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THE ROLE OF KLOTHO IN MINERAL METABOLISM AND INFLAMMATION

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Cover image: Immunofluorescence staining shows Klotho expression (red) in the distal tubules. DAPI (blue) stains the cell nuclei.

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I would like to dedicate this thesis to Sweden and Bangladesh

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LIST OF ABBREVIATIONS

1,25(OH) ₂ D	1,25 dihydroxyvitamin D
ADHR	Autosomal dominant hypophosphatemic rickets
CaSR	Calcium-sensing receptor
CKD	Chronic kidney disease
CVD	Cardiovascular disease
CYP24A1	1, 25-dihydroxyvitamin D 24-hydroxylase
CYP27B1	25 dihydroxyvitamin D 1-alpha hydroxylase
ESRD	End-stage renal disease
FGF	Fibroblast growth factor
FGF23	Fibroblast growth factor-23
FGFR	Fibroblast growth factor receptor
GFR	Glomerular filtration rate
HFTC	Hyperphosphatemic familial tumoral calcinosis
IF	Immunofluorescence
IHC	Immunohistochemistry
mKL	Membrane-bound Klotho
cKL	Shedded full-length Klotho
sKL	Truncated Klotho
<i>Ksp-KL</i> ^{-/-}	Distal tubule-specific <i>Klotho</i> knockout mice
Npt	The type II sodium-dependent phosphate co-transporter
Pi	Inorganic phosphorus
PTH	Parathyroid hormone
PTH1R	Parathyroid hormone receptor 1
qPCR	Quantitative real-time polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system
SHPT	Secondary hyperparathyroidism
<i>Six2-KL</i> ^{-/-}	Renal <i>Klotho</i> knockout mice
TIO	Tumor-induced osteomalacia
TRPV5	Transient receptor potential cation channel subfamily V, 5
VDR	Vitamin D receptor
XLH	X-linked hypophosphatemic rickets

APC	Antigen presenting cell
BM	Bone marrow
HSCT	Hematopoietic stem cell transplantation (HSCT)
Bu	Busulfan
CD	Cluster of differentiation
Cy	Cyclophosphamide
FISH	Fluorescence in situ hybridization
GI	Gastrointestinal
aGVHD	Acute graft versus host disease
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
IL	Interleukin
IP	Intraperitoneal
LPS	Lipo poly saccharide
NK	Natural killer
SP	Spleen
TGF- β	Transforming growth factor-beta
Th	T helper
TNF	Tumor necrosis factor
IGF-1	Insulin-like growth factor 1

1 INTRODUCTION

1.1 KLOTHO, A MULTIFUNCTIONAL ANTIAGING PROTEIN

1.1.1 Discovery of the Klotho gene

In ancient Greek mythology, Klotho (Clotho, Nona) is the goddess who swirls the strands of life and presides over birth and death. In biology, the name Klotho was initially considered as an ‘anti-ageing’ gene that was unintentionally identified by Kuro-o and colleagues in 1997[1]. Upon studying on transgenic mice with random mutations in a sodium–proton exchanger, these investigators discovered a mutant mouse that exhibited characteristic phenotypes similar to human aging (e.g., a shorter lifespan, atherosclerosis, vascular calcification, osteoporosis, diabetes mellitus type 2 and skin atrophy) [1]. Since all these phenotypes appeared with 100% penetrance only in mice homozygous for the transgene, they coined “Klotho” as the name for the involved gene [1]. In contrast to the Klotho-defective mutant mice, Klotho-overexpressed transgenic mice exhibited an extended lifespan, speculatively through the suppression of insulin/IGF1signaling [2]. Therefore, the *Klotho* gene is considered to be an anti-aging gene [2].

1.1.2 Structure and isoforms of Klotho

The human *Klotho* gene is located on chromosome 13, whereas in the mouse and rat, the locus of the *Klotho* gene is located on chromosome 5 and 12, respectively [3]. In these three species, the *Klotho* gene contains five exons and four introns, which transcribe 3036 (humans), 3042 (mice), and 3042 (rats) nucleotide mRNAs [3]. In the mouse and human, the third exon of *Klotho* gene contains an alternative splicing donor site which allows the generation of two different transcripts: one that encodes a transmembrane form consisting of 1014 amino acids (full length transcript) and the

another one that encodes a secreted form of 550 amino acid (truncated transcript) (Figure 1) [1, 4, 5]. The former form of Klotho is a 130 KDa membrane-bound protein (mKL) consisting of an N-terminal signal sequence, two large extracellular domains (KL1 and KL2), a single transmembrane domain and a short intracellular domain (Figure 1). It is worth noting that the circulating isoform of Klotho (cKL) is cleaved from the cell membrane isoform by proteolytic cleavage (Figure 1) and released into extracellular fluids, including blood, urine, and cerebrospinal fluid. This process is mediated by the alpha-secretases (ADAM10 and ADAM17) as well as the beta-secretase, BACE [6]. Once shedded, cKL exerts endocrine effects on its own, although the underlying mechanisms are not fully clarified.

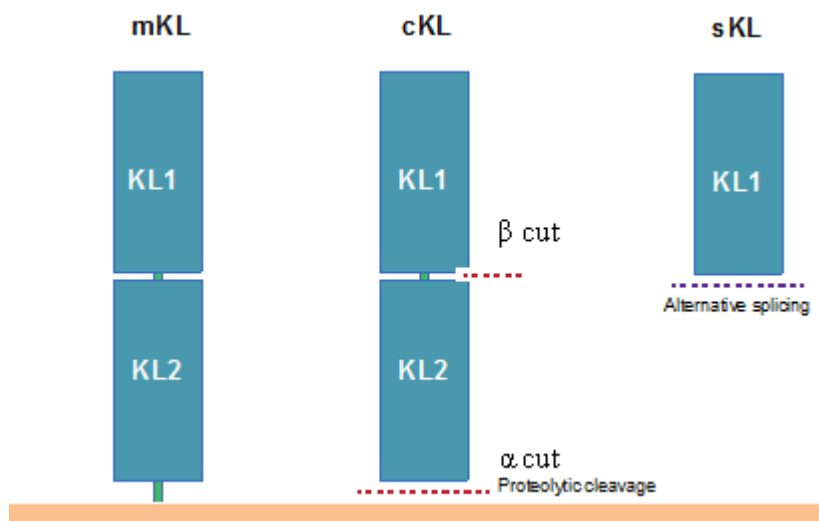


Figure 1. Klotho has three isoforms: a membrane-bound form (mKL), a circulating form (cKL) and a truncated form generated through alternative splicing (sKL).

1.1.3 Organ localization of Klotho expression and source of soluble Klotho

In the mouse, the membrane-bound Klotho is predominantly expressed in the kidneys, parathyroid glands and choroid plexus of the brain and at lower levels, in the placenta,

skeletal muscle, urinary bladder, aorta, pancreas, testes, ovary, colon, and thyroid gland [1]. The expression of Klotho in human choroid plexus has not been investigated yet. Based on the degree (intensity) of expression, tissue expression of Klotho is generally classified into three categories, i.e., the kidneys, parathyroid glands, choroid plexus, and sinoatrial node are classified as highly Klotho-expressing tissues [5]. On the other hand, the brain, eye, inner ear, endocrine system, lungs, parts of the gastrointestinal and genitourinary tracts, and placenta are nominated as intermediate-low Klotho-expressing tissues; and finally, the bones, skin, adipose tissue, liver, spleen, heart, blood and immune cells are considered as low-absent Klotho-expressing organs [5]. There are conflicting data regarding Klotho expression in arteries [7, 8].

Since the membrane-bound Klotho is highly expressed in the kidney, it has been suggested that the same organ is the main source for cKL and sKL as these isoforms of Klotho appear in the circulation, urine and CSF as a result of either cleavage of membrane-bound Klotho, or alternative gene splicing [9, 10]. In the kidney, Klotho is mostly expressed in the distal tubules and to a low extent in the proximal tubules. In fact, it has been shown that proximal-tubule knockout mice do not exhibit any significant reduction in the serum Klotho levels [11]. A possible reason for this could be that the deletion in the proximal tubule might not be sufficient to lower the serum levels of this protein as other Klotho-expressing tubules in the kidneys or extra renal tissues might trigger a compensatory up regulation to maintain a constant shedding rate of Klotho.

1.1.4 Mode of action of Klotho isoforms

The three Klotho isoforms can have distinct functions. mKL is identified as a permissive co-receptor/co-factor for fibroblast growth factor 23 (FGF23); upon interaction with this hormone, mKL plays a key role in the regulation vitamin D

biosynthesis and in phosphate and calcium homeostasis [3, 12]. In contrast to mKL's role as a co-receptor, cKL functions as an independent endocrine factor [13, 14]. Recent studies have revealed that cKL antagonizes TGF β , Wnt, tumour necrosis factor alpha (TNF- α) and insulin/IGF1 signaling and thereby protects against renal and cardiovascular stress, amongst other disorders [13, 15-18]. With respect to sKL, the biological relevance of this isoform is largely unexplored.

1.1.5 Regulation of Klotho

Klotho regulation remains to be comprehended entirely. However, it has been shown that the promoter region of the-Klotho gene contains vitamin D-responsive elements which suggest that Klotho expression can be upregulated by vitamin D. Indeed, in line with this suggestion, *in vivo* and *in vitro* studies have shown that treatment with vitamin D increases the expression of Klotho while decreasing aortic calcification in mice with chronic kidney disease [19, 20]. Similarly, mice on a low phosphate diet show enhanced Klotho expression [21]. From this observation, it can be inferred that a high amount of phosphate would actually reduce Klotho expression. Similarly, Klotho expression can be reduced under the effect of several other factors, such as calcium, inflammation, uremic toxins, FGF23 and oxidative stress [15]. The upstream regulators of Klotho shedding and alternative splicing is yet to be resolved.

1.2 THE ROLE OF KLOTHO IN FGF23 HORMONE SIGNALLING

1.2.1 Structure and function of FGF23

The fibroblast growth factors (FGFs) are a highly conserved family of proteins that by their function can be divided into intracellular, canonical, and hormone-like FGFs. The human/mouse FGF gene family consisting of 22 members, from FGF1 to FGF23. These 22 genes encode molecules that can bind to one or several of the fibroblast

growth factor receptors (FGFRs) [22]. The human FGF23 gene is located on chromosome 12p13 and composed of three exons. It encodes a 32kDa glycoprotein, which consists of 251 amino acids [23]. In contrast to the location of the human FGF23 gene, the mouse FGF23 gene is located on chromosome 6 and shares 72% amino acid identity [23]. Due to the lack of a heparin binding sequence in the FGF23 gene, it functions as an endocrine factor [24]. The FGF23 is a circulating protein mainly produced in the skeleton, more specifically in the osteocytes and osteoblasts and expressed in the bone and, to a much lower extent, in the thymus, brain, and heart [23, 25, 26]. In contrast to most other FGFs, it is a secreted factor with endocrinal actions mainly targeted at the kidneys and parathyroid glands. In the kidneys, FGF23 inhibits tubular reabsorption of inorganic phosphate (Pi) by reducing the expression level and increasing the internalization of sodium-dependent Pi co-transporters (Npt2a and c), thereby leading to increased phosphaturia and a subsequent reduction in systemic Pi level [25]. Additionally, FGF23 is a potent regulator of key enzymes in vitamin D metabolism, leading to diminished synthesis and enhanced degradation of vitamin D, which results in reduced circulatory levels of 1,25-dihydroxy vitamin D₃. In the parathyroid glands, FGF23 inhibits mRNA synthesis and protein secretion of the parathyroid hormone (PTH) [27, 28]. Accordingly, FGF23's net effect is a reduction of extra-cellular concentrations of Pi, vitamin D, PTH, and, indirectly, calcium.

1.2.2 Animal models and diseases-related to FGF23

When administered intravenously or when transgenically overexpressed in mice, FGF23 leads to hypophosphatemia due to increased wastage of renal phosphate, accompanied by a decrease in the levels of circulating 1,25(OH)₂D (as well as PTH in the short term) [29-31]. Patients with enhanced levels of FGF23 demonstrate a similar

phenotype, either in the form of abnormal processing (for instance, in autosomal dominant hypophosphatemic rickets, OMIM #193100 and ADHR), regulation (X-linked hypophosphatemic rickets, OMIM #307800 and XLH) or ectopic production (tumor-induced osteomalacia, TIO) [25, 32, 33]. Along with this, overexpression of FGF23 in mice has been observed to lead to a reduction in bone mineral density and to retardation in growth [34]. Conversely, FGF23 knockout mice or humans with homozygous missense mutations in FGF23 (for instance, hyperphosphatemic familial tumoral calcinosis in HFTC OMIM #21900) demonstrate the adverse effects of an opposing phenotype with hyperphosphatemia, hypercalcemia, high serum 1,25(OH)₂D levels and soft-tissue calcifications [35, 36].

1.2.3 Fibroblast growth factor receptors

In case of the kidneys and parathyroid glands, FGF23 binds with FGFRs and activates the MAPK/ERK pathway. FGFR binding requires the presence of Klotho, as an FGF23 co-receptor [37]. Klotho enhances FGF23's affinity to multiple FGFRs— FGFR1, 3 and 4 being most important among them—by forming Klotho–FGFR binary complexes. The fact that Klotho knockout mice express a phenotype identical to that of the FGF23 knockout mice amply illustrates Klotho's importance as a co-receptor; they also exhibit hyperphosphatemia, high serum 1,25(OH)₂D levels, infertility, growth retardation, shortened lifespan and ectopic calcifications, a 1000-fold or more increase in circulating FGF23 levels notwithstanding [1] .

1.2.4 Klotho and FGF23 signaling

In order to understand how as a hormone, FGF23 exerts its biological activities, it is necessary to elucidate the operative receptors that mediate FGF23 signaling. Although initial studies failed to determine a specific receptor for FGF23, a significant

development made by Urakawa et al., who showed that membrane-bound Klotho by interacting with FGF-receptor 1 functions as an actual FGF23-receptor [38]. Being more specific, Klotho acts as a permissive cofactor for FGF23 and designates the tissue specificity of this hormone. In fact, the significance of Klotho in mediating FGF23 signaling is exhibited in Klotho null mice, i.e., these animals despite having high levels of circulatory FGF23, show biochemical characteristics which are identical to those of FGF knockout mice [38]. Thus, current data support that FGF23, at least in physiology, is unlikely to exert biological effects without the presence of Klotho.

1.2.5 The role of FGF23/Klotho axis in mineral metabolism and homeostasis

1.2.5.1 Mineral metabolism and homeostasis

Calcium and phosphorus are crucial to life, and they are involved in many biological and physiological processes, including maintenance of bone structure, intracellular signaling and energy metabolism. Mineral homeostasis is preserved through a balanced influx and efflux from the intestine, bone and kidney, and orchestrated by several hormones in a complex set of feedback loops. Abnormalities in mineral metabolism are usually a consequence of either hereditary or acquired dysfunctions in any of these mentioned organs. The kidney is a pivotal organ regulating calcium and phosphorus metabolism and disrupted homeostasis of these elements are therefore almost universal in patients with chronic kidney disease (CKD) [39]. Overwhelming, epidemiological evidence links these abnormalities to adverse clinical outcomes, most prominently cardiovascular morbidity and mortality in patients with CKD [40, 41].

1.2.5.2 Mineral disturbances in chronic kidney disease

CKD is characterized by the abnormalities of the kidney as this organ is incapable to adequately maintain mineral homeostasis, which initiates a series of events that predictably lead to biochemical changes, altered bone metabolism, vascular calcification and increased mortality (CKD – MBD). Most importantly, the capacity to secrete Pi is compromised leading to a positive net Pi balance. With regard to FGF23, current evidence favors a ‘phosphate-centred’ view, supporting that the increased Pi load is the initial trigger for FGF23 synthesis. Indeed, FGF23 levels are profoundly elevated in CKD and strongly correlated to renal function [42]. The rise in FGF23 is detected already in the earliest stage of CKD when glomerular filtration rate (GFR) falls below 60 ml/min, even in individuals without any clinically evident renal damage. In contrast to FGF23, Klotho expression is dramatically reduced in CKD patients, and it is possible that Klotho deficiency *per se* could partially explain the exceptionally high morbidity and mortality in CKD that are accompanied by an accelerated ageing process and cardiovascular (CV) disease. In line with this statement is the finding that *Klotho* null mice display a shortened life span whereas transgenic over-expression of Klotho prolongs life span both in wild-type and Klotho-deficient mice [1, 2]. Human data also suggest that functional polymorphisms in the *Klotho* gene which result in lower expression of Klotho, are associated with reduced survival both in hemodialysis patients and in the general population as well as manifest CV disease and atherosclerotic plaque burden [43-45].

The cause of reduced Klotho expression in CKD is not understood, but several different factors may contribute including

- High levels of FGF23 that directly reduces Klotho.
- Inflammation.

1.2.6 The role of FGF23/Klotho axis in Inflammation

A recent study has shown that Klotho is also expressed in the bone marrow (BM), spleen and fetal liver cells [46]. Moreover, other studies have convincingly demonstrated that knocking out the Klotho gene in mice results in the suppression of B lymphopoiesis in the bone marrow and atrophy of the spleen and thymus in mice [1, 47]. Together, these observations strongly suggest that Klotho has a role in hematopoiesis and can exert regulatory effects on the immune cells and their functions. This suggestion is further supported by the results from several studies. For instance, it has been shown that the expression of Klotho in resting human CD4⁺ lymphocytes is significantly decreased proportionally to advancing aging [48]. Moreover, it has been demonstrated that the expression of Klotho protein is heavily suppressed in the CD4⁺ cells of rheumatoid arthritis (RA) patients and similarly in the immune cells of the patients with inflammatory bowel disease (IBD) [49, 50]. Interestingly, Klotho hypomorphs phenotypes are consistent with defective Ca²⁺ and P_i homeostasis leading to developed osteoporosis is frequently associated with human IBD and T-cell dysfunction [50]. By extension, renal Klotho expression is repressed in mice with inflammatory bowel diseases implicating that T helper type 1 (Th1-type) cells and their inflammatory cytokine including TNF- α and interferon gamma (IFN- γ) are possibly involved in the down-regulation of Klotho expression. Accordingly, it was shown that the inhibitory phenotype was ameliorated by the treatment with antibodies against TNF- α [50]. Members of the TNF- α superfamily cytokine, TNF- α and TNF-like weak inducer of apoptosis (TWEAK) have shown to exert adverse effects on the glomerular and tubular cells and thus, contribute to renal damage [51, 52]. A neutralizing antibody rescues the down-regulation of Klotho expression in TWEAK-KO mice [53].

Therefore, it is tempting to speculate that the pathophysiological roles of FGF23/Klotho axis include but not limited to regulation of mineral homoeostasis i.e., this axis may play an important role in the development and potentiation of inflammation.

1.3 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION AND ITS RELATED INFLAMMATORY COMPLICATIONS; GRAFT VERSUS HOST DISEASE AND ACUTE KIDNEY INJURY

Allogeneic hematopoietic stem cell transplantation (HSCT) is globally used to treat many cancer patients as well as patients with congenital metabolic disorders, immunodeficiencies, severe aplastic anaemia, and autoimmune diseases [54]. However, the application of HSCT has limitations as it induces complications [55]. Among these complications, acute graft-versus-host diseases (aGVHD) and acute kidney injury (AKI) belong to the life-threatening conditions [56-58]. aGVHD is an immune-mediated inflammatory disorder that develops 1-14 weeks after HSCT; affects 40-60% of HSCT patients and involves multiple organs including the skin, gastrointestinal tract and liver [59, 60]. AKI is also an HSCT-associated inflammatory condition that develops within 12 weeks after HSCT affecting 27-66% of the transplanted patients and predominantly targets the kidneys [61]. The exact mechanisms underlying the development of aGVHD and AKI are not well understood. However, regarding aGVHD, it has been suggested that the disease is mainly mediated by donor-derived T cells which are activated by the recipient antigen presenting cells (APCs) and polarized into type 1 T cells (Th1 cells) [56, 62, 63]. Furthermore, it has been suggested that donor-derived activated Th1 cells produce inflammatory cytokines, in particular, TNF- α and IFN- γ that severely damage multiple organs [57, 63]. Regarding HSCT-associated AKI, it has also been proposed that aGVHD can directly contribute to the development of AKI through inflammatory cytokines or

immune cell-mediated tissue injury [61]. In line with this proposal, we have previously shown that in mice with aGVHD, the kidney is infiltrated with the donor-derived T cells and that the expression of several inflammatory and immune response genes was significantly up-regulated in this organ [64, 65]. Furthermore, in a rat model of aGVHD, it has also been shown that during the progression of severe inflammation, the donor-derived leukocytes including helper (CD4⁺) T cells, cytotoxic (CD8⁺) T cells and macrophages (CD68⁺ cells) infiltrated and expanded into different tissue compartments of the kidney [66]. In line with these animal studies, clinical studies have revealed that the renal tubules are also the target tissue in patients with aGVHD [67]. Thus it is conceivable that the kidney is also a target organ for aGVHD and that a common inflammatory or immune mechanism might be responsible for the emergence of aGVHD and HSCT-associated AKI.

2 AIMS

The overall aim of the present thesis is to refine the understanding of renal Klotho function and its role in regulating mineral metabolism and inflammation.

The specific objectives are to:

- Elucidate the role of distal tubular Klotho in renal phosphate handling and on the regulation of FGF23 (Study I).
- Characterize the contribution of renal Klotho to the development of aging-like phenotype (Study II).
- Investigate the role of Klotho/FGF23 axis in the development of cardiovascular disease, a complication that occurs in patients with chronic kidney disease (Study III).
- Elucidate the role of Klotho/FGF23 axis in the development of acute graft versus host disease and acute kidney injury (Study IV).

3 MATERIAL AND METHODS

3.1.1 Ethical approval

All animal studies presented in this thesis were pre-approved by the Stockholm Southern Ethics Committee for Animal Research and were conducted in accordance with the animal welfare law, the Animal Protection Regulation and the Regulation of the Swedish National Board for Laboratory Animals (approval numbers S68-10, S116-12, S118-12, S67-74 and S56-14)

3.1.2 Cre-Lox recombination

Cre-Lox is a recombination system constitutes a sophisticated site-specific recombinase technology used to introduce inducible gene deletion in mice. Basically, the system contains two components: a P1 bacteriophage-derived enzyme, the Cre enzyme that recognizes and splices specific 34 base-pair DNA sequences called LoxP sites. When LoxP sites are inserted into the genome of the mice (floxed mice), recombination occurs between the LoxP sites in cells expressing Cre recombinase, resulting in either activation or repression or exchange of a specific DNA sequence or gene (Figure 3).

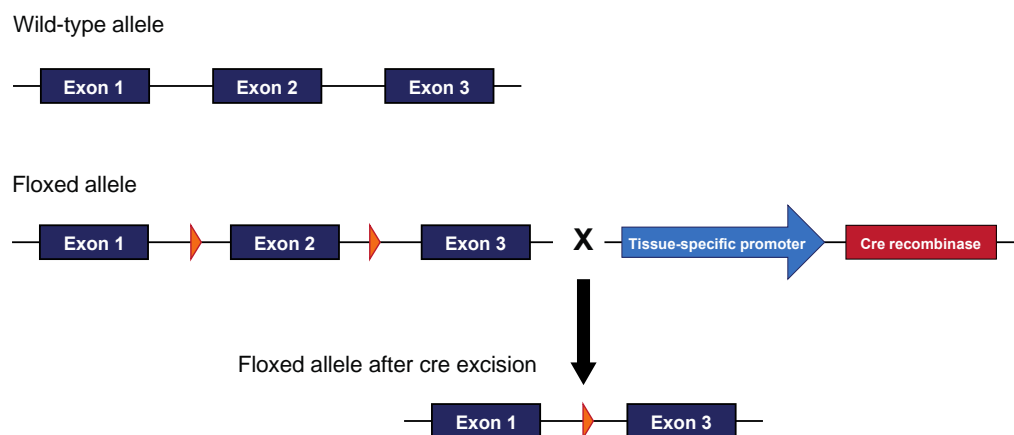


Figure 2. The target gene is flanked by LoxP sequences either as a whole or a part. This aims for either tissue-specific or organ-specific deletion and disruption of the gene function.

There are a few limitations and pitfalls to using Cre-Lox recombination. First, the lack of specificity of certain Cre promoters results in recombination in non-specific tissues, which can increase the chances of a confounding result or, at the very least, unexpected cellular phenotypes. Using a reporter strain is the most common method to confirm that the Cre expression is restricted to the intended target tissue. Second, Cre-Lox recombination is usually less efficient as compared to conventional knockout techniques. Third, the excessive cellular accumulation of Cre recombinase may lead to Cre toxicity. It can damage the DNA and cause cell death, which may affect the phenotype of mouse strains. In light of the observation that *Klotho*^{-/-} mice exhibit extensive morphological abnormalities and altered mineral metabolism, we aimed to utilize floxed *Klotho* mice to enable the dissection of tissue-specific effects of *Klotho* in a more physiological setting. Therefore, in the study I, we used mice with LoxP sequences introduced in the flanking regions of exon 2 of the *Klotho* gene, resulting in disrupted gene function in tissues expressing Cre recombinase (Figure 5B). In a study I, mice with a distal tubule-specific deletion of *Klotho* were produced using mice expressing Cre recombinase under the Ksp-cadherin promoter (B6.Cg-Tg(Cdh16-cre)91Igr/J; Jackson laboratory, ME, US) 102. For comparative studies, we also generated *Klotho* null like mice in which the *Klotho* gene was deleted globally using mice expressing Cre under the control of a human beta-actin promoter (FVB/N-Tg(ACTB-cre)2Mrt/J, Jackson laboratory). We used the same technology in study II as well to generate nephron specific knockout mice (*Six2-KL*^{-/-} mice) using a Cre-LoxP recombination as described in paper I. In study III, we generated knockout mice in which the *Klotho* gene was deleted in vascular smooth muscle cells (*Sm22-KL*^{-/-}) using a Cre-Lox recombination as previously described in paper I and II.

All Cre strains had earlier been crossed to reporter mice to validate tissue specificity of Cre expressions. The mice with a C57BL/6 background were used for maintenance breeding.

3.1.3 Allogeneic hematopoietic stem cell transplantation

BALB/c (H-2Kd) and C57BL/6 (H-2Kb) mouse strains (8-12 weeks old) were used as donors and recipients. Animals were kept under a pathogen-free condition in an animal facility with controlled temperature, humidity and 12 hours light/dark. They were fed with autoclaved standard mouse chaw and tap water ad libitum and allowed to acclimatize to their environment prior to performing the experiments.

For hematopoietic stem cell transplantation (HSCT), female BALB/c recipient mice were first conditioned with busulfan (Bu, 20 mg/kg/day, intraperitoneally (i.p.) for 4 days and subsequently, cyclophosphamide (Cy, 100 mg/kg/day, i.p.) for 2 days. Thereafter, the animals were allowed to rest for one day. At the day of transplantation (day 0), the recipient mice were injected intravenously (i.v.) with 20×10^6 bone marrow cells combined with 30×10^6 spleen cells from either syngeneic (BALB/c) or allogeneic (C57BL/6) donor mice. As control groups, a group of female BALB/C mice received only Bu-Cy, and another group was left untreated and non-transplanted as the control group.

3.1.4 Assessment of acute graft-versus-host disease

After the transplantation, recipient mice were monitored daily for the development of aGvHD. The degree of systemic aGVHD was assessed employing a well-established standard scoring system for five clinical features of aGVHD including weight loss, hunching, activity, fur texture and skin integrity [68].

3.1.5 Induction of lipopolysaccharide-induced acute kidney injury

For the induction of acute kidney injury, male C57BL/6 mice (6-8 weeks of age) were first injected i.p. with lipopolysaccharide (LPS) derived from *Escherichia coli* O111:B4 (5mg/Kg). Five hours after LPS injection, the animals were euthanized, and the kidneys and blood were collected for further analyses.

3.1.6 Immunological, histopathological, biochemical and molecular methods

3.1.6.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a widely used molecular method designed for detecting and quantifying peptides, proteins and antibodies in the body fluids. In the studies explained in this thesis, we used commercially available ELISA kits to quantify the serum levels of FGF23, PTH and 1,25 (OH)₂D. Serum levels of TNF- α and IFN- γ , were measured by a Cytometric Bead Array (CBA) Mouse Th1/Th2 Cytokine kit (BD Biosciences, San Diego, CA, US) in accordance with the manufacture's instruction.

3.1.6.2 Measurement of donor-recipient chimerism

The levels of donor-recipient chimerism in the spleen and bone marrow cells of transplanted mice were measured using flow cytometry. Spleen and BM cells were stained with Alexa Fluor 647-conjugated mouse anti-mouse H-2K^b and phycoerythrin (PE)-conjugated mouse anti-mouse H-2k^d (both purchased from BD PharMingen, San Diego, CA) to identify the donor- or recipient-derived cells, respectively. The cells were analyzed with a 4-colour BD FACSArray (BD Biosciences, San Jose, CA) flow cytometer. The percentage of chimerism was calculated from the ratio of Alexa Fluor 647 positive cells of all PE positive cells.

3.1.6.3 Immunohistochemistry and immunofluorescence

Upon dissection, tissues were fixed in 4% paraformaldehyde (overnight), and thereafter embedded in paraffin, and four-micrometer sections were prepared. After deparaffinization, the sections were rehydrated and incubated with low-pH solution to unmask the antigen. For immunohistochemistry, the sections were then immersed in 3% H₂O₂ in methanol, treated with 4% normal serum, and blocked with avidin and biotin. All sections were incubated with primary antibodies at 4°C overnight. Thereafter, the slides were incubated with biotinylated secondary antibodies and developed with 3,3'-diaminobenzidine substrate.

For immunofluorescence, after employing the primary antibodies, Alexa Fluor conjugated secondary antibodies were used for visualization the antigen (e.g., Klotho).

For nuclear staining, 4',6-diamidino-2-phenylindole was used.

3.1.6.4 Histopathological analysis

Paraffin-embedded sections were stained with either of the following stainings: hematoxylin and eosin (H&E), picosirius red for fibrosis (stains collagen I and III fibers), von Kossa (to detect calcification), periodic acid–Schiff (PAS) (to detect polysaccharides) and periodic Schiff-methenamine silver (PASM) (to detect carbohydrates) using related histological staining procedures. In all cases, the stained sections were examined in a blinded fashion by a skilled pathologist.

3.1.6.5 Serum biochemistry

Serum calcium, phosphate, urea, albumin and creatinine were measured using quantitative colorimetric assay kits (BioAssay; BioChain Institute, Inc., Newark, CA) or an autoanalyzer (Konelab 20XTi (Thermo Scientific, Vanta, Finland).

3.1.6.6 *In situ* hybridisation

In situ hybridisation (ISH) is a technique that allows the locating of positions of specific nucleic acid sequences (mRNA) within structurally intact cells or a morphologically preserved tissue section through the use of specific nucleic acid-labelled probes. These probes can then be detected by using a specific antibody to identify the mRNA expression and the location of the mRNA. Fluorescence *in situ* hybridisation (FISH) is a kind of ISH that uses fluorescent probes to detect mRNA or DNA sequences. In study IV, we used FISH to see the mRNA expression in the tissue slide.

3.1.6.7 *Immunoblotting*

Immunoblotting (also called western blotting) is the golden-standard for specific protein detection and quantification in a protein mixture (e.g., a cell, tissues lysate and body fluids). Kidney extracts were prepared, and protein levels were quantified using a BCA protein assay kit in accordance with manufacture's instruction. For each sample, an equal quantity of protein was separated by SDS-PAGE and electrotransferred to nitrocellulose. After blocking, the membranes were sequentially incubated with primary antibodies (anti-Klotho, anti-pan-14-3-3 or anti-actin antibody) and secondary antibodies conjugated with IRDye (LI-COR Bioscience, Lincoln, NE). The proteins were visualized using ODYSSEY imaging system (LI-COR Bioscience, Lincoln, NE). The intensity of the protein bands was quantified by densitometry using Image J software, and the results were expressed as fold decrement of the control.

3.1.6.8 Real-time qPCR analysis

Real-time quantitative PCR (qPCR) is a universal method used to amplify and quantify specific gene products. DNA binding dyes are used such as SYBR Green, or sequence-specific fluorescent reporter probes. In this thesis, total RNA was extracted from the kidney tissues and reverse-transcribed into cDNA, and real-time qPCR was subsequently performed using gene-specific primers in an SYBR green-based assay. The relative gene expression was calculated with the $2^{-\Delta\Delta Cq}$ or Ct method, normalizing the gene of interest to a reference gene in the same sample. GAPDH was used as the reference gene in the study I and β -actin in study II and III.

3.1.7 Statistical analysis

The D'Agostino and Pearson omnibus normality test was used to evaluate normal distribution. Upon verification of normal distribution, variables were compared employing a two-tailed t-test. Mann–Whitney test used to compare with non-normally distributed variables. The differences were considered statistically significant when $p < 0.05$. Regression analysis was performed using linear regression. In a study I, *Ksp-KL*^{-/-} mice with relative Klotho levels > 70% were excluded during comparisons between groups. All of the mice were included in correlation tests. For comparing several groups, one-way ANOVA test was used, and the differences were considered statistically significant when $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 MINERAL METABOLISM AND FGF23 LEVELS IN DISTAL TUBULE-SPECIFIC KLOTHO-KNOCKOUT MICE (STUDY I)

By the time we started to perform this study, it had been well established that in mice, the absence of functional Klotho could lead to development of several phenotypic characteristics which resembled those of human aging (e.g., shortened life span, arteriosclerosis, growth retardation, hearing loss, infertility, skin atrophy, thymic atrophy, osteoporosis, pulmonary emphysema and motor neuron degeneration [69]. It had also been demonstrated that renal Klotho could control the phosphate and calcium homeostasis by direct interaction with their corresponding contra-transporters and receptors Npt2a/c (in the proximal tubules) and transient receptor potential vanilloid-5 (TRPV5) (in the distal tubules), respectively [14]. Moreover, it had been shown that Klotho/FGF23 signaling could also inhibit phosphate re-absorption by internalizing the NPT2a/c contra-receptors and stimulating cyp24a1, an enzyme required for vitamin D metabolism [14]. This information and the fact that while phosphate re-absorption was confined to the proximal tubules, Klotho expression was shown to occur predominantly in the distal tubules, led to the question as how and to what extent the expression of Klotho in the renal distal tubules contributes to the regulation of mineral metabolism and the development aging-related disorders. In order to answer this question, distal tubule-specific Klotho knockout (*Ksp-KL^{-/-}*) and systemic Klotho (*β-KL^{-/-}*) knockout mice were generated using the Cre-Lox recombination. Thereafter, the phenotypic characteristics of these two knockout mice were compared to each other as well as to their wild-type littermates.

As expected, mice bearing a systemic deletion of Klotho gene (*β-KL^{-/-}*) possessed phenotypic characteristics very similar to the previously described Klotho null mice [1],

i.e., they exhibited a significant reduction in the life span, weakened mobility, kyphosis and severe growth retardation (Figure 3A). Furthermore, these mice were hyperphosphatemic, hypercalcemic and had high levels of circulating FGF23. These findings clearly indicate that floxed-Klotho allele is a function and that systemic targeting of the Klotho gene leads to the development of premature aging.

In contrast to β - $KL^{-/-}$ mice, Ksp - $KL^{-/-}$ mice were viable, fertile, had normal growth, body-size (Figure 3A) and life span. Analysis of mRNA expression and protein abundance of renal Klotho revealed that knockout efficiency is partial, but not complete. Furthermore, dual immunofluorescence staining showed that Klotho is mainly colocalized with TRPV5, a marker of distal tubuli and that the protein levels of this marker is reduced in Ksp - $KL^{-/-}$ mice (Figure 3B).

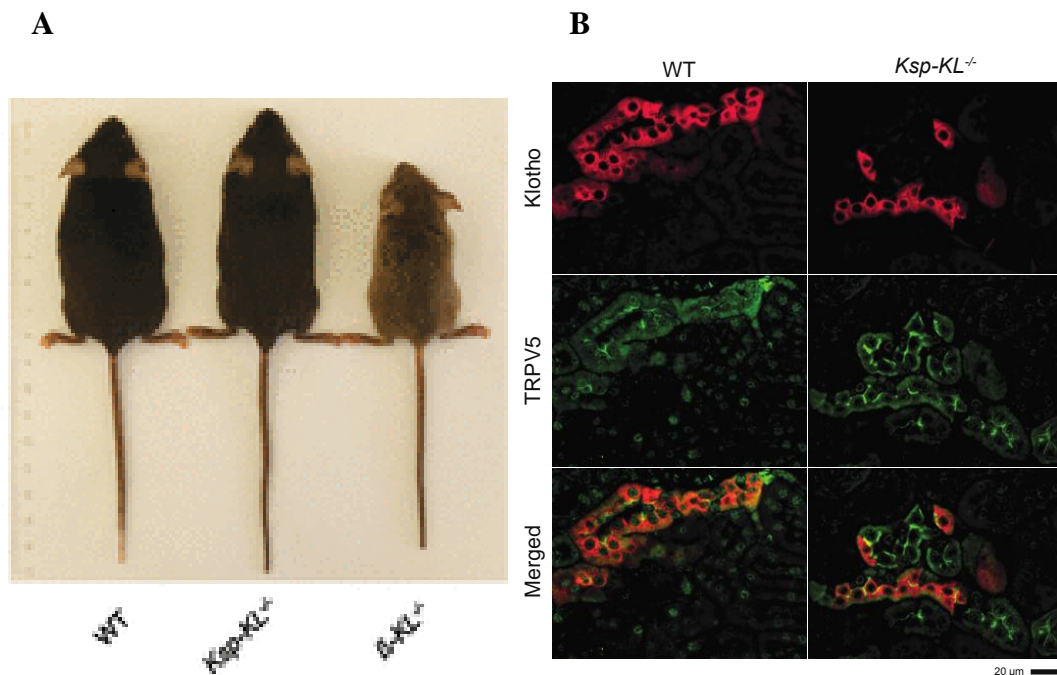


Figure 3. A) Deletion of renal distal tubular Klotho gene in Ksp - $KL^{-/-}$ mice does not alter the body size. B) Immunofluorescence staining reinforced partial deletion of distal tubular Klotho in Ksp - $KL^{-/-}$ mice. Klotho co-localized with TRPV5 in wild-type mice (WT), and partially in Ksp - $KL^{-/-}$ mice.

In addition to the reduction in the protein levels of Klotho and TRPV5 in the renal distal tubules, *Ksp-KL^{-/-}* showed hyperphosphatemia accompanied with a substantial increase in the serum levels of FGF23 and a slight increase in serum calcium levels, but decreased levels of circulating PTH. Since the efficacy of Klotho expression was variable in *Ksp-KL^{-/-}*, we analyzed the Klotho transcript level as a continuous variable and found that circulating FGF23 was the only serum parameter that correlated with the expression of Klotho. Furthermore, we found a marked threshold effect for FGF23, i.e., when the relative expression of Klotho was <30%, the serum levels of FGF23 reached 30-250 times higher than normal levels. This finding implies that there might be a resistance response when the circulatory levels of FGF23 reach certain high levels. A surprising observation showed that urinary phosphate excretion was unaltered in *Ksp-KL^{-/-}* mice. In contrast, there was a marked increase in brush-border membrane expression of Npt2a, possibly explaining the observation of hyperphosphatemia. Moreover, increased renal levels of VDR were also confirmed via immunoblotting. *Ksp-KL^{-/-}* mice displayed increased transcript levels of renal Cyp27B1, with Klotho transcript levels correlating to those of Cyp27B1, VDR, Npt2a, and FGFR1.

The theory that factors other than serum Pi contribute to FGF23 regulation was supported by elevated FGF23 levels observed in a subgroup of *Ksp-KL^{-/-}* mice with normal serum Pi. To this end, it can be hypothesized that end-organ resistance or soluble Klotho is involved. Histological analysis of *Ksp-KL^{-/-}* mice revealed a normal renal morphology. The direct paracrine effects of Klotho are disproven by lack of renal fibrosis and vascular calcification, at least during low cellular stress. Future studies could undertake the testing of these characteristics by inducing renal failure or with ageing mice (that have compromised renal function).

Stating that partial deletion of Klotho in the distal tubules significantly impacts how renal phosphate is handled in the proximal tubules, this study is the first to present genetic and functional evidence to support its hypothesis. While currently unknown, the factor(s) responsible for this proposed distal-to-proximal tubule signaling could speculatively involve soluble Klotho (Figure 4). The results of this study indicate a limited effect of distal tubular Klotho on vitamin D metabolism, even though it is apparently essential to renal phosphate handling.

More significantly, future studies could examine how a role for Klotho in the proximal tubule may not be excluded, as confirmed by our data.

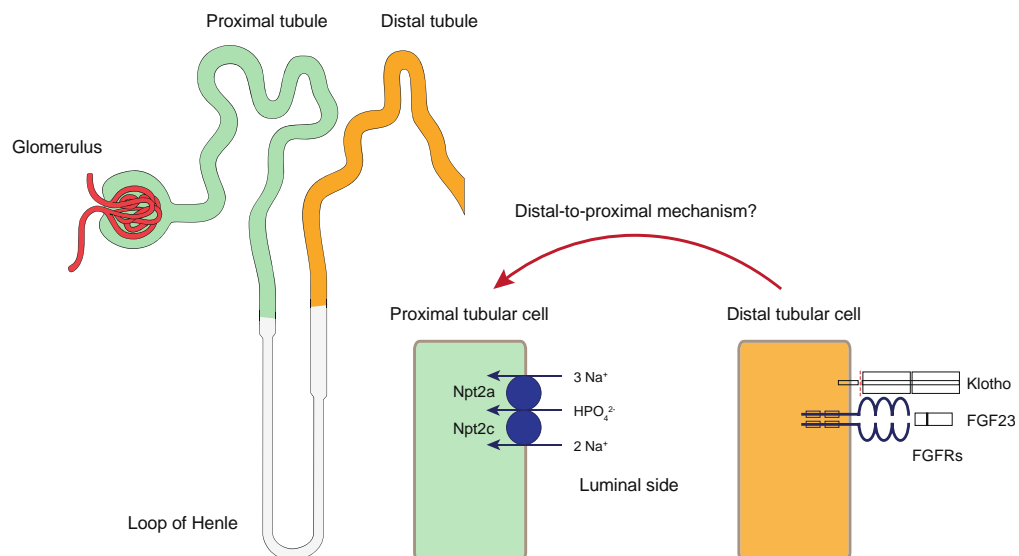


Figure 4. Renal phosphate handling in the proximal tubules is impacted by Klotho deletion in the distal tubules, suggesting the presence of a distal-to-proximal mechanism.

One unexpected finding in this study was that unlike \square -KL^{-/-} mice, Ksp-KL^{-/-} mice did not show any aberrant gross phenotype or severe renal abnormalities. Two possibilities can explain these observations. First, the incomplete knockout efficacy of the Klotho gene in the distal tubules might not be sufficient for the induction of early pathological conditions that observed in Klotho null mice. In this connection, as Klotho is considered as an anti-aging protein, it is of high interest to study the pattern aging as well as the development of age-related diseases in these animals.

4.2 THE KIDNEY'S CONTRIBUTION TO SYSTEMIC KLOTHO AND KLOTHO EFFECTS (STUDY II)

Our findings in study I revealed that specific deletion of the Klotho gene in the renal distal tubules despite inducing a severe dysregulation in mineral metabolism and increasing the circulatory levels of FGF23, did not lead to any immediate pathological symptoms. Thus, the question is, to what extent renal Klotho contributes to the development of age-related pathological manifestations remained unanswered. In order to explore the answer to this question, we generated a knockout mouse model in which the Klotho gene was deleted throughout the nephron (*Six2-KL^{-/-}*) employing the Cre-Lox combination. Immunofluorescence and western blotting showed successful deletion of renal Klotho (Figure 5A). In comparison to wild-type mice, renal Klotho transcript levels in the specimen were reduced by approximately 80%.

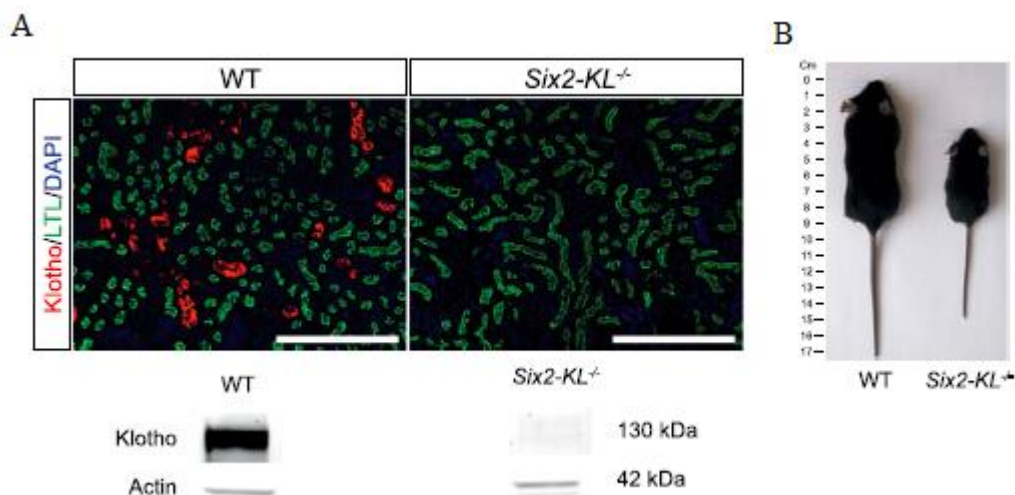


Figure 5. A) Efficient deletion of renal Klotho (red) in *Six2-KL^{-/-}* mice, as confirmed by immunofluorescence. Lotus tetragonolobus lectin (LTL, green) is a marker for proximal tubule-specific, and DAPI (blue) stains cell nuclei. **B)** *Six2-KL^{-/-}* mice were smaller in size compared to WT mice

Phenotypic characterizations of the *Six2-KL^{-/-}* mice showed that these animals were infertile and kyphotic, displaying growth retardation (Figure 5B) and a decreased life span; these phenotypes closely resembled those of our systemic Klotho knockout mice (*β-KL^{-/-}*) [70] and Klotho null mice [1]. Moreover, these knockout mice exhibited severe biochemical alterations including: low serum levels of PTH, hyperphosphatemia, hypercalcemia, hyperaldosteronism, and elevated serum levels of 1,25-dihydroxy vitamin D. These biochemical alterations were accompanied with a striking increase of circulating FGF23 indicating that FGF23 signalling is highly dysregulated in *Six2-KL^{-/-}* mice. Assessment of circulating Klotho revealed that in *Six2-KL^{-/-}* mice, serum levels of Klotho reduced up to 80%, an observation that validates the suggestion that the kidney is the principal contributor to circulating Klotho. To further supports this suggestion, kidney explants were used to evaluate the shedding of renal Klotho *ex vivo*. The results showed that the kidneys of *Six2-KL^{-/-}* mice had undetectable Klotho protein in conditioned media as compared to those of wild-type mice. Preserved expression of Klotho in extra-renal organs such as the parathyroid glands and choroid plexus in *Six2-KL^{-/-}* mice was confirmed by immunostaining. All these observations reinforce the kidney as the principal source of soluble Klotho; they also confirm that extra-renal tissues can not correct a systemic deficiency of Klotho.

Proceeding to examine the impact of renal Klotho ablation on the kidney histology, we detected the following abnormalities in the structure of renal tissue of *Six2-KL^{-/-}*: a higher proliferation rate, loss of differentiation between proximal and distal convoluted tubules, mild interstitial fibrosis, widespread nephrocalcinosis and loss of cuboidal Bowman's epithelium of glomeruli in male *Six2-KL^{-/-}*. An extensive histological comparison between *Six2-KL^{-/-}* mice and systemic *Klotho* knockout mice was conducted to evaluate the impact of local Klotho expression on the phenotype

resembling ageing. Pulmonary emphysema, reduced subcutaneous fat and skeletal hypomineralization constituted the histological findings in *Six2-KL^{-/-}* mice. It must be noted that *Six2-KL^{-/-}* and systemic *Klotho* knockout mice showed no differences in renal or extra-renal abnormalities.

To conclude, our findings in this study demonstrate that the kidney is most likely the primary source of circulating Klotho and mediator of Klotho function and thus, providing several implications for the field of kidney research. Results of this study also support the possibility that therapeutic Klotho delivery in patients with chronic kidney disease (CKD), a state characterized Klotho deficiency, may mitigate uremic complications, thereby slowing the disease progression.

4.3 ROLE OF FGF23/KLOTHO AXIS IN IN CARDIOVASCULAR SYSTEM (STUDY III)

Cardiovascular disease (CVD) is a significant burden in patients with CKD. Significant attempts have been made to understand the cause of the raised prevalence of cardiovascular morbidity and mortality in CKD. With respect to this, FGF23-Klotho represents a novel hormonal axis that has recently attracted significant attention because observational studies unequivocally associate high circulating FGF23 levels to vascular dysfunction; also, FGF23 is predictive of all-cause and cardiovascular mortality in longitudinal outcome studies [71-77]. Further, membrane-derived circulating Klotho itself functions as a true hormone to prevent vascular calcification and protect endothelial properties. Similar findings with a Klotho-deficient state confer increased susceptibility to vascular damage [78]. However, despite the fact that most of the clinical data clearly show a link between the elevation of FGF23 and CVD, experimental data supporting direct vasculotoxic effects of FGF23 is lacking. Recent studies mainly support the FGF23-Klotho endocrine axis' role in cardiovascular pathology, though the responsible mechanisms remain largely unexplained. This study

aimed to elucidate the role of the Klotho/FGF23 axis in the development of CVD, a complication that occurs in patients with CKD. To this end, we generated mice with tissue-specific deletion of Klotho in vascular smooth muscle cells (*Sm22-KL^{-/-}*) to analyze the expression of the FGF23 co-receptor Klotho in the mouse arteries.

Sm22-KL^{-/-} mice were viable, fertile, had normal body size and weight and exhibited no significant alterations in the serum levels of phosphate, FGF23, calcium, and creatinine as compared to those in their corresponding wild-type mice. This indicated that vasculature is not a major target tissue for FGF23. Very low levels of arterial Klotho transcripts were detected with quantitative real-time PCR. In contrast, corresponding Klotho levels were undetectable in *Sm22-KL^{-/-}* mice. Intravenous delivery of recombinant FGF23 elicited an increase in the expression of Egr-1 (a Klotho-dependent FGF23 signaling marker) in the kidney but not in the artery. Furthermore, FGF23 treatment did not alter the structure, calcification in bVSMCs or dilatory and contractile behaviors of the arterial specimen *ex vivo*.

In summary, our findings show that FGF23-Klotho signaling is abolished in mouse arteries and that treatment with FGF23 does not affect the vascular response. Further, vascular calcification and endothelial function were unaffected by FGF23 treatment, thus casting doubt on the assumption that direct FGF23 vascular toxicity can cause cardiovascular pathology in CKD, at least not in these mice. Yet, the possibility of species differences in terms of vascular Klotho expression must be acknowledged, warranting more extensive evaluations of the human arterial specimen.

4.4 ROLE OF FGF23/KLOTHO AXIS IN AGVHD AND ITS RELATED AKI (STUDY IV)

Our studies have clearly shown that partial or systemic Klotho gene ablation results in an intensive increase of circulatory FGF23 [8, 70, 79, 80]. High circulating levels of FGF23 in combination with low serum or urine levels Klotho have been found in

patients with CDK [81] AKI [82] as well as in animal models for these diseases [82]. Since in both diseases, renal cell injury and its related products can provoke inflammatory responses [83, 84], it is likely that dysregulation in FGF23/Klotho axis plays an important role in inflammation. Thus, in study IV, we aimed to examine this possibility by investigating the role of FGF23/Klotho in the development of aGVHD and HSCT-associated AKI, two main inflammatory reactions that emerge after allogeneic HSCT. Thus, recipient female BALB/c mice were first conditioned with Bu-Cy and thereafter, transplanted with bone marrow and spleen cells from the allogeneic (C57BL/6) or syngeneic (BALB/c) donor mice. Subsequently, the transplanted animals were monitored for the development of aGVHD. At disease onset, the animals were euthanized and thereafter, aGVHD- and AKI-related parameters were measured. For comparative studies on the role of FGF23/Klotho in AKI, we also used a LPS-induced AKI model.

We found that in addition to developing aGVHD symptoms (e.g., body weight loss, ruffled fur, hunched posture and diarrhoea), mice receiving allogeneic cells also exhibited full donor cell chimerism with high serum levels of TNF- α , IFN- γ and FGF23 (Figure 6A). These characteristics were accompanied with elevated serum levels of urea and phosphate and decreased serum level of albumin. Along with these phenotypic biochemical alterations, mice with aGVHD exhibited a marked down-regulation in mRNA and protein expression levels of renal Klotho (Figure 6B). With respect to the underlying mechanism for the down-regulation of Klotho expression, it has been shown that renal Klotho expression is repressed in mice with inflammatory bowel diseases and implicates the role of Th1-type cells and their inflammatory cytokine including TNF- α and IFN- γ in the mechanism of inhibition in a dose-dependent manner via activation of NF κ B [53]. The inhibitory phenotype was ameliorated by anti-*tumour necrosis factor* (TNF) antibody [49]. Member of the TNF- α superfamily of cytokine TNF- α and TNF-

like weak inducer of apoptosis (TWEAK) has a pleiotropic function on glomerular and tubular cells that contribute to renal damage [51, 52]. A neutralizing antibody rescues the Klotho expression in TWEAK-KO mice indicating that these cytokines regulate Klotho at the transcriptional level. Other important findings in this study also fit with our initial hypothesis that the dysregulation of FGF23/Klotho axis in mice with aGVHD can simultaneously lead to the development of AKI. Firstly, our results showed that in addition to the increased serum levels of FGF23 and reduced expression of renal Klotho, aGVHD mice exhibited elevated serum levels of urea, phosphate (Hyperphosphatemia), and decreased serum level of albumin (hypoalbuminemia) which are common phenotypic characteristics in patients with AKI [85-87]. In fact, loss of body weight in aGVHD mice may be caused by hypoalbuminemia. Accordingly, in these mice, histopathological analysis of the kidney revealed intense cytoplasmic vacuolization of the renal proximal tubule epithelial cells, which is suggestive of lysosomal proliferation. Finally, phenotypic characteristics of mice with aGVHD were found to be indistinguishable from those of lipopolysaccharide- (LPS)-induced AKI mice. Thus, dysregulation of FGF23/Klotho appears to play a key role in the development of aGVHD and AKI. It can thus be inferred that therapeutic targeting of this axis might help minimize the development of aGVHD and AKI, thereby enhancing the treatment efficacy of HSCT.

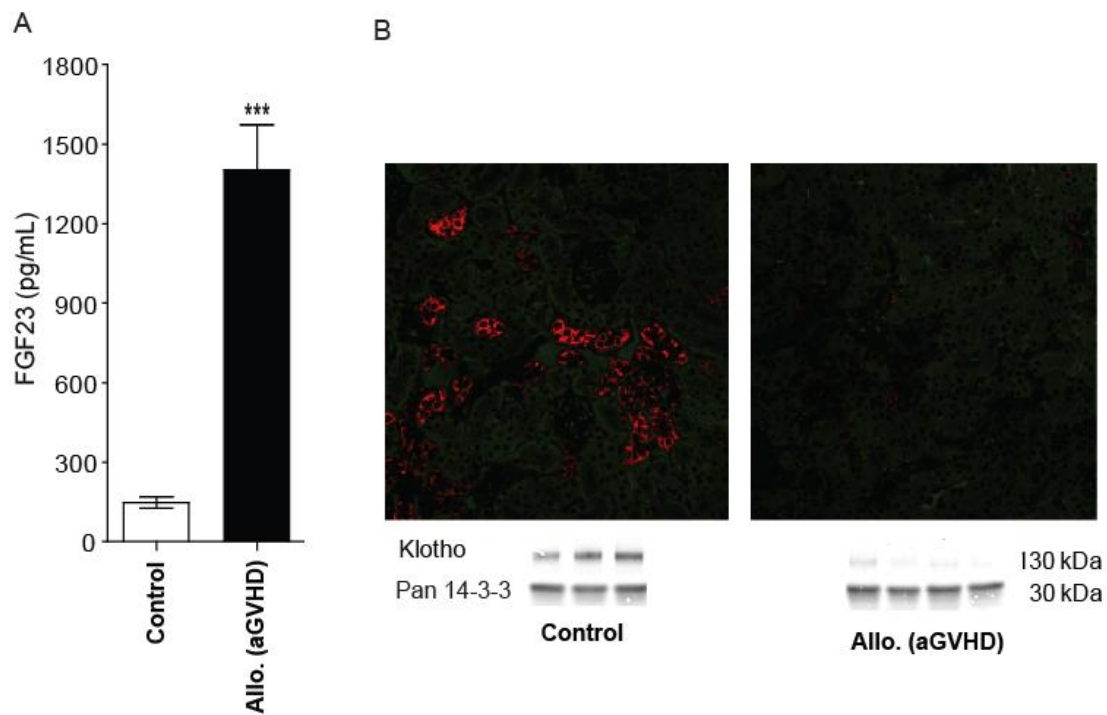


Figure 6. **A)** FGF23 level was measured in the control and allogeneic (aGVHD)-transplanted groups. **B)** mRNA expression of Klotho in control and aGVHD mice correlated with renal Klotho expression in this groups visualized by Fluorescent *in situ* hybridization and western blotting.

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