the currently available observational studies and the mechanistic in vivo and in vitro studies. Our hypothesis is that serum FGF23 concentrations can be effectively and sustainably lowered with a combination of a phosphate restricted diet and dietary phosphate binder. This topic is addressed in part 1 of this thesis.

Data suggest that serum FGF23 concentrations can be reduced with restriction of phosphate uptake from the gastro-intestinal tract. However, data on the potency of several dietary measures and various types of phosphate binders to influence FGF23 in different stages of CKD are scattered. Therefore, in chapter 2 we summarize current data on dietary and pharmacological interventions to modulate serum phosphate and FGF23 concentrations.

Even more, to test if lowering of FGF23 may reduce cardiovascular risk, it is important to know whether FGF23 can be effectively, predictably, sustainably and safely reduced in CKD patients by lowering gastro-intestinal phosphate uptake. Therefore, we started an investigator-initiated, prospective, multi-centre, open label, single arm trial in subjects with CKD stage I-IV with normal serum phosphate levels and not taking any phosphate binder therapy aiming to demonstrate the feasibility of lowering FGF23 concentrations with a phosphate restricted diet in combination with the phosphate binder sevelamer using a forced uptitration treatment regimen. In chapter 3 we describe the study protocol of this trial (Sevelamer on FGF23 Trial (SoFT)).

Part 2: Interventions to increase serum α-Klotho concentrations in patients with CKD

While FGF23 plasma concentrations rise exponentially, both membrane-bound and soluble Klotho levels decrease as CKD progresses. As outlined, loss of Klotho is associated with increased cardiovascular morbidity and mortality. A possible explanation for this increased cardiovascular risk is the involvement of Klotho in endothelial integrity and vascular calcification, two typical
features involved in CKD. Therefore, in chapter 4, we review current understanding of Klotho deficiency on the endothelial function and vascular calcification.

For the intervention studies of this part of the thesis, we planned to measure α-Klotho levels in serum and urine. α-Klotho concentrations in urine were already reported in different studies (71, 78, 79). However, these studies did not report the reliability of urinary α-Klotho measurements. For this reason, we examine the stability of α-Klotho in both fresh catheter and fresh voided urine (chapter 5), prior to proceed with performing clinical studies using this as an endpoint.

Oxidative stress is increased in CKD and is associated with downregulation of α-Klotho expression (59). Some in vitro and in vivo animal studies showed that therapeutic reduction of oxidative stress upregulates the expression of α-Klotho (80-84). If applicable to human CKD this could be a method to restore α-Klotho concentrations. Therefore, we measured α-Klotho concentrations in serum samples of a clinical cohort with mild to moderate CKD, exposed to anti-oxidative therapy or placebo (ATIC study (85)), as described in chapter 6.

Another possible mechanism to upregulate α-Klotho concentrations was suggested by the study of Sze et al. They found extremely high α-Klotho concentrations in patients with acromegaly. These α-Klotho concentrations returned to normal after surgical removal of the pituitary adenoma (86). This could imply that in turn upregulation of α-Klotho concentrations might be achieved by exogenous growth hormone (GH) or insulin-like growth factor 1 (IGF-1). Some preliminary data support the assumption of a crosstalk between Klotho and the GH-IGF1 system. For instance, two studies found that Klotho inhibits the insulin and IGF-1 pathways (48, 50). Moreover, Klotho-deficient mice are hypoglycemic and extremely sensitive to insulin (50, 87), whereas Klotho-overexpressing mice are insulin resistant (50). Two recent publications demonstrated increased α-Klotho concentrations after GH therapy in adults and children with a growth hormone insufficiency (88, 89). We hypothesized that exogenous GH administration can increase α-Klotho concentrations, also in the absence of primary GH deficiency, in both healthy and CKD patients. This will be studied in chapter 7.
References


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