Pathways to proteinuria

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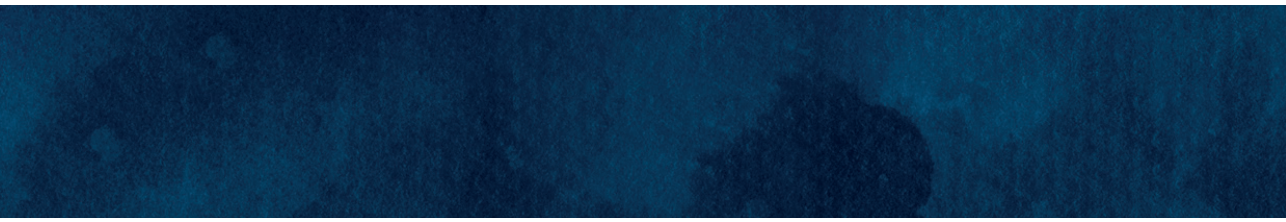

CHAPTER 1

General introduction and outline

Proteinuria and chronic kidney disease

Proteinuria is an independent predictor of the progression of kidney injury, cardiovascular morbidity, and overall mortality (1). Under physiological circumstances, no sustained proteinuria is present. Proteinuria only occurs when serum proteins are able to pass the glomerular filtration barrier and when tubular reabsorption mechanisms are saturated or fail. Damage to any of these components can result in proteinuria. Moreover, although the mechanisms of damage and disease vary across the different types of renal diseases, many still lead to proteinuria and can result in chronic kidney disease (CKD). CKD is classified according to the remaining glomerular filtration rate and the amount of proteinuria in the Kidney Disease Improving Global Outcomes (KDIGO) guidelines (2).

The prevalence of CKD is expected to rise further due to an aging population. The 2019 'Global burden of disease study' reports that chronic kidney disease rose from rank 29 to 18 between 1990 and 2019 as a cause for disease-adjusted life years (3). In the Netherlands, an estimated 12% of the population suffers from chronic kidney disease (CBS/nierstichting). Treatment of underlying disease and general cardiovascular risk management such as treating hypertension and dyslipidaemia are still the cornerstone of management of chronic kidney disease (CKD) and attenuating proteinuria. A specific treatment for proteinuria might become feasible when the pathways leading to proteinuria are elucidated further. For this, a closer investigation of the structures and mechanisms that normally prevent proteinuria from occurring is required.

The glomerular filtration barrier

Figure 1 shows an overview of human renal anatomy with each image going into more detail. Most humans have two kidneys as seen in the top left image. They are located in the retroperitoneal space. The parenchyma of the kidney is usually divided in the outer cortex and inner medulla. Each human kidney contains around one million functional units called nephrons. Each nephron consists of a glomerulus and a tubular apparatus, shown in the detail of the sagittal image of the human kidney. The glomerulus, seen in the top right, is a specialized capillary bed that starts with the afferent arteriole which comes from the renal artery, which in turn directly sprouts from the abdominal aorta. 20 to 25% of cardiac output is routed to the kidneys, where it first passes the glomerulus. There, around 20% of passing serum is filtered by passing the glomerular filtration barrier, and the other 80% continues through the efferent arteriole to the peritubular capillaries where both active and passive secretion and reabsorption take place. The entire glomerular capillary bed is lined by the glomerular filtration barrier, shown in the middle image. Below, the detail shows a schematic overview of the distinct structures

of the glomerular filtration barrier. The glomerular filtration barrier is made up of the glomerular endothelial glycocalyx layer (purple), fenestrated endothelial cells (red), glomerular basement membrane (grey), and the visceral epithelial cells (yellow) – or podocytes – with interdigitating foot processes (green).

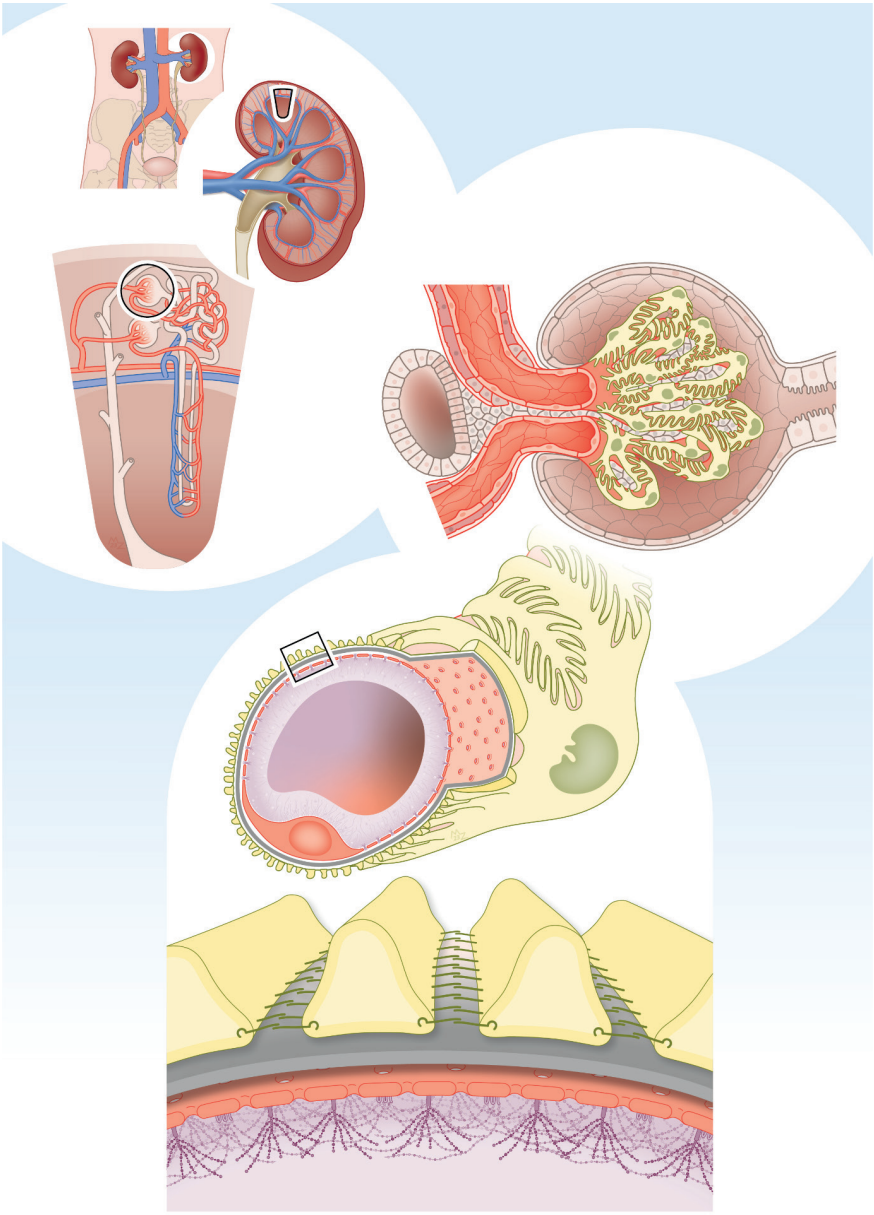

Figure 1. The human kidney, nephron, glomerulus, glomerular capillary, and glomerular filtration barrier

Glycocalyx and fenestrated endothelial cell

The first layer of the glomerular filtration barrier is the glycocalyx. As the name implies, it consists mostly of various 'sugary' chains, which consist of negatively charged heparan sulphate glycosaminoglycan chains attached to heparan sulphate core proteins and hyaluronan. The glycocalyx has a role in mitigating inflammation, and coagulation.(4) The glycocalyx lines the specialized endothelial cells of the glomerulus. These endothelial cells have the distinguishing feature of being fenestrated with 60 nm pores. The negatively charged glycocalyx covering these pores is considered to be the first barrier between the vascular lumen and the ultrafiltrate that serum proteins such as albumin encounter. In end-stage renal disease, damage to the structural integrity and composition of the glycocalyx is observed.(5) Also, in experimental models where damage to the glycocalyx is induced, proteinuria occurs. As such, it is thought to be a vital part of the glomerular filtration barrier in the protection against proteinuria.

Glomerular basement membrane

The next layer is the glomerular basement membrane (GBM). It is an extracellular matrix that is proximally deposited by the glomerular endothelial cells and the visceral epithelial cell on the distal end. On electron microscopy, three GBM layers can be distinguished. These are the lamina rara interna on the vascular side, the lamina densa, and the lamina rara externa adjacent to the epithelial side. The GBM mainly contains laminin, collagen type IV, and heparan sulphate proteoglycans. It normally has a thickness between 300 and 400 nm. The GBM has an overall negative charge due to the sulphated glycosaminoglycan chains of the heparan sulphate proteoglycan aggregates.

Heparan sulphate glycosaminoglycans

Glomerular filtration occurs with both size and charge selectivity (6). Maintaining this selectivity and GFB integrity has long been attributed to heparan sulphate glycosaminoglycans (7, 8). As stated above, they are localized in both the glycocalyx and GBM. Heparan sulphate glycosaminoglycan chains consist of repeating disaccharide motifs that contain a uronic acid and a glucosamine derivative. Theoretically, up to 48 different motifs can be formed. Heparan sulphate synthesis starts with chain initiation, where a tetrasaccharide linkage region is formed and is covalently bound to a core protein. The next phase of HS synthesis consists of chain polymerization, which is dependent on enzymes encoded by the *EXT1* and *EXT2* genes. These enzymes form a co-polymerase that adds repeating disaccharide units consisting of D-glucuronic acid (GlcAβ4) and *N*-acetyl-glucosamine (GlcNAcα4). During chain polymerization, the polymer is also modified

by various sulfotransferases and an epimerase leading to GlcNAc N-deacetylation/N-sulfation, epimerization of GlcA to L-iduronic acid (IdoA), and 2, 3, and 6-O-sulfation. These modifications not only result in an overall negative charge, but also the formation of ligand binding sites, such as FGF-2, antithrombin, chemokines, and cytokines. Thus, when chain polymerization is impaired due to a lack of EXT1 or EXT2, no modification can take place and hence the heparan sulphate is functionally impaired.(9)

Podocyte and slit diaphragm

Lastly, the distal end of the GFB is covered by visceral epithelial cells or podocytes. Podocytes gained their name from the Greek words podos (ποδος), which means foot, and kutos (κύτος), meaning jar or vessel, and used as a term to describe cells. One of the characteristics of podocytes is the presence of interdigitating foot processes called pedicles. The remaining space between these processes creates so-called filtration pores or slit diaphragms. The diaphragm is not an open connection to Bowman's space. Adjacent foot processes are connected by nephrin (*NPHS1*) and NEPH1 (*KIRREL1*).(10) These proteins connect adjacent foot processes by spanning the slit diaphragm and attaching to the actin cytoskeleton of podocytes.

The actin cytoskeleton and dynamin

When podocytes are damaged, loss of architectural organization of the cytoskeleton occurs, which leads to retraction and effacement of the podocyte and its foot processes. The pathways and proteins responsible for podocyte cytoskeletal organization are steadily being uncovered. One of these proteins is dynamin, a small GTPase that is primarily known for its role clathrin-coated vesicle budding in neurons. It has now been described to be involved in the turnover of nephrin, regulation of actin, and endocytosis of albumin by podocytes, making it a potential future therapeutical target for preventing proteinuria. (11-14)

Tubular reabsorption mechanisms

After passing the glomerular filtration barrier, the ultrafiltrate enters Bowman's space. Afterwards, the ultrafiltrate passes various parts of the tubule, where its content is altered through re-absorption and secretion which occur in both active and passive manners. Normally, when serum proteins such as albumin are indeed able to pass the glomerular filtration barrier, they are re-absorbed by proximal tubular epithelial cells. Tubular reabsorption mechanisms are not only responsible for the re-absorption of filtered protein, but also that of for example urea, bicarbonate, phosphate, glucose, and

sodium. The first part of ultrafiltrate is isotonic to serum, and as such, active processes are required to reabsorb these solutes. Hence, the proximal tubule epithelial cells possess many mitochondria to facilitate these active processes. On the tubular luminal side, these cells are lined with microvilli which form the brush border, which significantly increases the luminal surface area. It is here that solutes and colloids are reabsorbed. Some groups have provided evidence that albumin does in fact pass the glomerular filtration barrier in much greater quantities than previously thought. They claim that tubular reabsorption mechanisms thus play a larger role in preventing proteinuria than has been attributed to them before.(15) Although the relative contribution to preventing proteinuria is controversial, there is a general consensus that adequate tubular reabsorption mechanisms are required to prevent, at least in part, the loss of protein in the urine. A complete loss of tubular reabsorption function results in Fanconi syndrome. This syndrome is characterized by proteinuria and severe acid-base and electrolyte disorders. In children, the most common cause of Fanconi syndrome is nephropathic cystinosis. Nephropathic cystinosis is a lysosomal storage disorder that results in cystine crystal deposition in various tissues. The proximal tubule is usually the first affected site.

As stated above, pathological processes in any one of the GFB layers can lead to proteinuria. Although this has long been attributed to the individually affected layer, it is now deemed more likely that the glomerular filtration barrier functions as a whole unit and requires all components to properly function. Moreover, the GFB can be seen as a dynamic barrier with various repair and compensation mechanisms rather than a static barrier that only sieves particles based on size, weight, and hydrostatic pressure.

Identifying new targets

Identifying which components of the glomerular filtration barrier are needed to maintain proper barrier function is essential to identifying potential therapeutic options. Historically, the function of the various components of the glomerular filtration barrier has been discovered in patients with genetic defects leading to proteinuria.(16) More recently, experimental animal models have been used to identify genes and their encoded products that might be important in GFB function. For example, genetic association studies in proteinuric rats have identified a panel of various genes potentially involved in the development of proteinuria.(17) After identifying these genes in a previously described array, the candidate genes and their encoded proteins were then investigated further to establish whether a direct relation to the development of proteinuria is present. In multiple studies presented here, the method to examine whether a protein has a significant role in the development of proteinuria consisted of assessing whether knocking down mRNA

translation resulted in proteinuria in a zebrafish embryo model. Next, differential mRNA and protein expression are investigated in spontaneously proteinuric rats. In this step, the temporal relationship between the onset of proteinuria and relative loss of expression is assessed. Furthermore, the translation to human proteinuric kidney disease is made by examining the protein expression of the investigated targets in kidney biopsies from patients with proteinuric renal diseases.

These methods were used both to investigate known proteins, such as dynamin, but has also uncovered previously unknown proteins to be involved in the development of proteinuria, such as transmembrane protein 14A.

Aim and outline of the thesis

In this thesis, the pathways leading to proteinuria are explored. To identify potential pathways, elements considered essential are revisited, known pathways are explored further, and new players in the field of proteinuria are identified.

First, a zebrafish embryo model to assess both glomerular filtration barrier function and tubular reabsorption mechanisms is presented in **Chapter 2**. The use of this model for developing new therapeutic options for the rare but devastating disease of nephropathic cystinosis is presented.

In **Chapters 3** and **4**, the loss of heparan sulphate glycosaminoglycans is investigated. Heparan sulphate glycosaminoglycans have long been considered essential for adequate glomerular filtration function. In **Chapter 3,** a global heparan sulphate glycosaminoglycan deficiency on the development of proteinuria was shown to not affect glomerular filtration barrier function nor tubular reabsorption mechanisms. The used *dackel* zebrafish embryo mutant has a biallelic germline mutation in the zebrafish homologue of *EXT2*, resulting in truncated and functionally impaired heparan sulphate glycosaminoglycan chains. **Chapter 4** continues with investigating the loss of heparan sulphate glycosaminoglycans in multiple osteochondroma patients, who have a heterozygous mutation in either EXT1 or EXT2. Here, no proteinuria, a specific renal phenotype, or changes to the glomerular endothelial glycocalyx were observed.

In **Chapter 5**, the role of dynamin in proteinuric conditions and human disease is explored. Dynamin has been identified to play an important role in maintaining glomerular filtration barrier structure and function. In this chapter, this role is further specified as a dynamically regulated protective mechanism against the development of proteinuria.

A previously unknown factor in the development of proteinuria is transmembrane protein 14A, which is discussed in **Chapter 6**. It is described as a protective element in the development of proteinuria through experiments in cell culture, a zebrafish embryo model, proteinuric rats, and human proteinuric kidney diseases.

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