Studies on the pathogenesis of chronic kidney disease

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Chapter 1

General introduction

Scope of this thesis

Chronic kidney disease

The scope of this thesis is to study the pathogenesis of chronic kidney disease. Chronic kidney disease is a general term for kidney diseases with a gradual loss of renal function over a long period [1]. The definition of chronic kidney disease is: the occurrence of kidney damage with gradually decreased kidney function (GFR<60mL/min/1.73 m^2) for three months or more [2]. Kidney damage is characterized by abnormalities in urine or blood tests, and/or defects observed in kidney morphology. In 2017, chronic kidney disease affected almost 700 million people globally, and it is recognized as a worldwide public health problem [3]. Understanding the underlying mechanisms of the development of chronic kidney disease will pave the way for the development of preventative and therapeutic strategies. Based on the location in the kidney where the primary injury is present, chronic kidney disease can be divided into two categories: glomerular diseases (such as diabetic nephropathy, glomerulonephritis, and focal segmental glomerulosclerosis) and tubular diseases (such as nephropathic cystinosis, polycystic kidney disease and pyelonephritis). This thesis focuses on the pathogenesis of diabetic nephropathy and nephropathic cystinosis.

The kidney

Kidneys are paired bean-shaped organs located on the left and right of the posterior abdominal wall (**Figure. 1A**). The main functions of the kidney are: 1) excretion of excess fluids and waste products out of the blood into the urine; 2) controlling homeostasis by regulating fluid osmolality, acid-base and electrolyte balance; 3) synthesizing hormones, such as erythropoietin and renin.

The basic functional unit of the kidney is called the nephron, consisting of a glomerulus and a renal tubule **(Figure. 1B)**. Each human adult kidney contains around 1 million nephrons [4].

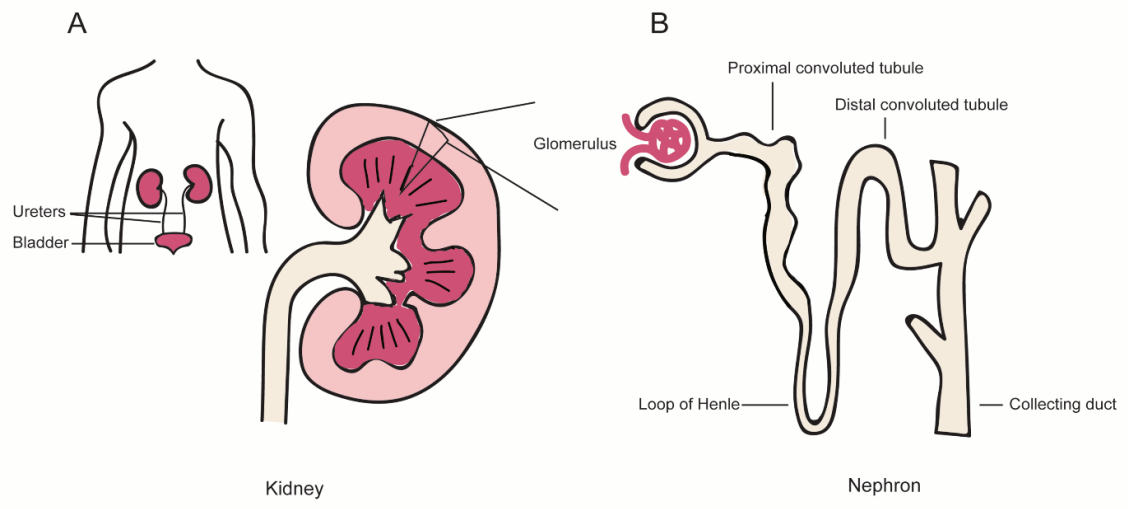

Figure 1. The gross anatomy of the kidney and the structure of the nephron

Glomerulus

The glomerulus is a specialized filter unit with a tangle of capillaries (**Figure. 2A**). Afferent arterioles give rise to glomerular capillaries which merge to exit the glomerulus as efferent arterioles. Bowman's capsule is a spherical structure surrounding the glomerulus. Bowman's capsule encloses the capillary tufts creating Bowman's space. Bowman's capsule is covered by parietal epithelial cells. The visceral epithelial cells, also called podocytes, coat the outer surface of the glomerular capillaries. Podocytes together with glomerular basement membrane (GBM) and the fenestrated endothelial cells constitute the glomerular filtration barrier (GFB) that the ultrafiltrate passes through (**Figure. 2B**). The GFB separates the vascular from the urinary space, and it is crucial for ultrafiltration. Under healthy conditions, only water, small solutes, and small proteins can pass through the intact GFB because of its size-selective and

charge-selective properties. However, defects in at least one of the layers of GFB can cause leakage of larger plasma proteins into the urine, resulting in proteinuria.

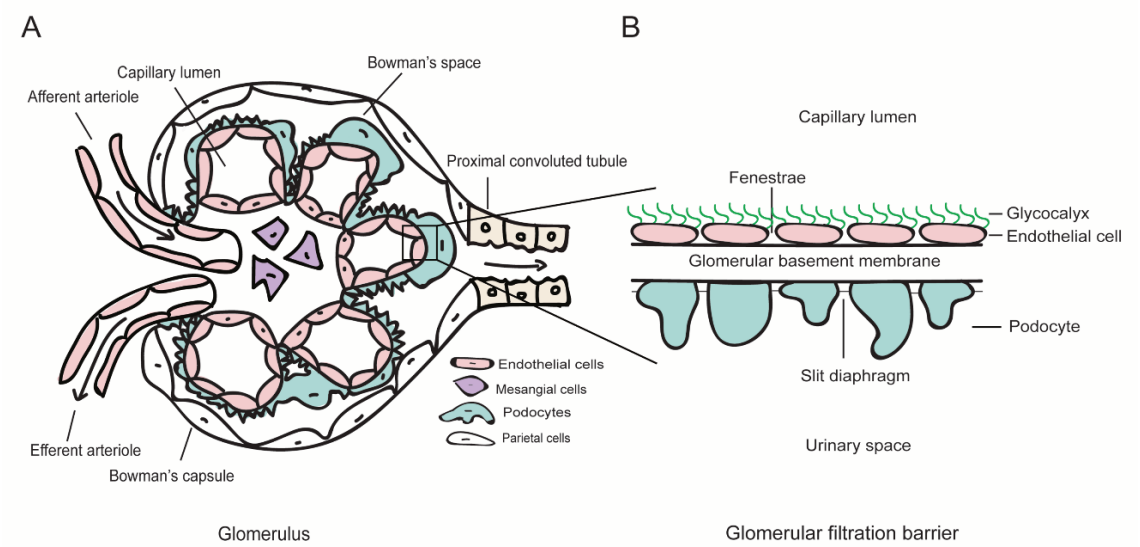

Figure 2. The structure of the glomerulus and the glomerular filtration barrier

Glomerular endothelial cells are highly specialized cells lining inside of the glomerular capillaries. The transcellular windows of glomerular endothelium are referred to as fenestrae. It has been demonstrated that a glycocalyx, a network composed of glycoproteins, coats the luminal surface of the glomerular capillaries and bridges the fenestrations of glomerular endothelial cells [5]. It has been reported that the glycocalyx is vital to the permselectivity (selective permeability to certain proteins and molecules) of the glomerular capillary [6,7]. Alterations in endothelial glycocalyx components increase microvascular permeability and cause albuminuria in glomerular diseases [8-10].

The GBM is a sheet-like structure that consists of extracellular matrix molecules, and it is the only uninterrupted layer in the GFB. It comprises collagens and no collagenous glycoproteins, such as type IV collagen, laminin, nidogen, fibronectin, and heparan sulfate proteoglycan [11]. The network of collagen IV is regarded as a crucial scaffold integrating other components in the GBM. Mutations in collagen IV α3 result in Alport syndrome, which leads to deafness and kidney failure in children [12]. Ernst Pöschl et al. demonstrated that collagen IV is dispensable for the initiation of GBM assembly. It is essential to maintain the integrity and stability of GBM at later stages of development [13]. Laminin gene mutations can also cause GBM defects. Mutation of laminin β2 in humans results in a congenital manifestation called Pierson syndrome, a disorder characterized by congenital ocular and neurologic abnormalities and nephrotic

syndrome [14]. An experimental study demonstrated that insufficient levels of laminin α5 to maintain GBM integrity leads to the development of polycystic kidney disease [15]. These studies collectively suggest that collagen networks and laminin components are crucial to support the standard structure and function of GBM. The GBM is negatively charged [16]. Heparan sulfate proteoglycans are assumed to contribute significantly to the net negative charge of GBM [17]. It is generally accepted that the net negative charge of GBM can repel the plasma albumin, which is also negatively charged, therefore prevents the passage of albumin through the GFB. Recent studies have aroused debate on the concept of charge selectivity. Researchers found that the disruption of the GBM charge by mutating the heparan sulfate proteoglycans, such as agrin or perlecan-heparan, does not alter glomerular permselectivity and does not cause proteinuria [18-20].

Podocytes are terminally differentiated epithelial cells. Podocytes consist of a large cell body, thick primary processes, and intricate interdigitating foot processes. Podocytes can counteract the distensions of GBM and stabilize the architecture of the glomerulus due to their contractile foot processes [21]. The specialized cell-cell junction connecting the foot processes from adjacent podocytes called “slit diaphragm” or “slit pore” [22], which is crucial for the maintenance of healthy podocytes. Several proteins are essential for keeping the intact function of this slit diaphragm, such as nephrin, podocin, transient receptor potential canonical (TRPC) proteins, Kin of IRRE-like protein 1 (NEPH1), CD2-associated protein (CD2AP) and Tight junction protein-1 (ZO-1). Dysfunction of slit diaphragm results in disconnecting the foot process of adjacent podocytes and flattening of foot processes, termed “foot process effacement”, which is the most common pathological change in podocyte diseases. Recently, it was demonstrated that the slit diaphragm comprises a complex dynamic signaling hub as well as a filter due to their anatomical features to prevent proteinuria [23]. Disruption in any of the slit diaphragm signaling pathways is tightly related to podocyte loss and the filtration defect, directly or indirectly contributing to the development of proteinuria in several glomerular diseases, including focal segmental glomerulosclerosis, diabetic nephropathy and immune-mediated glomerulonephritis.

Mesangial cells are specialized pericytes located on the lumen side of the GBM. They structurally support the glomerular capillaries. Besides, the mesangial cells' contractile potency enables them to alter ultrafiltration surface area and capillary flow in the glomeruli and subsequently regulate the single-nephron glomerular filtration rate (SNGFR) [24]. The mesangial cells synthesize the mesangial matrix consisting of different types of collagen (mainly of type IV and V collagen), laminin, and fibronectin. Mesangial cells and matrix are called mesangium

[25]. The mesangial cells also produce cytokines, chemokines, growth factors and vasoactive factors, which participate in the cross-talk with the adjacent endothelial cells and GBM. Abnormal proliferation of mesangial cells can be found in diabetic nephropathy, IgA nephropathy and lupus nephritis [26]. The mesangial matrix expansion is an early hallmark of diabetic nephropathy, which inversely correlates with glomerular filtration rate [27].

Renal tubule

The renal tubules transport and process the ultrafiltrate on its way to the renal pelvis. The renal tubule can be divided into several segments based on morphology and function, including proximal convoluted tubule, loop of Henle, distal convoluted tubule, and collecting duct. The glomeruli, together with the proximal tubules, form the renal cortex. The renal medulla consists mainly of distal tubules and the collecting ducts. The glomerular ultrafiltrate first enters the proximal convoluted tubule, where it reabsorbs the majority of substances in the ultrafiltrates, such as electrolytes, proteins, glucose, phosphates, amino acids, uric acid, and bicarbonate. After passing through the proximal convoluted tubule, the filtrate continues to the loop of Henle, the distal convoluted tubule, and the collecting duct. Finally, the remaining filtrate moves as urine via the pelvis and the ureter to the bladder (**Figure. 1B**).

Progression of chronic kidney disease

Once the kidney has been damaged, it may continue to get worse over time. The progression of chronic kidney disease involves both glomerular and tubular injury and interstitial fibrosis development, which parallels the decline in renal function [28].

Glomerular injury increases the filtration load by increasing SNGFR of the remaining functioning glomeruli, which leads to glomerular hyperfiltration. Meanwhile, increased filtration pressure in the capillary tufts results in glomerular hypertension. Glomerular hyperfiltration and glomerular hypertension together cause the remaining nephron hypertrophy by increasing the size of the Bowman's capsule, the glomerular tuft, and Bowman's space. The hypertrophy of the remaining nephrons is an adaptive response to the elevated intraglomerular pressure that increases the filtration rate in the remaining intact glomeruli. However, persistent glomerular hyperfiltration and glomerular hypertension accelerate the loss of remnant nephrons. In turn, abnormal function and structure in nephrons further exacerbate the glomerular injury.

The GFB is maintained as long as podocytes cover the enlarged filtration surface area. However, podocyte hypertrophy may fail to do so when it is beyond a certain threshold. In that situation, the slit-diaphragm are disrupted, which will cause dysfunction of GFB and protein leakage into the tubular fluid resulting in proteinuria. Endothelial dysfunction, leading to a proinflammatory and prothrombotic state, is also thought to play a pivotal pathophysiological role in glomerular injury [29]. Endothelial cells synthesize a variety of proinflammatory molecules, including vascular cell adhesion molecule and intercellular adhesion molecule, which contribute to inflammatory processes [30]. During endothelial dysfunction, endothelial cells also trigger platelet adhesion, platelet aggregation, and fibrin formation [31]. Recent years have witnessed a growing appreciation that endothelial-podocyte bidirectional cross-talk may play a pivotal role in the pathogenesis of glomerular injury [32]. Endothelial dysfunction likely elicits podocyte dysfunction [33]. It is well known that the vascular endothelial growth factor (VEGF) signaling axis is critical for maintaining the endothelium's normal function. VEGF is produced by various types of cells, and it acts predominantly on endothelial cells. The differentiation, function and survival of glomerular endothelial cells are highly dependent on VEGF, which is typically produced by podocytes [34,35]. In preeclampsia, soluble fms-like tyrosine kinase-1 (sFlt-1), a splice variant of VEGF receptor Flt-1 and a natural inhibitor of VEGF-A, is overexpressed and causes glomerular endotheliosis and proteinuria [36]. The hypertrophy of mesangial cells, increased mesangial cellularity, and increased mesangial matrix expansion jointly lead to mesangial expansion during the development of glomerular injury. With time, the accumulation

of the mesangial matrix, along with mesangial cells, are increasing. This lesion can progress to segmental glomerulosclerosis and eventually leads to global glomerulosclerosis [37].

The interaction of dysfunctional tubular cells with interstitial tissue results in tubulointerstitial injury, which is the primary causal event associated with the progressive kidney function loss in chronic kidney disease [38]. It has been suggested in the literature that the passage of plasma proteins in the ultrafiltrate plays an essential role in chronic tubulointerstitial injury. The increased filtration of plasma proteins can induce inflammation by upregulation of chemoattractant and adhesive molecules via activation of NF-κB-independent and NF-κB-independent pathways [39]. The interstitial infiltrates of mononuclear cells, such as monocytes and macrophages, contribute to the fibrogenic process via the production of several profibrotic molecules [40]. The abnormal interstitial accumulation of inflammatory cells, fibronectin, and extracellular matrix collagens (I, III, V, VII, and XV) result in interstitial fibrosis [41,42]. Furthermore, the ultrafiltered protein load can promote complement activation, which aggravates tubular and interstitial injury progression [39].

An excess accumulation of extracellular matrix and a disproportional renal parenchyma loss contribute to renal fibrosis [43]. Renal fibrosis is an inevitable outcome of progressive chronic kidney disease [44], which leads to a progressive loss of renal function and ultimately results in end-stage renal failure.

Diabetic nephropathy

Diabetes Mellitus

Diabetes Mellitus is a metabolic disorder characterized by prolonged hyperglycemia. Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM) are the most common types of diabetes. T1DM is referred to as “insulin-dependent diabetes mellitus”. The loss of beta-cells, mainly attributed to autoimmune destruction, fails to produce sufficient insulin to maintain normal blood glucose levels in the pancreas. It is mainly attributed to autoimmune destruction. T2DM is referred to as “non-insulin-dependent diabetes mellitus”. The cells of the patients with T2DM fail to respond appropriately to insulin. T2DM is the most prevalent type in all populations. Maturity onset diabetes of the young (MODY) is caused by a genetic defect that disrupts insulin production. Gestational diabetes occurs in pregnant women who have hyperglycemia without a previous history of diabetes mellitus. It is caused by reduced insulin production or insulin resistance, which may improve or disappear after delivery.

Diabetic vascular complications

Hyperglycemia-induced diabetic vascular damage may involve the following five underlying mechanisms:1) increased polyol pathway flux, 2) increased formation of advanced glycation end-products, 3) activated the ligands of advanced glycation end products and increased expression of its receptor, 4) increased protein kinase C activation, 5) and increased the hexosamine pathway flux [45]. One hypothesis is that all five mechanisms are activated by one upstream event: the overproduction of reactive oxygen species. The condition that the overproduction of reactive oxygen species exceeds the cell's antioxidant response is known as oxidative stress [46], which can cause cellular damage [47,48] and lead to diabetic complications [45]. Most patients with long-lasting diabetes develop both macrovascular and microvascular complications. Macrovascular diseases include coronary heart disease, cerebrovascular disease, and peripheral vascular disease. Microvascular diseases include retinopathy, neuropathy, and nephropathy. However, it is still unknown why a majority of patients with diabetes do not develop these complications.

Diabetic nephropathy: clinical features

Diabetic nephropathy is a major complication of diabetes and the leading cause of end-stage renal disease. As diabetes itself is a prerequisite of diabetic nephropathy, the diagnosis of diabetic nephropathy mainly bases on diabetes history combining with physical examinations, laboratory evaluation, and imaging of the kidneys [49]. Clinical diabetic nephropathy is diagnosed by increased urinary albumin excretion and reduced glomerular filtration rate, often accompanied by hypertension and dyslipidemia. According to the natural history of diabetic nephropathy, five stages of progression are recognized [50]. Stage 1 is characterized by hyperfiltration and hypertrophy of the glomeruli and increased kidney size. The morphological changes of the kidney are still reversible at this stage. Stage 2 is characterized by glomerular structural alterations like GBM thickening and mesangial matrix expansion without clinical signs. Stage 3 is characterized by the incipient rise in microalbuminuria (urine albumin excretion rate, UAER: 30~ 300 mg/24h) in diabetic patients. Stage 4 is characterized by persistent macroalbuminuria (UAER> 300 mg/24h), which finally progresses to end-stage renal disease (stage 5) due to advanced diabetic nephropathy.

Diabetic nephropathy: histopathology

Identifying the renal histological abnormalities through a renal biopsy is also crucial to the diagnosis of diabetic nephropathy (**Figure. 3A-3B**). A uniform international accepted

pathological classification has been developed, which improves communication among clinical nephrologists and renal pathologists, and is also useful for the research activities on diabetic nephropathy. The widely used histopathological classification of diabetic nephropathy is as follows: Class I: GBM thickening alone (GBM thickness >430nm in men and >395nm in women); Class II: mesangial expansion (mesangial expansion present in > 25% of the mesangium; mild-IIa; moderate-IIb); Class III: Nodular sclerosis (the presence of Kimmelstiel-Wilson lesion, but < 50% diffuse global glomerulosclerosis); Class IV: Advanced diabetic glomerulosclerosis (>50% diffuse global glomerulosclerosis with or without nodules) [51].

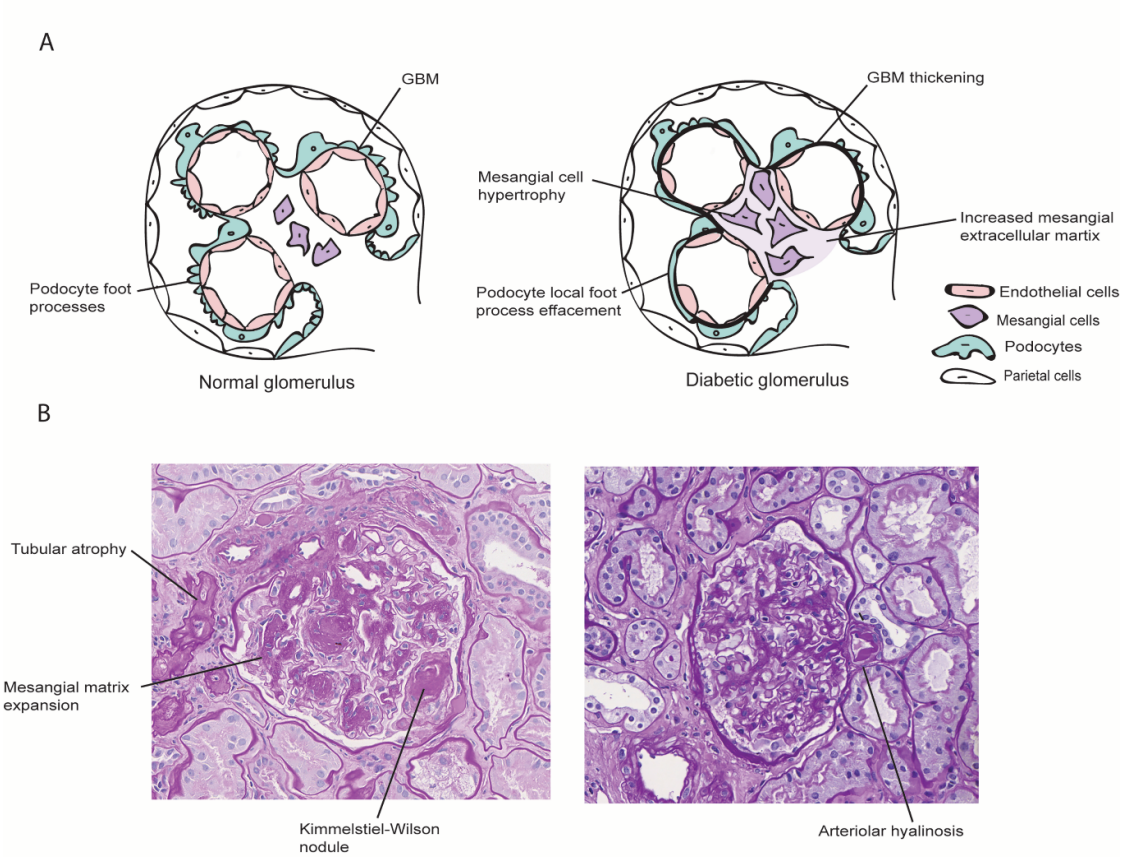

Figure 3. The renal histological abnormalities in diabetic nephropathy

A renal biopsy is not routinely taken in patients with diabetic nephropathy because the diagnosis is assumed to exist when proteinuria is present in a patient with diabetic retinopathy. Klessens et al. demonstrated in a large autopsy cohort that diabetic nephropathy is underdiagnosed. They found that 20 of 106 subjects, who had renal histopathological changes characteristic for diabetic nephropathy, did not present any diabetic nephropathy associated clinical features during their lifetime [52].

Diabetic nephropathy: genetic predisposition

A genetic predisposition, also called genetic susceptibility, is an increased likelihood of developing a particular disease based on the genetic background under the influence of environmental conditions. Despite the increasing prevalence of diabetes, only a minority of individuals with diabetes develop diabetic nephropathy (30%~50%), suggesting that genetic determinants could influence the development and progression of diabetic nephropathy. Krolewski et al. found that the incidence rate of diabetic nephropathy rises within the first fifteen years and then declines in patients with type 1 diabetes [53]. Familial clustering of the development of diabetic nephropathy has also been observed [54-56]. These studies jointly provide evidence for a genetic predisposition in diabetic nephropathy. Additionally, a meta-analysis reported that the variants of genes, such as *ACE* (the gene coding for the angiotensin-converting enzyme), *APOC1* (the gene coding for apolipoprotein C1), *APOE* (the gene coding for apolipoprotein E), EPO (the gene coding for erythropoietin), *VEGFA* (the gene coding for vascular endothelial growth factor-A), associated with diabetic nephropathy [57]. Therefore, understanding the role of the genetic factors in diabetic nephropathy is crucial since it could provide a new sight on early diagnosis or potential therapeutic strategies.

Genes in diabetic nephropathy

Clusterin (*CLU*) has protective properties in diabetic nephropathy

Clusterin is a disulfide-linked heterodimeric protein encoded by *CLU*. Clusterin is widely expressed in various tissues, and it participates in several physiological processes, including cell differentiation [58], lipid transport [59,60], complement inhibition [61], and regulation of apoptosis [62,63]. The *CLU* gene produces three different protein isoforms through alternative splicing and post-transcriptional modifications. These three isoforms of clusterin play different roles in the regulation of apoptosis. For instance, the nuclear isoform localizing to the nucleus is proapoptotic, while the cytosolic isoform, localizing to the cytoplasm, and the secretory isoform being secreted out of cells are anti-apoptotic [62,64].

Clusterin is involved in many processes, including ageing [65], inflammatory diseases [66], neurodegenerative diseases [67], and cancer [64]. A Japanese study reported that polymorphisms of the *CLU* gene were associated with T2DM [68]. Using microarray analysis, our group found that glomerular *CLU* mRNA expression is significantly higher in diabetic nephropathy patients than in healthy individuals [69]. Moreover, Nakatani et al. found that glomerular clusterin protein levels are 2.42-fold higher in diabetic nephropathy patients than non-diabetic controls in an

autopsy cohort [70]. These studies suggested that glomerular clusterin is increased and might play a role in the development of diabetic nephropathy.

In diabetic patients, increased intracellular reactive oxygen species levels lead to oxidative stress, damaging all cell components, including DNA, proteins and lipids [71]. As mentioned previously, the overproduction of the reactive oxygen species can induce diabetic vascular damage [45]. Numerous studies have shown that clusterin could protect cells against oxidative stress-induced damage. For instance, it has been reported that clusterin protects porcine proximal tubular cells against H_2O_2-induced damage [72]. Kim et al. demonstrated that exogenous clusterin protects retinal endothelial cells and astrocytes against H_2O_2-induced apoptotic cell death [73]. They also reported that clusterin protects retinal pigment epithelial cells against oxidative stress via the PI3K/Akt signalling pathway [74]. Besides, Jun et al. found that clusterin could protect cardiomyocytes from oxidative stress-induced apoptosis by inhibiting the Akt/GSK-3beta signalling pathway [75].

Podocytes are terminally differentiated and highly specialized epithelial cells in the glomerulus, which serve as a critical component of the GFB. It has been reported that the reduced numbers of podocytes are associated with the development of diabetic nephropathy in patients with type 2 diabetes [76], and changes in the structure and density of podocytes occurred in the early stages of DN [77]. Moreover, Weil et al. reported that podocyte detachment is correlated with albuminuria in these patients [78]. Zheng et al. found that the specific overexpression of metallothionein (an antioxidant protein) in podocytes reduces the podocyte damage/loss and attenuates the pathogenesis of diabetic nephropathy, which provides evidence that oxidative stress in podocytes contributes to the development of diabetic nephropathy [79]. Rastaldi et al. demonstrated that biotinylated clusterin can bind to podocytes via the LDL receptor and prevent PKC activation in membranous glomerulonephritis [80].

<u>The role of carnosinase-1 (*CNDP1*) overexpression in the progression of diabetic nephropathy</u>

Carnosinase-1 is a dipeptidase that belongs to the M20 metalloprotease family. It is an enzyme that hydrolyses carnosine (β-alanyl-L-histidine). Carnosine has been suggested to have a renal protective effect with anti-oxidative [81], anti-glycation [82], and carbonyl scavenging [83] properties. Two forms of carnosinase, serum carnosinase (CN-1) and non-specific carnosine dipeptidase (CN-2), are encoded by the *CNDP1* (*CN1*) and *CNDP2* (*CN2*) genes, respectively [84]. *CNDP1* and *CNDP2* genes are both located on chromosome 18q22.3. Vardarli et al. demonstrated the susceptibility to type 2 diabetic nephropathy to be located at chromosome 18q22.3-q23 in a Turkish family-based linkage study [85]. Further research revealed an association between the

variants (five, six, or seven leucine repeats) in the leader peptide of the *CNDP1* gene and the susceptibility for diabetic nephropathy. The homozygous five-leucine allele is associated with a lower carnosinase activity, and six and seven leucine alleles are associated with a higher carnosinase activity [86,87]. The serum carnosinase form is present in the serum in humans but not in rodents [84,88]. In an experimental study, Sauerhöfer et al. generated a hCN1 transgenic *db/db* mice model by overexpression of human *CNDP1* in *db/db* mice, an animal model for T2DM. They found higher human carnosinase-1 levels and lower L-carnosine levels in the serum and significant renal hypertrophy in hCN1 transgenic *db/db* mice compared to non-transgenic *db/db* mice, which confirmed an association of the *CNDP1* variants with diabetic nephropathy [89].

It has been suggested in the literature that aerobic exercise training could ameliorate renal function and attenuate renal lesions in the diverse diabetic rodent models [90-93]. Physical activities are also suggested to be beneficial to lower microalbuminuria excretion in type 2 diabetic patients [94,95]. A review from Culbertson et al. elucidated that human athletes involved in anaerobic sports have higher concentrations of carnosine in muscle, and exercise performance also increases resting muscle carnosine levels [96]. A higher carnosine content in the circulation was also found in the animals when physically active [97,98]. Carnosine is mainly present in skeletal muscle and has lower amounts in the renal tissue. The supplementation of carnosine has been shown to protect against the progression of diabetic nephropathy in different diabetic animal models [83,99-101].

The role of leptin in the development of diabetes and diabetic nephropathy

The leptin gene was discovered in 1994 [102]. Human leptin is a 16-kDa protein hormone encoded by the *LEP* (*ob*) gene. It is predominantly synthesized and secreted by white adipose tissue. Other organs, including placenta, stomach and skeletal muscle can also be leptin synthesis sources [103]. Except as a regulator of the energy homeostasis via suppressing appetite, leptin also participates in diverse physiological processes, including immune regulation [104,105], endocrine regulation of the energy metabolism [106], reproduction [107,108], and lipid metabolism [109,110].

In humans, patients lacking leptin have evident obesity and glucose tolerance impairments [111,112]. Leptin deficient (*ob/ob* mice) [113,114] and leptin receptor-deficient (*db/db* mice and Zucker diabetic fatty rats) rodents [115,116] are characterized by hyperphagia, obesity, hyperglycemia and insulin resistance. Growing evidence suggests that leptin therapy has a beneficial effect on glucose metabolism and insulin resistance, and it could be a promising therapy for diabetes. The initial thought of a glucose-lowering function of leptin is attributed to the secondary effects of attenuating obesity. Recent research has shown that leptin treatment could improve glucose

metabolism aberrations independent of altering body weight. For instance, Pelleymounter et al. found that a low dose of leptin (0.1mg/kg/day) can normalize the serum glucose levels without lowering body weight in *ob/ob* mice [114]. Furthermore, researchers demonstrated that the acute disruption of leptin signaling using the polyethylene glycosylated mouse leptin antagonist could elevate hepatic glucose production and decrease whole-body insulin sensitivity without significantly altering the body composition in C57BL/6 mice [117]. Yu et al. reported that leptin could reverse hyperglycemia and ketosis by suppressing the action of glucagon on the liver and improving the utilization of glucose in the skeletal muscle in insulin-deficient diabetic rodents [118]. Leptin also can improve insulin sensitivity through both central and peripheral systems [119-121]. Morton et al. found that leptin-receptor gene therapy directed at the area of the hypothalamic arcuate nucleus improves glucose intolerance and insulin sensitivity via the phosphatidylinositol-3-OH kinase signaling pathway [122]. These studies collectively indicated that perturbances in the leptin signaling pathway might be crucial for the development of type 2 diabetes.

Leptin is highly homologous among different species. For instance, human leptin is 83% ~84% identical to rodents' leptin. Meanwhile, the basic structural features and intracellular signaling mechanisms of leptin and its receptor also appear to be conserved throughout vertebrates [123]. Several studies reported that administering exogenous leptin in fish reduces food intake [124-126], which has also been shown in *ob/ob* and diet-induced obsess mice [127], indicating the conservation of the leptin signaling system's function throughout vertebrates.

Olsen et al. demonstrated that intraperitoneal streptozotocin injection could induce hyperglycemia in adult zebrafish; these streptozotocin-induced diabetic zebrafish exhibited thickening of GBM in the kidney [128]. Anti-diabetic drugs effective for diabetic patients could ameliorate the hyperglycemia in the overfed zebrafish, suggesting that the glucose homeostasis pathways are conserved between zebrafish and human [129]. In zebrafish, there are two paralogous genes encoding leptin, called *lepa* and *lepb* [130]. In a previous study, our group found that the *lepb* gene, but not the *lepa* gene, is significantly downregulated in zebrafish larvae under an insulin-resistance state caused by acute hyperinsulinemia, suggesting the lepb might play a crucial role in insulin homeostasis in zebrafish [131].

Nephropathic Cystinosis

Cystinosis is a genetic disorder that follows an autosomal recessive inheritance pattern, and it belongs to the group of lysosomal storage disease disorders [132]. The incidence of cystinosis is 1/100,000 to 1/200,000 in live births. It is caused by deleterious mutations in the *CTNS* gene

encoding for cystinosin protein, which has an indispensable role in transporting cystine out of the lysosomes [133]. Defective cystine function results in the intralysosomal accumulation of cystine, which causes cell damage and organ dysfunction. The kidney, eye, muscle, liver, endocrine tissues, reproductive system and central nervous system all can be affected by cystinosis. Nephropathic cystinosis is the foremost clinical characteristic of cystinosis, known as renal Fanconi syndrome [134]. The inadequate reabsorption in the renal proximal tubule leads to leaking abnormal amounts of glucose, uric acid, amino acid, bicarbonate and phosphate into the urine. The proximal tubular damage can further progress to progressive glomerular damage and end-stage renal failure, eventually requiring renal replacement treatment.

Until now, the underlying mechanisms of nephropathic cystinosis remain unclear. An appropriate animal model resembling the features of human nephropathic cystinosis is crucial to understand this disease. Histopathologic features of human nephropathic cystinosis are: 1) Light microscopy: cystine crystal deposition in glomerular and tubular epithelial cells, and the interstitium; multinucleated glomerular and tubular epithelial cells, particularly involving podocytes; the unique "swan-neck" deformity in the proximal tubule. 2) Electron microscopy: cystine crystals (hexagonal, rhombohedral, or polymorphous in shape) within the interstitial macrophage and tubular epithelial cytoplasm [135]. Different animal models have been developed to recapitulate the features of human nephropathic cystinosis. The first *Ctns-/-* mouse model was generated in a mixed 129Sv x C57BL/6 strain [136]. In this *Ctns-/-* mouse model, cystine accumulation was found in kidney, liver, and muscle. However, these mice failed to develop renal proximal tubulopathy and glomerular dysfunction. Nevo and colleagues found that *Ctns-/-*mice with C57BL/6 background had an accumulation of cystine in tissues and pronounced histological renal lesions in the proximal tubules, and eventually developed chronic renal failure [137]. Recently, Shimizu et al. established a novel congenic Ctnsugl mutation on the F344 standard rat strain [138]. This Ctns mutant rat presented with renal lesions and cystine accumulation in renal tissue. Moreover, the cystine crystals in the lysosomes of the renal cortex were observed. These murine models are beneficial in revealing the pathogenic aspects of nephropathic cystinosis. However, murine models are usually time-consuming, expensive, and limited to a small number of experimental subjects. Therefore, generating less time- and cost-consuming animal models would be helpful to investigate the underlying molecular mechanism and find novel treatments for nephropathic cystinosis. In our previous study, we generated *ctns* mutant zebrafish which displayed glomerular and tubular dysfunction at the larvae stage and had significantly increased cystine levels in the kidney at the adult stage [139].

Animal models for chronic kidney disease

The best model to study human diseases is man itself. Since most experiments cannot ethically and practically be carried out on humans, animal models are extensively used as a representative alternative. Animal models are essential components of medical research because they provide the opportunity to understand the underlying mechanisms of diseases and assess potential novel therapeutic targets before testing in humans. Utilization of the datasets and resources from both human and animal models can lead to a better understanding of human diseases, including chronic kidney disease. A good animal disease model should meet several conditions. For instance, it has representative features of the particular human disease, is time-effective and cost-effective, and is easy to handle. Besides, the availability of molecular and genetic tools for this animal model also should be considered. Different animal models facilitate studying various aspects of a given disease since no perfect animal model for each disease exists. Choosing an optimal animal model is highly dependent on the specific hypotheses of the research.

Lacking reliable preclinical models is the major obstacle in dissecting the pathogenesis of disease and developing novel therapies for chronic kidney disease. Hence, developing new animal models of chronic kidney disease remains highly needed. Nowadays, murine models have been most widely used in studying chronic kidney disease. Since zebrafish are becoming a promising disease model [140], in this thesis, we investigate whether zebrafish can be used as a disease model for chronic kidney disease.

Murine models

The genome's similarity to the human genome (99% of human and mouse genomes are conserved) makes murine models extensively used in modelling human diseases for decades [141]. Human and murine have many similar aspects, including anatomy, cell biology and physiology. The genetics and biology in murine models are well known, and numerous diverse tools for studying the murine models have been established. It is also possible to replace a particular mouse gene with its human counterpart, which allows the specific transgenic mouse to produce the human version of the protein [142].

To date, the murine models are widely used to study the genetics and pathophysiology of chronic kidney disease (**Figure. 4A-4A"**) [143]. For instance, a diabetic murine model can nicely mimic many features of human diabetic nephropathy[144]. The albuminuria, serum creatinine, and creatinine clearance can be measured in those murine models. Based on the latest criteria published by the nephropathy subcommittee of the Animal Models of Diabetic Complications

Consortium, desirable rodent models of diabetic nephropathy should meet the following criteria: 1) The decline in GFR is greater than 50%. 2) The increase in albuminuria compared with controls for that strain at the same age and gender is greater than 10-fold. 3) Pathological features in the kidneys, advanced mesangial matrix expansion (with or without nodular sclerosis), arteriolar hyalinosis, tubulointerstitial fibrosis, podocyte loss, and demonstration of thickening of GBM by electron microscopy [145-147].

However, the promising findings in murine models cannot always effectively translate into clinical trials [148]. Hence, developing new animal models of chronic kidney disease remains highly needed.

Zebrafish model

In 1930, zebrafish (*Danio rerio*) were used as a classical developmental and embryological model due to their rapid development and optical clarity of the embryos and larvae. Nowadays, zebrafish are gaining popularity for modelling several human diseases. Approximately 70% of human genes have orthologs in zebrafish [149]. Furthermore, a lot of critical molecular pathways are highly conserved between humans and zebrafish [150]. Therefore, zebrafish can theoretically be utilized to understand the pathogenic mechanisms of many human diseases. Zebrafish eggs are laid and fertilized externally. Therefore, they can be efficiently manipulated with various gene-editing tools. For instance, microinjection of early fertilized embryos with antisense morpholino or mRNA to change gene expression, or using CRISPR/Cas9 approach to generate knock-out or knock-in zebrafish [151]. Besides, zebrafish have a high fecundity; 50~300 eggs can be produced at a time [152]. Lower requests on space and cost for maintenance are the advantages of zebrafish compared to murine models. Another advantage of zebrafish is that they are ideal for large-scale genetic and therapeutic screening, which is limited in murine models. Naturally, mammalians can form three distinct excretory organs during renal development, called pronephros, mesonephros and metanephros. Like other fish, zebrafish only develop the pronephros and mesonephros. However, the segmental anatomy of the nephron is conserved among vertebrates (**Figure. 4B-4B"**) [153], which offers the possibility to study kidney diseases in zebrafish. Until now, there are not many studies using adult zebrafish as an animal model for chronic kidney disease. Considering that zebrafish might help get insights into the mechanisms and potential therapies for chronic kidney disease, a better understanding of the renal pathology of adult zebrafish is required.

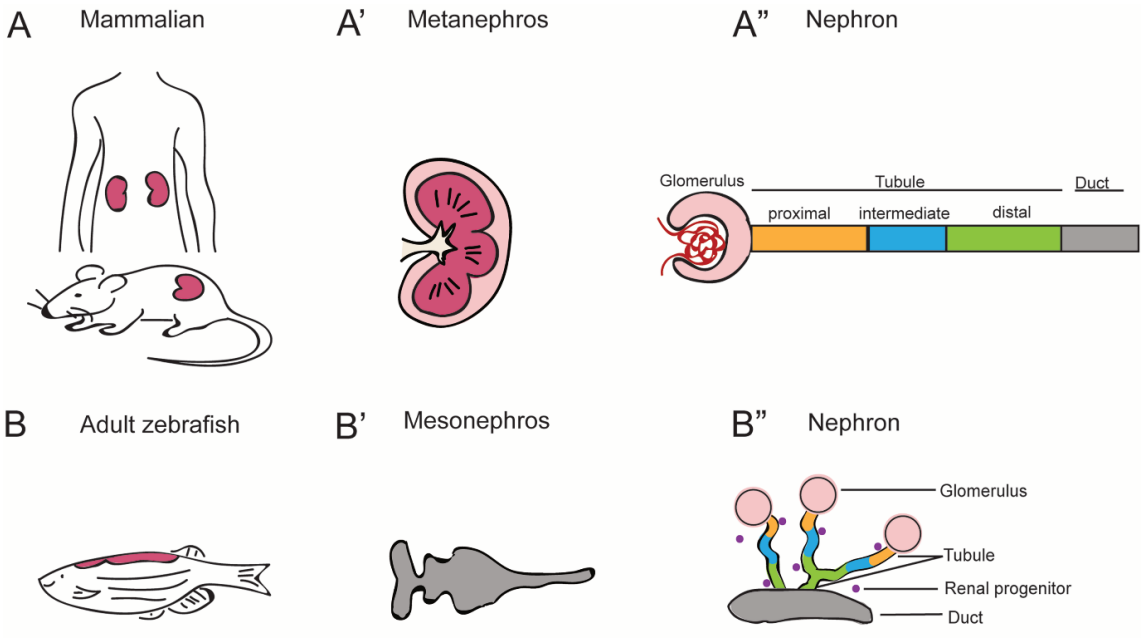

Figure 4. The segmental composition of the nephron is conserved between vertebrates

Aims and outline of this thesis

The aim of this thesis is to evaluate the role of protective factors in the development of diabetic nephropathy both in clinical studies and in animal models. Understanding the role of these factors is vital for developing prevention and treatment strategies for diabetic nephropathy. Moreover, generating good animal models could provide the opportunity to understand the underlying mechanisms of chronic kidney diseases and assess potential novel therapeutic targets before clinical testing in humans. We are the first who investigate whether the *lepb* mutant and *ctns* mutant adult zebrafish model can be used for chronic kidney disease studies.

Clusterin, a glycoprotein, is upregulated in the glomeruli of patients with various forms of kidney disease. Using microarray analysis, our group found that glomerular *CLU* mRNA expression is significantly higher in diabetic nephropathy patients than in healthy individuals [69]. Several *in vitro* studies have demonstrated that clusterin could protect against oxidative stress-induced apoptosis. Therefore, we hypothesized that increased glomerular clusterin might protect podocytes against oxidative stress-induced damage in diabetic nephropathy. In the study described in **Chapter 2**, we examined glomerular clusterin expression in both a large cohort of patients with diabetic nephropathy and a diabetic mouse model. Also, we examined the regulation of clusterin expression in a human podocyte cell line cultured under various diabetic conditions. Finally, we investigated whether the recombinant clusterin protein can protect the podocytes against oxidative stress *in vitro*.

Carnosinase is an enzyme that hydrolyses carnosine. Previous studies have revealed an association between the variants of the *CNDP1* (*CN1*) gene and the susceptibility for diabetic nephropathy. The supplementation of carnosine has been shown to protect against the progression of diabetic nephropathy in different diabetic animal models. Compared to non-transgenic *db/db* mice, hCN1 transgenic *db/db* mice have higher human carnosinase-1 levels and lower L-carnosine levels in the serum, and significant renal hypertrophy. A higher carnosine content in the circulation was also found in the animals when physically active. Based on these findings, we hypothesized that 1) overexpressing the hCN1 or 2) chronic aerobic exercise training would have effects on the development of diabetic nephropathy. BTBR *ob/ob* mice develop pronounced diabetic nephropathy rapidly, compared to *db/db* mice [154]. In the study described in **Chapter 3,** we investigated the impact of these two interventions on the development of diabetic nephropathy in BTBR *ob/ob* mice.

Leptin is a hormone, which functions in the regulation of energy homeostasis via suppression of appetite. In humans, patients lacking leptin have evident obesity and glucose tolerance impairments, the hallmarks of type 2 diabetes. In a previous study, our group found that the *lepb* gene, *but* not the *lepa gene*, is significantly downregulated in zebrafish larvae in an insulin-resistance state caused by acute hyperinsulinemia, suggesting that *lepb* might play a crucial role in insulin homeostasis in zebrafish [131]. Based on mouse studies of leptin- or leptin receptor-deficient mice, we hypothesized that *lepb*-deficient zebrafish develop the pathogenic features of diabetes and diabetic nephropathy. In the study described in **Chapter 4**, we investigated whether deletion of *lepb* via CRISPR-CAS9 techniques influences the glucose homeostasis and adiposity in zebrafish and whether *lepb* deficiency contributes to the development of type 2 diabetes and diabetic nephropathy in adult zebrafish.

Cystinosis is a rare and incurable lysosomal storage disease. Mutations in the *CTNS* gene encoding for cystinosin cause defective function in lysosomal cystine transport. Some cystinosis murine models have been regenerated; however, no one resembles all the features of human nephropathic cystinosis. A *ctns* mutant zebrafish displayed glomerular and tubular dysfunction at the larvae stage and had significantly increased cystine levels in the kidney at the adult stage [139]. We hypothesized that the renal histopathological features of *ctns* mutant adult zebrafish resemble that of human nephropathic cystinosis. In the study described in **Chapter 5**, we characterized the *ctns* mutant adult zebrafish' renal features and compared them with the age-matched control adult zebrafish by performing histopathological examinations, including HE and PAS staining, immunohistochemistry, toluidine blue staining and transmission electron microscopy.

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