# Pathways to proteinuria

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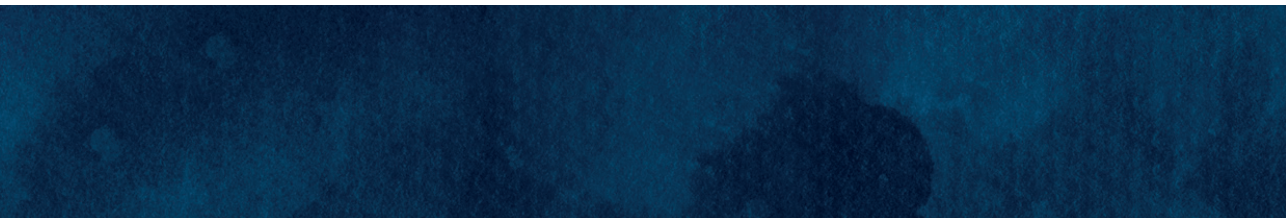

# CHAPTER 7

## General discussion and future perspectives

Patients with proteinuria can suffer from a myriad of diseases that cause protein to pass the glomerular filtration barrier and being insufficiently reabsorbed by the proximal tubule apparatus. These diseases include those that are histopathologically characterized by scarring and fibrosis of glomeruli, such as both primary and secondary focal segmental glomerulosclerosis, and diabetic nephropathy. Other types of diseases leading to proteinuria could be classified as auto-immune mediated, such as lupus nephritis and membraneous nephropathy. Moreover, many monogenetic diseases that cause proteinuria have been identified. Most of these display either a podocytopathy or a defect in tubular reabsorption. Although the underlying pathophysiological mechanisms differ between all of these diseases, they can share some elements in their respective pathways leading to proteinuria. As proteinuria is an independent risk factor for the progression of renal disease, cardiovascular morbidity, and overall mortality, treatments attenuating or relieving proteinuria are needed. Current treatment is mainly focused on the underlying disease and consists of reducing glomerular filtration pressure through inhibition of the renin-angiotensin-aldosterone system and, depending on whether an auto-immune or auto-inflammatory disease is involved, the addition of immunosuppressive drugs such as corticosteroids.

Elucidating the pathways leading to proteinuria is required to identify novel potential therapeutic targets for the treatment of proteinuria. Historically, the main constituents of the glomerular filtration barrier were identified through analysis of hereditary proteinuria syndromes, as also reviewed by Tryggvason *et al.*(16) For example, the slit diaphragm proteins of Nephrin (*NPHS1*) and Podocin (*NPHS2*), glomerular basement membrane protein Laminin (*LAMB2*), and transcription factors that influence podocyte gene expression (*WT1* and *ACTN4)* were all identified by investigation of monogenetic proteinuric diseases.

As eloquently said by Iain Drummond: 'unravelling the molecular pathogenesis of human disease presents many experimental challenges, not the least of which is that experiments on humans are generally frowned upon.(105)' Although experimentation on animals is also increasingly frowned upon and must rightfully adhere to rigorous ethical standards, it is currently still an indispensable element of pathophysiological research. In this thesis, a combination of cell culture, experimental animal models, histopathological examination of human tissue, and a patient cohort investigation were all employed to investigate pathways leading to proteinuria.

## Nephropathic cystinosis

In chapter 2, an experimental zebrafish embryo model for the autosomal recessive disease of nephropathic cystinosis is introduced. Nephropathic cystinosis is a lysosomal storage disease where the *CTNS* gene is mutated, which leads to the accumulation of cystine in lysosomes. If left untreated, the disease is fatal.(106, 107) Currently, specific treatment is limited to cysteamine, which prevents further cystine accumulation but does not reverse the damage. Moreover, drug compliance is relatively low due to adverse effects of bad breath, skin odour, gastro-intestinal complaints such as nausea, vomiting, diarrhoea, and abdominal pain.(108-110) A *Ctns* knockout mouse has been developed, but this model lacks the glomerular changes also seen in nephropathic cystinosis.(111-113) The *ctns* -/- zebrafish mutant introduced in this study is presented as a promising model for the investigation of new therapeutic options and the pathophysiology of nephropathic cystinosis. The model displays a phenotype similar to that of the human disease, including cystine accumulation, increased glomerular permeability, and decreased proximal tubular reabsorption. They have higher mortality than wild-type animals. These last symptoms were preventable by treating mutant embryos with cysteamine. Renal and extrarenal manifestations of cystinosis have also been described in the adult model of this mutant.(114) The zebrafish cystinosis model has already been used to test novel treatment strategies for nephropathic cystinosis, such as luteolin, disulfiram, and bicalutamide-cysteamine.(115-118)



## Heparan sulphate glycosaminoglycans

Chapters 3 and 4 discuss the previously held paradigm that heparan sulphate glycosaminoglycans are essential to glomerular filtration barrier function. This hypothesis was formulated by Kanwar and Farquhar several decades ago and was based on the finding that enzymatic removal of HS-GAG resulted in the loss of GFB integrity.(7, 8) Also, HS-GAG expression has been found to be reduced in various proteinuric renal diseases.(22) However, based on the results presented in chapters 3 and 4, homozygous germline mutations in zebrafish and, respectively, heterozygous mutations in humans of HS backbone elongating enzymes are shown not to result in proteinuria, nor a renal phenotype. The role of HS-GAG has long been thought to provide the GFB its charge selectivity due to the negatively charged sulphate groups of heparan sulphate. In chapter 3, we show that a significant reduction in negatively charged sites in the glomerular basement membrane does not result in proteinuria. Results from other experimental animal models with HS-GAG deficiencies are in line with this notion.(23-25, 27, 119) In chapter 4, the effect of heterozygous germline mutations on the backbone elongating

enzymes of heparan sulphate glycosaminoglycans was investigated in patients with multiple osteochondromas. Multiple osteochondroma is an autosomal dominant disease caused by a mutation in either EXT1 or EXT2 leading to the formation of, as the name implies, multiple osteochondromas.(59, 60, 69) We investigated a cohort of multiple osteochondroma patients in a cross-sectional manner and found that they did not exhibit proteinuria or an altered endothelial glycocalyx. Also, we investigated a historic cohort of patients who had both an osteochondroma resection and kidney biopsy in their medical history. Upon re-examination of the slides, no specific glomerular morphological changes were observed. One patient did show a glomerular phenotype on electron microscopy similar to that of a described case of 'MO glomerulopathy' with focal fibril deposition. (56) The rare cases of MO glomerulopathy are hypothesized to be caused by local loss of heterozygosity.

In conclusion, the results from these studies support the growing body of evidence that loss of heparan sulphate glycosaminoglycans does not result in loss of glomerular filtration barrier integrity, despite resulting in loss of negatively charged sites.

## Dynamin and GTPases

One of the most promising potential therapeutic targets for the treatment of proteinuria is dynamin. Dynamin is known for its role clathrin-mediated endocytosis and synapse junction vesicle budding. Dynamin is a GTPase that forms a helical polymer around the neck of budding vesicles and causes membrane scission (120). In the kidney, it has been identified to be involved in the turnover of nephrin, direct interaction with actin and actin-regulatory proteins, and the endocytosis of albumin by podocytes. (11, 12, 14, 74) Its function depends on its oligomerization state and on whether it is cleaved by cathepsin L. (13, 14, 87, 121, 122) Schiffer *et al.* and Ono *et al.* demonstrated the potential of dynamin as a therapeutic target by treating several proteinuric animal models with Bis-T-23, which stimulates dynamin oligomerization. After administration, proteinuria decreased and the ultrastructure of podocyte foot processes was restored.(75, 76) In chapter 5, we show that glomerular dynamin mRNA expression increases before the onset of proteinuria and that both Dynamin and Cathepsin L protein expression is increased in proteinuric patients with various different underlying diseases. These results further support the suggested protective and dynamic role of dynamin in preventing the development of proteinuria through its interaction with the actin cytoskeleton and nephrin before the onset of proteinuria. As this mechanism also seems to play a role in proteinuric patients, this study further propagates the concept that dynamin and its regulation are potential therapeutic targets for the treatment of proteinuria.

Podocyte actin cytoskeletal regulation not only depends on dynamin, which is classed as a large GTPase, but also on the Rho-family of small GTPases like RhoA, Cdc42, and Rac1. They are involved in podocyte foot process motility and junctional and cytoskeletal interactions. Imbalances to the Rho GTPases are described to result in either hypo- or hypermobility of foot processes which both result in the progression of podocytopathy. (123) Rho GTPase signaling can be influenced by circulation factors such as soluble urokinase-type plasminogen activator receptor (suPAR), which activates Rac1. Inhibiting suPAR has been shown to inhibit podocyte injury *in vitro*. (124)

The results described in this thesis, combined with other literature on actin cytoskeleton regulation, expand the understanding that the GFB is not the static barrier it was once presumed to be, but rather an intricate apparatus that is dynamically regulated depending on local circumstances and circulating factors.

## Transmembrane protein 14A

In chapter 6, transmembrane protein 14A (TMEM14A) is reported as another important protein in the preservation of adequate GFB function and integrity. It was previously implied to be involved in suppressing Bax mediated apoptosis.(95) Other than that, TMEM14A is a relatively unknown protein. Here, we identified it to be involved in the development of proteinuria by examining the results of a microarray study in spontaneously proteinuric Dahl SS rats. There, it was found to be significantly downregulated compared to spontaneously hypertensive, non proteinuric rats. To establish whether TMEM14A plays a direct and essential role in the development of proteinuria, a zebrafish embryo knockdown model was utilized. Results from this study shows that knocking down TMEM14A translation results in loss of GFB integrity without affecting tubular reabsorption capacity. Next, we show that both mRNA and protein expression of TMEM14A is reduced before onset of proteinuria. This study also reveals that glomerular TMEM14A expression is increased in proteinuric kidney disease, except in diabetic nephropathy. This result corresponds with *in vitro* findings, where inducing podocyte damage also increases TMEM14A expression. A protective mechanism by TMEM14A is proposed with a potential action mechanism through inhibiting podocyte apoptosis. Further studies are required to assess whether this is indeed the case. It would be of particular interest to identify up- and downstream modulators of TMEM14A expression and function.

## Zebrafish embryo model

Zebrafish (*Danio rerio*) are freshwater fish originally from Southern Asia. They have become a widespread scientific model for the investigation of various pathophysiological

processes, including renal physiology. They are even part of the aquatic habitat on the International Space Station and are one of the few vertebrates to have lived a full life cycle in space.(125)

In chapters 2, 3, 5, and 6, an experimental zebrafish (*Danio rerio*) embryo model is used to assess whether knocking down mRNA translation of a single gene results in the development of proteinuria and whether tubular reabsorption mechanisms remain intact. Using this model presents several advantages compared to other experimental animal models. First, zebrafish embryos develop rapidly. Most major organs are formed within 40 hours post-fertilization. Due to their mostly transparent appearance, this development can be visualized relatively easily. Secondly, a single pair of adult zebrafish can lay over 200 eggs. Thus, in controlled conditions, it is possible to create high throughput models. The zebrafish embryo kidney consists of a pronephros with two nephrons that share a fused glomerulus in the midline of the body. Despite its simple structure compared to the more complex human metanephros, the zebrafish kidney shares many similar features with the kidneys of higher vertebrates and as such, is increasingly used as an experimental model for the study of cellular and molecular mechanisms of renal pathophysiology.(105, 126) Because of these characteristics, these animals are highly suited for investigating individual components of the pathways leading to proteinuria. (39, 40, 43, 82, 105, 126, 127)

In chapters 2 and 3, genetically mutated zebrafish were used as experimental models. In chapters 5 and 6, gene knockdown was effectuated by injecting zebrafish embryos with morpholino constructs. These constructs bind to mRNA and thus inhibit translation, leading to a functional knockdown of the targeted gene and its mRNA. In all these models, functional assays of glomerular filtration barrier integrity and tubular reabsorption were assessed by injecting a mixture of TRITC-labelled 3 kDa and FITC-labelled 70-kDa dextrans. As 3 kDa dextrans can freely pass the glomerular filtration barrier, they are reabsorbed in endosomes in the proximal tubule under physiological conditions. On the other hand, 70 kDa dextrans do not readily pass the GFB and as such, are only reabsorbed when GFB integrity is compromised. Thus, the presence of 3 kDa droplets was used to assess whether tubular reabsorption mechanism functions properly. The presence of 70 kDa was used to assess the loss of GFB integrity. This model was developed in these studies after adapting it from Hentschel *et al.* (40)

Other methods to assess GFB integrity in zebrafish embryos have also been developed by others. For example, a transgenic zebrafish expresses its main serum protein, vitamin D binding protein, bound with green fluorescent protein (EGFP). This model removes

the necessity to inject a dextran mixture but has no simultaneous assessment of tubular reabsorption function. With the introduction of this model, measuring the loss of fluorescence intensity in the zebrafish eye was also established as an indirect measurement of loss of GFB integrity. (45)

## Spontaneously proteinuric rat model

Laboratory rats (*Rattus norvegicus domestica)* are perhaps the most well-known experimental animal model, after mice. The first documented experiment on rats was performed in France back in 1856 and consisted of the examination of the effects of adrenalectomy.(128) Rats were also ahead of zebrafish regarding space travel, as they had joined Soviet space dogs Belka and Strelka aboard the Sputnik 5 in 1960. In this thesis, the spontaneously proteinuric Dahl salt-sensitive rat strain was compared with nonproteinuric spontaneously hypertensive rats in chapters 5 and 6. Although these two strains have similar blood pressure levels, the Dahl rats become progressively proteinuric as they age. The cause of early onset albuminuria in Dahl rats was previously found to be a polygenic trait.(79) In that study, genome-wide linkage, and quantitative trait loci (QTL) mapping analysis was performed. These QTLs were subsequently used to identify individual genes that are involved in the development of proteinuria. This was done by microarray analysis on purified Dahl and SHR glomeruli and comparing the differential regulation in time to the previously defined QTLs. Dynamin, which is discussed in chapter 5, was one of the cytoskeleton-related genes identified in this manner. TMEM14A was one of the most markedly downregulated genes in the comparison of relative expression prior to QTL correlation.



## Future perspectives

In conclusion, the work presented in this thesis adds to the knowledge of the pathways to proteinuria by both challenging the previously held tenet of a static filtration barrier and supporting the theories entailing a dynamically regulated interplay between the various layers of the glomerular filtration barrier in conjunction with the tubular reabsorption apparatus. As also reviewed by Comper *et al.*, the functionality of both the GFB and proximal tubular reabsorption seems to depend on whether proteinuric circumstances are present.(129) The expansion of comprehension of the pathophysiological mechanisms underlying pathways to proteinuria will be key to identifying new therapeutic targets. As described above, the novel zebrafish model of nephropathic cystinosis has already proven its worth for testing new therapeutic compounds whilst simultaneously offering new insights in the pathophysiology of cystinosis.

Regarding the role of negative charge and specifically, that of heparan sulphate glycosaminoglycans in the glomerular filtration barrier, it would be most interesting to investigate which proteins or circulating factors (next to the previously identified heparanase) influence its expression and degradation.(130, 131) Also, the changes in ligand binding ability of the glomerular glycocalyx might reveal more about the role of HS-GAG in maintaining GFB integrity.

Both large (dynamin) and small (Rho family) GTPases have already shown promise as therapeutic targets in preventing or even attenuating renal damage in proteinuric animal models. Compounds acting on these targets are yet to enter safety and efficacy testing for human trials but are an elegant example of translational medicine from a pathophysiological point of view. New potential targets, such as TMEM14A and its uncharted regulatory proteins, are being discovered at a high rate. As glomerular expression levels in human proteinuric kidneys in our TMEM14A experiments differed depending on etiology, it is conceivable that this particular pathway might not be of interest to all proteinuric diseases, but only a subset like diabetic nephropathy. It can be tentatively stated that the further identification of its protein-protein interactions including up- and downstream effects will reveal if this pathway to proteinuria is indeed a feasible therapeutic option.

The zebrafish experimental animal model has presented itself as an expedient model for both identifying and testing therapeutic targets. Further innovations in experimental animal models and especially in non-animal models such as organoids, will hopefully increase the rate of discovering potential targets and screening the effectiveness of therapeutic compounds. Thus, by further illuminating the pathways to proteinuria we hope to keep advancing the field towards targeted treatment of proteinuria for the benefit of our patients.

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