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IDENTIFICATION OF GENES AND PATHWAYS INVOLVED IN THE DEVELOPMENT AND PROGRESSION OF GLOMERULAR DISEASES

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Cover illustration: An insight into the glomerulus in health and disease, an illustration by Yana Dabaghie

IDENTIFICATION OF GENES AND PATHWAYS INVOLVED IN THE DEVELOPMENT AND PROGRESSION OF GLOMERULAR DISEASES

Thesis for Doctoral Degree (Ph.D.)

By

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To my family and loved ones

Mischief Managed

Popular science summary of the thesis

The kidneys are the filtration unit of the body. They are responsible for cleaning up the blood from waste products and excess water, regulation of blood pressure and production of hormones. In an adult human kidney, we have around 1 million tiny functional units called the nephrons. Each nephron is made of two parts: 1) A ball of tiny blood vessels called the glomerulus, where the blood gets filtered; 2) A long tube where the fluid flows called the tubule. We can imagine nephrons as a big urine production factory, made up of different departments and workers - in this case its different cell types. Each of them has its own function and structure but all of them need to communicate with each other to perform correctly.

In case of a disease, this balance is disturbed. Some of the cells can get injured, which make them stop functioning the right way and this leads to miscommunication which can end up with a chronic abnormality. This is what happens in chronic kidney disease (CKD), where the kidneys gradually lose their function. Due to the complicated nature of the kidney as an organ and of the disease itself, the treatment options for CKD are limited. Therefore, it is of a high importance to understand what happens in the diseased kidney to be able to find better treatment options and stop diseases from getting worse.

In order to produce functional units, we need a code that is present in form of genes in our DNA. This code is copied as instructions by RNA and taken to special translation systems to get the final functional units called the proteins. In the end different genes function in different sequential steps of a biological processes, which are known as genetic pathways.

In this thesis, we aimed to study what happens in the glomerulus in health and disease, through the study of RNA transcriptomes (through studying the instructions or what we call RNA fingerprints), with the goal of identifying which genes and pathways contribute to the disease progression.

In the first part of the thesis, we used a new technique, called single cell RNA- sequencing (scRNA-seq), where we can analyse the transcriptomes of each individual cell.

In **paper I** we studied transcriptomes in healthy conditions, in order to get a better understanding of the glomerulus in both mouse and human. We managed to capture rare cells, and to determine the specific codes for each different cell type. By knowing the code, we got new insights into how different cell types work and communicate in healthy kidney tissue. Moreover, we compared the different glomerular cells between mouse and human which gave us valuable information for future studies.

In **paper II** we studied a class of drugs called angiotensin converting enzyme inhibitors (ACEi). ACEi are usually the first drug to be prescribed for patients with CKD. We have treated mice that had glomerular damage with ACEi and studied what happens to transcriptomes under this drug therapy. We found out that one specific cell type, called mesangial cell, showed most changes under ACEi treatment. Mesangial cells are known to provide structural support for glomeruli by producing mesangial matrix (similar to pillars and blocks in a building). They also communicate with other cell types, all of which are disturbed in diseases. Treatment with ACEi attenuated this response in mesangial cells and decreased the activation of genes and pathways involved in matrix production and formation of fibrous tissue.

In the second part of the thesis, we looked in detail into some genes that we found interesting in previous transcriptome studies. We chose two genes (that we found to be highly produced by the glomerulus) for further studies: Retinoic acid receptor responder 1 (Rarres1) and Natriuretic peptide receptor 3 (NPR3). We studied what happens in the glomerulus in the presence and absence of these genes, as well as how they affect the progression of the disease.

In **paper III** Rarres1 was found to be increased in samples collected from patients with CKD. More specifically, Rarres1 was produced more by endothelial cells. Endothelial cells are the cells that line blood vessels and regulate the exchange between blood and surrounding tissue. Therefore, we developed genetically modified mice where Rarres1 was highly produced by endothelial cells and tested what happens in disease. After induction of kidney injury, these mice showed more glomerular damage. On the other hand, when we generated mice that lacked Rarres1 in endothelial cells, we saw an opposite effect: these mice were protected from glomerular damage. Further investigations connected Rarres1 to the activation of a specific pathway called NFκB. This pathway regulates important cellular functions such as cell growth, cell death, and response to inflammation. Thus, we propose Rarres1 as a possible therapeutic target in the future for CKD.

In **Paper IV** we looked into one of the players of natriuretic peptide (NP) system. NPR3 is a clearance receptor which is responsible for clearing excess NPs, thus playing a role in blood pressure regulation. We hypothesized that by inhibiting the clearance action of NPR3, we can protect glomeruli under disease conditions. Therefore, we treated two different glomerular disease models with a specific molecule that inhibits NPR3. We tested different doses and delivery methods of the inhibitor. The treatment showed some protective effects in certain, but not all conditions. We concluded that further optimization is needed to find the right dose and delivery method of the inhibitor to get the best protective outcomes.

All together in this thesis we have demonstrated the advantage of transcriptomic approach to gain detailed new knowledge on the glomerulus. Moreover, we provide new insights into the role of Rarres1 and NPR3 in kidney physiology and diseases. In the end, we can say that we have contributed into finding some of the puzzle pieces needed to understand the bigger picture of CKD and hopefully a step closer to treating it.

Abstract

Chronic kidney disease (CKD) affects millions of people worldwide and is characterized by a reduction in glomerular filtration rate and albuminuria resulting in a gradual loss of kidney function. The high prevalence of the disease, along with the limited treatment options available, makes it a major burden to the health care systems around the world. Current treatment strategies are directed towards slowing the progression and delaying the complications. The glomerulus is the filtration unit of the kidney and a major target of injury, making glomerular diseases one of the leading causes of CKD.

The kidney is a challenging organ to study due to tissue heterogeneity, complex disease phenotypes and morphologies. The lack of knowledge on the molecular mechanisms of disease pathogenesis limits the development of new diagnostic and treatment tools.

The main aim of this thesis was to gain a better understanding into genes and pathways involved in the development and progression of glomerular diseases. In a long run, our aim is to utilize the gained knowledge to develop new means to diagnose and treat CKD.

In the first part of the thesis (**paper I and II**), we used single cell RNA-sequencing (scRNA-seq) to get insights into the glomerular environment in health and disease, as well as under drug therapy.

Paper I: By profiling the glomerulus, we defined the true transcriptomic signatures of specific cell types, gained better insight into their functionality, and identified molecular profiles of rare cell types. By comparing the expression profiles between mouse and human glomerulus, we revealed significant cross-species differences in the main glomerular cells.

Paper II: By profiling the molecular signatures of Angiotensin Converting Enzyme-inhibitor (ACEi) in diseased glomerular tissue, we revealed mesangial cells (MCs) to be the main early responder to the treatment. MCs showed downregulation of genes and pathways related to extracellular matrix (ECM) production. Only few transcriptomic changes were detected in the other glomerular cell types.

In the second part of the thesis (**paper III and IV**), we investigated two candidate genes identified through transcriptomic studies. Retinoic acid receptor responder 1 (Rarres1) and Natriuretic peptide receptor 3 (NPR3) were analysed through various *in vitro* and *in vivo* experiments.

Paper III: We investigated the role of Rarres1 in the glomerulus using various transgenic mouse lines, molecular profiling of patient material and *in vitro* models. We identified Rarres1 as a possible therapeutic target and biomarker of injury for glomerular diseases. In diseases, an up-regulation of Rarres1 expression was observed in endothelial cells, in

which it aggravated the glomerular injury. This effect was potentially mediated by the activation of NFκB pathway via tyrosine kinase Axl

Paper IV: We analysed the role of NPR3 in the glomerulus, and especially explored the possibility of manipulating glomerular natriuretic peptide (NP) system through NPR3. Pharmacological inhibition of NPR3 showed variable reno-protective effects when profiled in two rodent models of glomerular injury, suggesting that the modulation of the glomerular NP system could be a potential therapeutic target for CKD. However, more studies are needed to optimise the treatment strategy and to further understand the role of NPR3 in kidney tissue.

To summarise in this thesis, we have demonstrated the power of transcriptomic approach in gaining new knowledge on the molecular biology of the glomerulus. Moreover, our studies with Rarres1 and NPR3 contribute to identification of possible novel therapeutic approaches and biomarkers.

List of scientific papers

I. Bing He, Ping Chen, Sonia Zambrano, **Dina Dabaghie**, Yizhou Hu, Katja Möller-Hackbarth, David Unnersjö-Jess, Gül Gizem Korkut, Emmanuelle Charrin, Marie Jeansson, Maria Bintanel-Morcillo, Anna Witasp, Lars Wennberg, Annika Wernerson, Bernhard Schermer, Thomas Benzing, Patrik Ernfors, Christer Betsholtz, Mark Lal, Rickard Sandberg, Jaakko Patrakka.

   Single-cell RNA sequencing reveals the mesangial identity and species diversity of glomerular cell transcriptomes.
   Nature Communications, 12(1), p.2141.

II. **Dina Dabaghie**, Liqun He, Emmanuelle Charrin, Katja Möller-Hackbarth, Gül Gizem Korkut, Christer Betsholtz, Jaakko Patrakka.

   Profiling glomeruloprotective mechanisms of angiotensin converting enzyme inhibitors (ACEi) using single cell RNA-sequencing
   Manuscript

I. Katja Möller-Hackbarth, **Dina Dabaghie**, Emmanuelle Charrin, Sonia Zambrano, Guillem Genové, Xidan Li, Annika Wernerson, Mark Lal, and Jaakko Patrakka.

   Retinoic acid receptor responder1 promotes development of glomerular diseases via the Nuclear Factor-κB signaling pathway.
   Kidney International 100, no. 4 (2021): 809-823.

II. **Dina Dabaghie***, Emmanuelle Charrin*, Pernilla Tonelius, Birgitta Rosengren, Anna Bjornson-Granqvist, Mark Lal, Jaakko Patrakka.

   Unravelling the role of natriuretic peptide clearance receptor (NPR3) in glomerular diseases
   Manuscript *Equal contribution

Scientific papers not included in the thesis

Emmanuelle Charrin, **Dina Dabaghie**, Ilke Sen, David Unnersjö-Jess, Katja Möller-Hackbarth , Mikhail Burmakin , Rik Mencke , Sonia Zambrano , Jaakko Patrakka, Hannes Olauson

Soluble Klotho protects against glomerular injury through regulation of ER stress response
Communications Biology, in press.

Contents

List of abbreviations

CKD	Chronic kidney disease
scRNA-seq	Single cell RNA-sequencing
ACEi	Angiotensin converting enzyme inhibitor
MCs	Mesangial Cells
ECM	Extracellular matrix
Rarres1	Retinoic Acid Receptor Responder 1
Npr3/A	Natriuretic peptide receptor 3/A
NP	Natriuretic peptide
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
ESRD	End-stage renal disease
IgAN	IgA Nephropathy
ARB	Angiotensin receptor blocker
RAAS	Renin-angiotensin-aldosterone system
AngII	Angiotensin II
DN	Diabetic nephropathy
TGF-β	Transforming growth factor beta
PAI-I	Plasminogen activator inhibitor 1
NFκB	Nuclear factor kappa B
SGLT2i	Sodium/glucose cotransporter-2 inhibitors
GCs	Glucocorticoids
MRAs	Mineralocorticoid receptor antagonists
MCP-1	Monocyte chemoattractant protein 1
ET-1	Endothelin 1
RNA-seq	RNA-sequencing
EGF	Epidermal growth factor
JAK	Janus kinase
STAT	Signal transducer and activator of transcription
UUO	Unilateral ureteral obstruction

Smad3	SMAD Family Member 3
WT	Wildtype
KO	Knockout
snucRNA-seq	Single nuclei RNA-sequencing
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
IFN	Interferon
IRI	Ischemia/reperfusion injury
TIG1	Tazarotene-induced gene 1
FSGS	Focal segmental glomerulosclerosis
ANP	Atrial natriuretic peptide
BNP	Brain natriuretic peptide
CNP	C-type natriuretic peptide
NPR1/A	Natriuretic peptide receptor 1/A
NPR2/B	Natriuretic peptide receptor 2/B
cGMP	cyclic GMP
cAMP	cyclic AMP
PI	Phosphatidylinositol
eNOS	Endothelial nitric oxide synthase
MAPK	Mitogen-activated protein kinases
DOCA	Deoxycorticosterone acetate
NEPi	Neprilysin inhibitor
UNx	Uninephrectomy
DEGs	Differentially expressed genes
siRNA	Small interfering RNA
PEC	Parietal epithelial cells
MDC	Macula Densa cells
MLC	Mesangial-like cells
BSA	Bovine serum albumin
Eng	Endoglin
Klf	Krüppel-like family of transcription factors

EMT	Epithelial–mesenchymal transition
ANCA Vasculitis	Anti-Neutrophil Cytoplasmic Antibody Vasculitis
$Cre^{+}/Rarres^{fl/fl}$	Rarres1 podocyte specific knockout
Rarres1_Tie2_KI	Rarres1 endothelial cells specific knock-in
Rarres1_Tie2_KO	Rarres1 endothelial cells specific knockout
NTS	Nephrotoxic serum
α-SMA	Alpha-smooth muscle actin
$NPR3^{PodKO}$	NPR3 podocyte specific knockout

1 Introduction

1.1 Kidney tissue and glomeruli

The kidneys are responsible for blood filtration, removal of waste products and urine formation. They maintain the body homeostasis by regulating the fluid volume and salt-balance essential for blood pressure regulation. Additionally, they are a major player in the excretion of metabolic end products as well as enzyme and hormone production[1].

Nephrons, the functional units of the kidney, are responsible for blood filtration and water-salt reabsorption. On average, there is about one million nephrons present in each adult human kidney, but the number of nephrons varies a lot between individuals[1]. The filtration unit of the nephron is the glomerulus, a ball of capillaries supported by the mesangium and covered with highly specialized epithelial cells named podocytes. The filtration of the blood and generation of primary urine happens through a three-layer capillary wall of the glomerulus formed by endothelial cells, the glomerular basal membrane (GBM), and podocytes. The primary urine generated by glomeruli is further processed in tubules. Tubuli are segmented with different components of tubular system having highly specialized functions leading to a series of modifications of primary urine, including reabsorption and secretion of solutes and fluids, before becoming the final urine[1]. Taken together, the kidney is an organ with an intricate, highly heterogenous structure, where numerous different cell types have distinct structural and functional features in both health and disease. This has made the kidney a challenging organ to study.

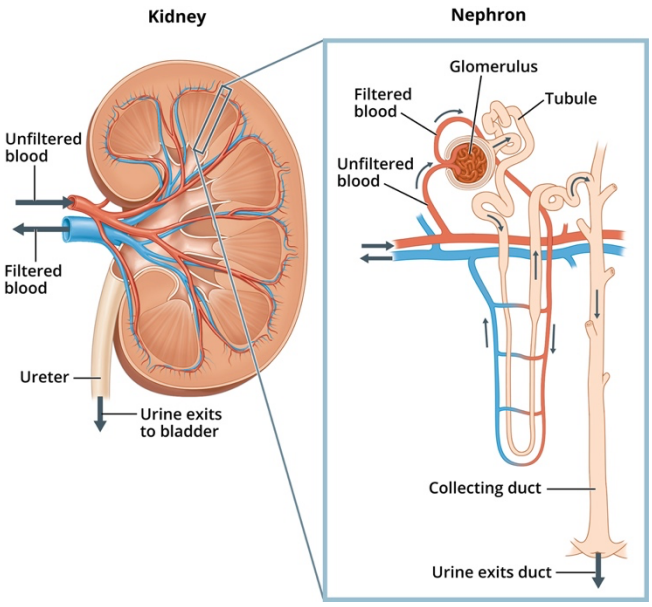

Figure 1 The kidney and the nephron.

Image adapted from National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, published under CC BY license.

1.2 Chronic kidney disease

Chronic kidney disease (CKD) affects about 10% of the global population. It is typically characterized by a reduction in the glomerular filtration rate (GFR) and albuminuria as secondary to the protein leakage through the glomerular filtration barrier[2,3]. Due to the crucial role of the kidneys in blood pressure and maintenance of body fluid homeostasis, CKD in all stages increases the risk for cardiovascular, hemodynamic, and metabolic diseases, and thus is a major cause of morbidity and mortality along with a decreased

quality of life. In many cases CKD leads to end-stage renal disease (ESRD), where the irreversible damage to the kidney tissue can only be treated with chronic dialysis or organ transplantation[2]. The high prevalence of CKD along with the limited treatment options makes it a major economic burden to the health care systems around the world[3].

1.2.1 Glomerular diseases and CKD

Glomerular diseases are one of the leading causes of CKD and ESRD. Glomerular damage occurs often secondary to systemic disorders like diabetes, hypertension, and lupus. In some cases, the glomerulus is the primary target of injury, such as in case of IgA nephropathy (IgAN), membranous nephropathy and anti-GBM (Goodpasture) syndrome.[4]

A common pathway in the progression of glomerulopathies is a protein leakage through the filtration barrier. In some cases, such as hypertension and diabetes, an increase in the capillary filtration pressure results in an increased glomerular permeability, consequently increasing the protein filtration and tubular protein reabsorption. Increased protein content in the primary urine leads to an overload on the glomerular and tubular cells, activating different fibrinogenic and inflammatory pathways. Similar responses are observed in disorders with normal glomerular filtration pressure including immune-mediated nephropathies[5–7]. Damage to the glomerulus is commonly characterised by a variety of histopathological changes, such as podocyte foot process effacement, podocyte loss, mesangial matrix expansion, MC proliferation and alterations in the GBM, ending with fibrosis and glomerular sclerosis[4,8].

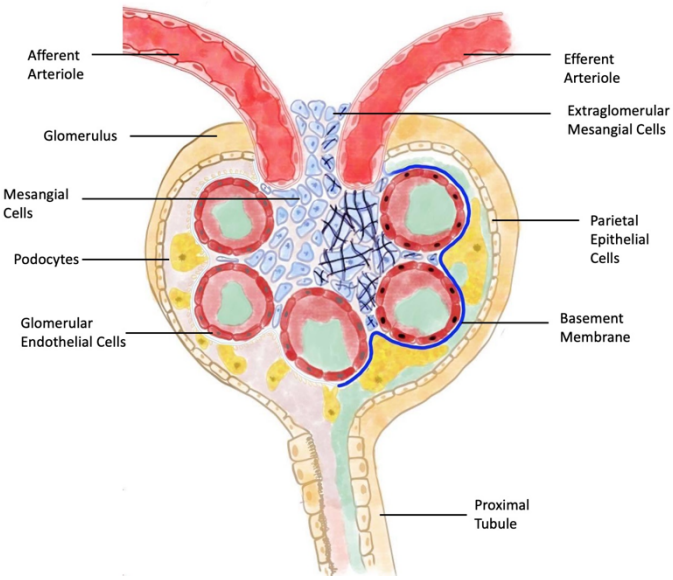

Figure 2 Glomerulus in health and disease

An illustration of glomerulus in the healthy state (on the left) and after the induction of disease (on the right). Illustration by Yana Dabaghie.

Glomerular and immune cells are critical for the maintenance of glomerular health and dysregulation in disease. Each cell type plays a unique role within the glomerulus, and they are specific targets of injury in different disease processes. Still, due to the proximity and cell-cell crosstalk, an injury to one cell population influences the other cell types[8]. This dynamic interaction environment and the presence of multiple cellular components contribute to the disease development and progression and makes glomerular tissue a challenging subject to study.

1.3 Treatment strategies of CKD

Treatment of CKD aims to slow the progression and prevent or delay complications. The asymptomatic nature of the disease in the early stages makes the early diagnosis and intervention very important, especially in high-risk patient populations. CKD is a complex disease, with a high prevalence in systemic disorders such as diabetes and hypertension. Thus, the interventions are mostly directed towards systemic factors, such as glycaemic and blood pressure control, through lifestyle changes and pharmacological interventions. The main classes of therapeutic agents used today are summarized in the following.

1.3.1 Angiotensin Converting Enzyme Inhibitors (ACEi) and Angiotensin Receptor Blockers (ARBs)

Angiotensin II (AngII), a key player in renin angiotensin aldosterone system (RAAS), is a peptide hormone mediating multiple effects on target organs. ACEis inhibit the generation of AngII, whereas ARBs block the receptor for AngII. Today, targeting RAAS via ACEi and ARBs is the standard care for most types of CKD and glomerular diseases. Clinical trials have shown the regression of injury and a decrease in proteinuria in patients with hypertension, diabetic nephropathy (DN), IgAN, membranous nephropathy and HIV-associated nephropathy. Even in patients with advanced stages of CKD ACEi/ARBs have shown to slow the progression to ESRD[9,10].

The reno-protective mechanisms of ACEis/ARBs are mainly attributed to their capacity to regulate the hyperfiltration in diseased kidney by decreasing glomerular filtration pressure. However, many non-hemodynamic effects have been reported and suggested to be important mediators of reno-protection[5,9,11]. For instance, AngII has been reported to act as a cytokine that is crucial for the progression of renal and glomerular injury through its profibrogenic and proinflammatory actions. Thus, ACEis/ARBs could suppress extracellular matrix (ECM) deposition and immune activation by targeting AngII. [12].

Throughout the years the mechanism of action of these agents have been explored using various experimental animal models and *in vitro* systems. *In vivo* studies have shown a regression in glomerular sclerosis and restoration of normal glomerular capillary structure after glomerular injury, as well as a decrease in tubulointerstitial and vascular lesions[13,14]. Importantly, these outcomes were seen with and without the effect on systemic blood pressure.

Mechanistically, anti-fibrotic/inflammatory effects of ACEi/ARBs have been linked to transforming growth factor-β (TGF-β)[13,15], plasminogen activator inhibitor (PAI)-1[16,17] and Nuclear factor kappa B (NF-κB) pathways[18]. The involvement all principal glomerular cell types have been investigated, including: 1) Suppression of mitogenic effect in MCs[14,19]; 2) Effect on endothelial repair proliferation, and stimulation of angiogenesis[14,20]; 3) Decreased podocytes loss[21–24].

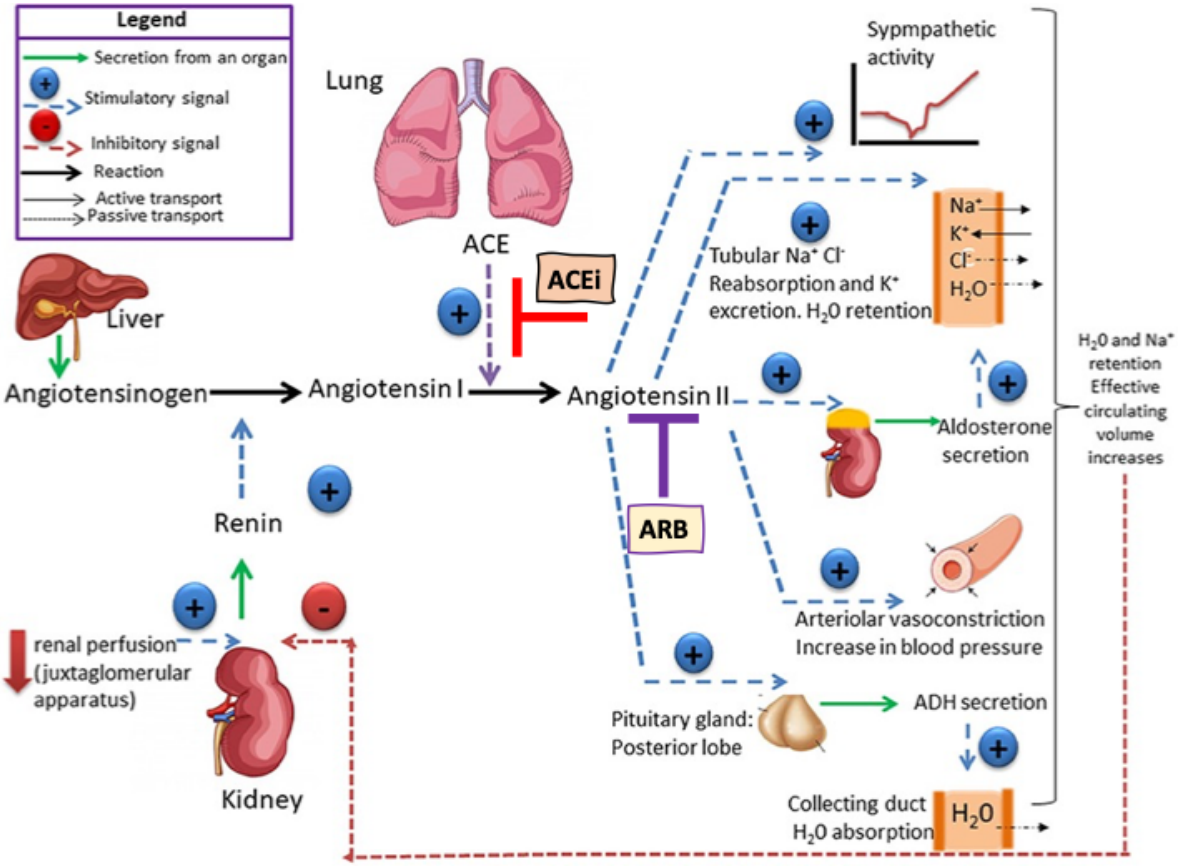

Figure 3 Renin Angiotensin Aldosterone System

Mechanism of action of angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blocker (ARB). Image adapted from de Almeida LF, Coimbra TM., Front Pediatr. 2019, published under CC BY license.

1.3.2 Sodium/glucose cotransporter 2 inhibitors (SGLT2i)

Over the last few years SGLT2i have emerged as a promising therapy for cardiovascular and renal disease. Recent clinical trials have shown the efficiency of SGLT2i in patients with different stages of CKD and a broad range of aetiologies regardless of the presence or absence of diabetes. The effect on GFR decline and decrease in renal-cardiovascular mortality were seen in these clinical settings[11,25].

In diabetes, the reno-protective effects are partially mediated by blood glucose-lowering effects. However, in non-diabetic conditions mechanisms are more difficult to understand. In proximal tubuli, by blocking glucose transport, SGLT2i inhibits also the transport of sodium, and thus increases the concentration of sodium in macula densa. This leads to the dilation of efferent arterioles and constriction of afferent arterioles, decreasing glomerular blood flow[26]. This can potentially explain part of the glomerulo-protective effects in non-diabetic setting. Animal studies have shown their effect on the reduction of inflammation, fibrosis, and oxidative stress in the renal environment. Additionally, extrarenal mechanisms such as blood pressure reduction, neurohormonal improvement and modulation of RAAS and the sympathetic nervous system are seen under SGLT2i treatment[26,27].

1.3.3 Glucocorticoids (GCs)

GCs have been used for a long time in the management of renal diseases, especially in glomerulopathies. Their main effect is believed to be through the suppression of immune function and the reduction of inflammation both systemically and locally in renal environment. However, there is evidence that GCs may have a direct effect on glomerular permeability and function. Studies have shown protective effects on podocytes both *in vitro* and *in vivo*. The main drawback of GCs are their side effects, which make them not suitable for treating many chronic disease processes [28,29].

1.3.4 Other treatment approaches in CKD

Mineralocorticoid-receptor antagonists (MRA) are used in the treatment of CKD due to their anti-inflammatory and antifibrotic effects. MRAs act on aldosterone levels and thereby regulate fluid volume, blood pressure and potassium balance[30]. Despite their beneficial effect in reducing the cardiovascular mortality and alleviating albuminuria, their low safety profile has limited the use of these agents. Nevertheless, the development of a new generation of nonsteroidal-MRAs is showing promising results with improved anti-inflammatory/fibrotic effects and less side effects. These agents are currently under investigation in different stages of CKD and especially in patients with DN[31,32].

Moreover, several novel treatments are in the development pipelines as promising therapies. For instance, inhibitors targeting renin, TGF-β pathway, monocyte chemoattractant protein-1 (MCP-1) and endothelin (ET)-1 are currently under testing for efficacy and safety[10,30].

1.3.5 Slowing progression yet no reversal of injury

It can be argued that CKD is often treated as a single entity regardless of the underlying cause of the disease. Current treatment options are broadly initiated across different CKD populations because they are inexpensive and with a low potential risk for side effects. The interventions slow the progression of CKD and decrease the incidence of ESRD. Yet, can we consider this decrease good enough, or is there more we can do?

The lack of new treatment options for CKD is highly connected to the poor knowledge of the molecular pathophysiological events in the disease. Moreover, our incomplete understanding of the mechanisms-of-action of currently used reno-protective drugs contributes to this challenge. Obviously, a better understanding of the specific cell types and pathogenic pathways involved is needed. The knowledge gained may help us to develop better strategies to prevent progression or even restore the renal function.

1.4 Transcriptomics approach in CKD

1.4.1 Next generation sequencing and transcriptomics

The development of high-throughput next-generation sequencing platforms has opened the door to genetic and transcriptomic studies of complex diseases, providing us with information needed to understand the molecular mechanisms behind disease pathogenesis, as well as providing insights into the protective mechanisms of current therapies. Transcriptomics approach through RNA-sequencing (RNA-seq) allows us to cover in high resolution the dynamic nature of the transcriptome, including unravelling detailed view of gene expression, identification of alternative splicing, discovery of novel transcripts and allele-specific detection[33]. Transcriptome profiling allows us to make a comparison between pathological and healthy states, leading potentially to the identification of pathogenic signalling pathways and unravelling cellular origins of the disease. Similarly, we can gain insights into the molecular networks and pathways that are activated or repressed in a response to a particular treatment. This has made RNA-seq one of the most utilized approaches to study disease processes at the molecular level[34]. The acquired knowledge can potentially be utilized in diagnostics, prognostics, therapy selection and novel therapy development.

1.4.2 Bulk RNA-profiling using microarrays

Within the field of nephrology, the transcriptomics approach has been embraced and implicated in screening of patient derived materials and animal disease models. For instance, microarray data from micro-dissected tubulointerstitial fractions of patient biopsies have pinpointed a number of genes which differential expression correlated to the GFR levels at the time of biopsy when compared to healthy donors. Within the study the mRNA levels of epidermal growth factor (EGF) was correlated to the protein levels of EGF detected in urine, both of which were shown to be correlated with CKD progression and GFR-decline rates, making urinary EGF a potential non-invasive biomarker in CKD patients[35].

Microarray study of biopsies from DN patients showed a significant upregulation of Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway[36]. This discovery laid a foundation for trials targeting JAK inhibition for DN. Moreover, the insights from this work were applied to other kidney diseases like focal segmental glomerulosclerosis, models of polycystic kidney disease and renal fibrosis[37]. In another microarray study, an unbiased comprehensive catalogue of differential gene expression in DN patients was generated for both glomerular and tubulointerstitial portions[39]. The study described many novel candidates that can serve as potential targets for future studies[38]. A similar approach was taken on lupus nephritis in both patient biopsies and murine models[39]. This approach generated data that can help in the selection of animal

models and provided a basis for new therapeutic strategies[39]. In studies on kidney fibrosis (in both mouse and human), the role of inflammation and metabolism has been shown in microarray studies [40,41].

1.4.3 Bulk RNA-profiling using RNA-sequencing (RNA-seq)

During the last decade, RNA-seq has taken over as a method of choice for global RNA profiling. In mouse models of immune-mediated kidney damage (using anti-GBM glomerulonephritis model) and non-immunologically associated kidney damage (Unilateral Ureter Obstruction (UUO)), the upregulation of inflammatory, immune response and cytoskeleton related genes was observed, along with a downregulation of genes involved in electron transport and metabolism[42]. Further studies using knock-out (KO) animals validated the role of specific mothers against decapentaplegic homolog 3 (Smad3) pathway in the development of glomerulonephritis[42].

An RNA-seq study comparing both glomerular and tubulointerstitial fractions collected from DN patients to samples from healthy living donors has been performed. DN-associated changes were identified in both tissue compartments, and pathways of interest were highlighted. These included for instance the upregulation of pathways related to ECM reorganisation and inflammation in the glomerular portion, and the upregulation of apoptotic pathways in the tubulointerstitial fraction[43].

As genome-wide RNA profiling studies generate massive amounts of data, a need for accessible platforms that integrate data from numerous sources has increased. Some platforms in the field of nephrology include Nephroseq (www.nephroseq.org), Renal Gene Expression Database[44] and the Genito Urinary Development Molecular Anatomy Project (GUDMAP)[45]. These interfaces allow users to mine data and visualize gene expression data from human material and animal models related to various kidney diseases. Easy access and practical comparison tools implemented provide an opportunity to identify new genes of interest in different kidney diseases and open up possibilities for researchers to investigate their genes of interest in depth and determine their specific role in the pathogenesis of the disease.

1.4.4 Single Cell RNA-Sequencing

Bulk tissue transcriptomics has provided novel insights into the pathogenesis of CKD, identified potential biomarkers to be used in diagnostics, and pinpointed attractive therapeutic targets. However, this method comes with limitations. Due to the heterogeneity of the kidney tissue, bulk RNA profiling does not allow the elucidation of transcriptional changes in specific cell types.

Single cell RNA- sequencing (scRNA-seq) adds another dimension to the transcriptomics studies. This unbiased, high throughput and high-resolution approach, unravels

transcriptional profiles from individual cells, and therefore provides insights into the composition of heterogenous tissues, the dynamics of transcription in different cell types, and regulatory relationships between genes in health and disease[46].

Multiple platforms to run scRNA-seq have been developed. The pros and cons of various scRNA-seq methods (fluidic, microdroplet, and non-microfluidic approach) differ in several parameters. A variation in the number of cells captured divides the platforms into low-throughput methods, such as Smart-seq and Cel-Seq2, and high throughput methods, such as 10x Chromium, Drop-seq and SPLiT- seq. Parameters like sensitivity, reproducibility, extent of multiplets and the ability to recover meaningful biological distinction in cell types varies as well. Smart-seq2/3 are the only platforms offering a full-length cDNA coverage, which is an advantage when studying RNA splicing isoforms and genetic variants. Other key considerations include the cost, time needed for each method, and obviously availability of each platform[47,48]. In the end, all parameters should be considered carefully and related to the scientific question that the experiment is trying to answer.

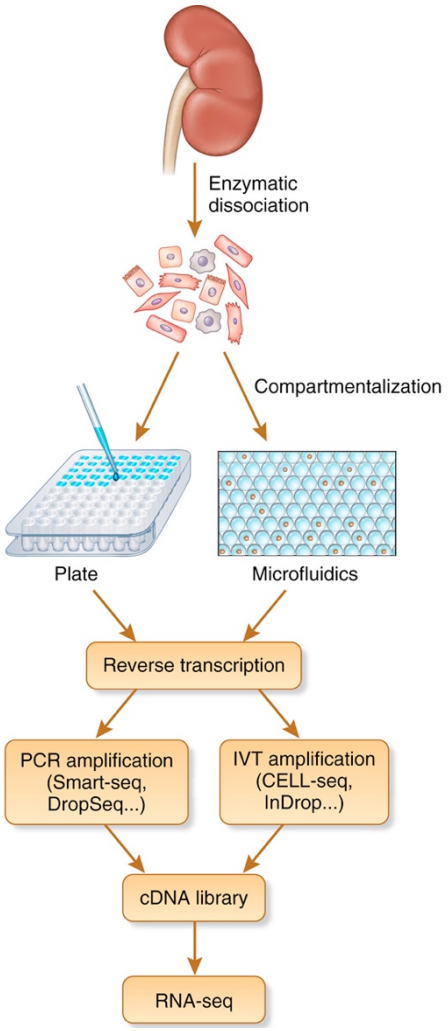

Figure 4 scRNA-seq experimental workflow

All scRNA-seq protocols share the following common steps: (i) Enzymatic dissociation of the sample into a single-cell suspension. (ii) Compartmentalization of single cells into an individual chamber (well or oil droplet). (iii) Reverse transcription. (iv) Library amplification via PCR or IVT depending on the protocol selected. (v) Library fragmentation/tagmentation. (vi) RNA-seq. IVT, *in vitro* transcription; PCR, polymerase chain reaction; scRNA-seq, single-cell RNA-sequencing. Image adapted from Wu, H. and Humphreys, B.D., *Kidney international*, 2017, published with permission from the author/publisher.

Single cell analyses can be challenging for many reasons. For instance, some tissues are hard to dissociate (brain, skeletal muscle, adipose), whereas others are preserved in a frozen form (such as many renal biopsies stored in biobanks). In these cases, an alternative approach of single nuclei RNA-sequencing (snucRNA-seq) has been used. Previous studies in different organs including the kidney have shown comparable results between scRNA-seq and snucRNA-seq, with a good performance regarding sensitivity, classification of cell types and genes detected[47,49].

Within the field of nephrology an array of scRNA-seq studies has been published in the recent years. First studies conducted on healthy murine and normal human kidney tissue successfully identified the main kidney cell types in both cortex and medulla[50,51]. In depth profiling resulted in an improved characterisation of different cell types and added a number of novel markers for each cell type[50,52]. Similarly to other organs, scRNA-seq helped in discovering novel subpopulations of cells, such as a subtype of collecting duct

cells with unexpected cell plasticity[50]. Additionally, the diversity and heterogeneity of different cell populations has been explored. Endothelial cells showed the highest heterogeneity in the glomerular compartment[53], and a surprising diversity of immune cells were discovered in normal kidneys[52,54]. Studies focusing on the spatial gene expression across different kidney compartments revealed a smooth transition in gene expression profiles of tubular cells. Gene expression changes followed the change of osmolarity and functionality across the different tubular compartments[55]. A combination of scRNA-seq with single nuclei ATAC-seq (Assay for Transposase-Accessible Chromatin) gave an insight into the transcriptional and chromatin accessibility landscape in normal human kidney, highlighting the heterogeneity of proximal tubule and thick ascending limb[56]. Studies on healthy kidney tissue generated several cell atlases accessible for researchers to explore and serve as an important base for future scRNA-seq studies.

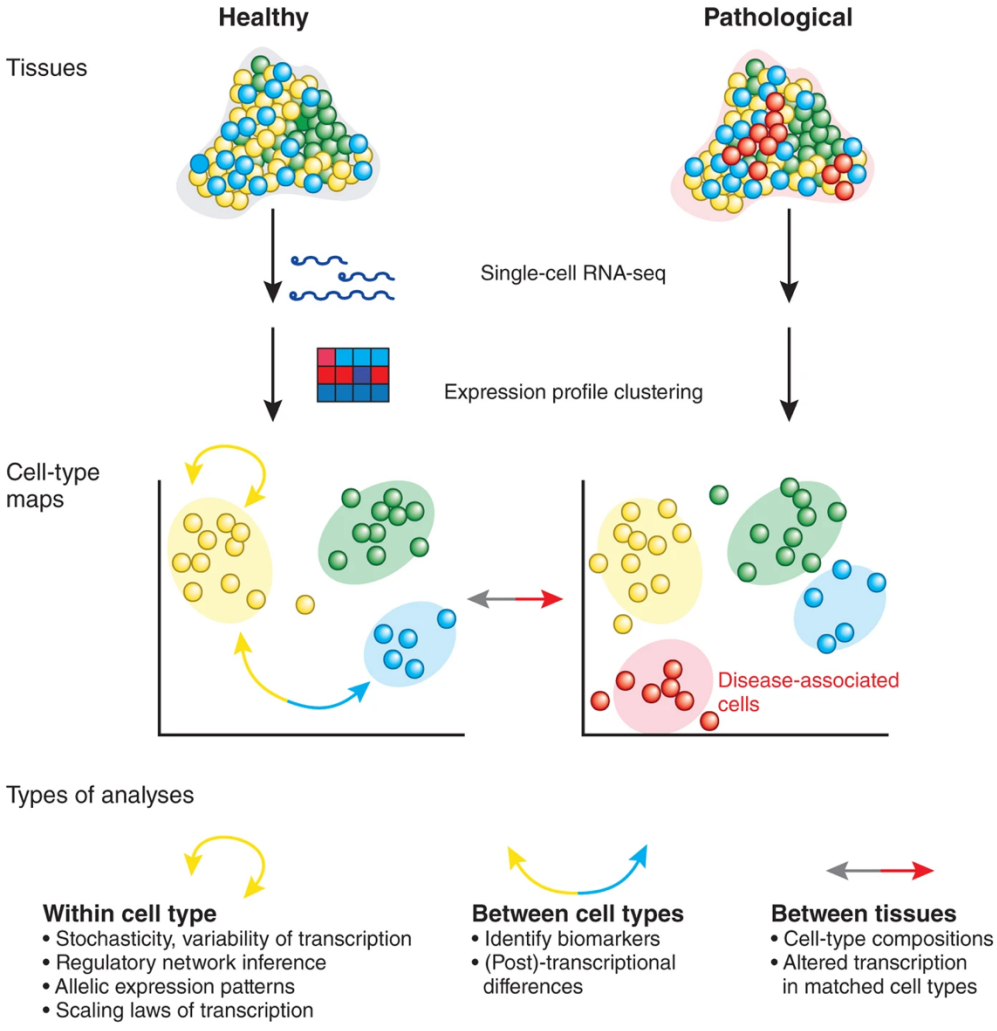

Figure 5 Single-cell transcriptome analyses of tissues and cell types

Types of analysis that can be done with data acquired by scRNA-seq. Image adapted from Sandberg, Rickard, *Nature methods*, 2014, published with permission from the author/publisher.

Obviously, profiling various kidney diseases in both murine models and patient derived biopsies has been a high priority. In an experimental model of DN (induced by streptozotocin), scRNA-seq showed a significant increase of immune cells (predominantly macrophages). Glomerular endothelial and MCs showed dynamic changes in gene expression patterns related to angiogenesis and regulation of translation and protein stabilization[57]. In another diabetic model (BTBR ob/ob), more limited changes were observed in endothelial cells, while both podocytes and MCs showed activation of pathways related to glucose and lipid metabolism[58]. In a small-scale study in early human DN, scRNA-seq analyses showed expressional changes involved in immune cell activation, ion transport and angiogenesis. Moreover, increased potassium signalling in the thick ascending limb and late distal convoluted tubule, was accompanied with an increased angiogenic signalling in the glomerulus[59].

Several studies have also applied scRNA-seq to kidney tissue collected from animal models/patients with IgAN. In a murine model of IgAN, the key role of endothelial cells was pinpointed in the early stages of glomerulopathy, especially in the immune cell recruitment and infiltration[60]. On the other hand, two studies in human IgAN biopsies highlighted the role of MCs. Pathways related to inflammation and ECM accumulation were identified in MCs, and an increase in the cross-talk between the mesangium and remaining cells was observed[61,62].

For lupus nephritis few studies have been performed with rather limited number of biopsies and analysed cells. Only populations of fibroblasts and tubular, endothelial, and T-cells were captured. Regardless of the small number of cells sequenced, authors could still identify the correlation between active interferon (IFN) molecular signatures in the tubular cells and the chronicity index / IgG deposition / quantity of proteinuria in different patients, suggesting that the quantification of mRNA level of IFN response could be a useful biomarker[63].

scRNA-profiling of glomerular tissue in nephrotoxic serum induced glomerulonephritis model showed in podocytes induction of genes associated with cell adhesion, cytoskeletal regulation, and inflammation. These changes were normalised when albuminuria was resolved. On the other hand, MCs and endothelial cells continued to show transcriptional changes regardless of albuminuria levels. MCs showed an increase in cell adhesion and cytoskeletal regulation, while endothelial cells had high expression of proangiogenic factors[58]. In doxorubicin-induced model of podocyte injury, a decrease in the number of podocytes and podocyte-related markers was seen, with an increase in oxidative stress signalling pathways[58].

Single cell transcriptomics has been applied also to study mechanisms of kidney fibrosis. Several murine models, such as ischemia reperfusion injury (IRI), UUO, Folic acid induced fibrosis, and chronic aristolochic acid administration, has been used. Studies focused on

the different proliferative and differentiating states of proximal tubular and stromal cells[49,64–66]. Human nephrectomy samples were, on the other hand, used to identify the source of scar formation during kidney fibrosis. Major contribution to ECM deposition was attributed to mesenchymal cells, whereas de-differentiated proximal tubular cells had a less significant role[67].

To gain insights into the reno-protective mechanisms of currently used drugs in CKD, scRNA-seq has been used to map the transcriptomic response to therapies in a murine model of DN. The study showed that different therapies targeted different cell types and resulted in non-overlapping transcriptomes. A clear effect was seen in both glomerular and tubular cells, with several targets of interest reported[68]. Although the study was rather descriptive in nature, the cell atlas generated offers a great resource for researchers to explore.

Besides profiling kidney tissue in CKD, two studies have shown the feasibility of the method to analyse urinary cells. Various cell types were captured and presented distinct transcriptional profiles that could be connected to specific kidney cell types. This opens up the possibility of using urine as a liquid biopsy to monitor kidney cell health[69,70]. Finally, scRNA-seq technique has been applied successfully to study kidney development[48], kidney allograft biopsies[71], kidney organoids[72,73] and even an insight on cross-species landscape of kidney tissue[39].

Single cell transcriptomic profiling has an immense potential but presents also new challenges and limitations. Due to the high sensitivity (and cost) of this method, extra consideration should be put in the experimental design and processing of samples. Batch effects and unwanted technical variations may jeopardize the reflection of the real transcriptional state of each cell before the dissociation, and therefore overshadow the true biological state[74]. Cell type bias is also one of the obstacles to face in an unbiased approach, as some cell types might be harder to dissociate, or they could be highly sensitive to the dissociation process, leading to the damage/loss of these cell types in the final analysis. This could be overcome by selective sorting of cells using fluorescence-activated cell sorting (FACS) or using the snucRNA-seq approach[74,75]. Finally, to analyse the large amount of data generated, a reproducible bioinformatics approach is needed, however, high variability of the data raises computational challenges. An increased popularity of the scRNA-seq approach has led to the generation of a large variety of computational pipelines, with thousands of them publicly available. However, best practices have not yet been established. Further development in the field of the bioinformatics is clearly needed to develop more efficient tools with a goal of generating an optimal computational pipeline[76].

1.4.5 Bulk vs scRNA-seq

It has been speculated that bulk RNA-sequencing might yield similar resolution as scRNA-seq as long as tissue samples have similar cell type composition. However, this is rarely a case and in practise would be hard to control. Additionally, signals might be lost or undetectable in bulk analysis if particular gene expression is limited to a small cell population. scRNA-seq/ snucRNA-seq approach has shown to be highly informative in distinguishing separate cell types, describing transcriptional changes across a cell population, and enabling a bioinformatic reconstruction of dynamic cellular processes like development, differentiation, and disease progression, which has not been possible with bulk transcriptomics profiling. Future perspective of the highly evolving single cell transcriptomic field is the combination with other multiomics (e.g., genomics, epigenomics, proteomics, spatial information) measurements.

1.4.6 Validation of transcriptomic findings

Bulk and single cell transcriptomics have contributed during the recent decade significantly to the understanding of pathogenic mechanisms in CKD. These insights provide an opportunity that can lead to discoveries of biomarkers for diagnosis and monitoring the disease progression, as well as identification of possible therapeutic targets or improvement of currently available treatments.

One of the main limitations of transcriptomic studies is the multiple layers of regulation between the mRNA expression and downstream changes in protein functions[37,77]. Thus, RNA profiling studies should lead to the next level of experiments that can verify the involvement of targets and pathways discovered. One option is to interrogate these discoveries in animal models. Generating gene-specific knockout (KO)/knock-in (KI) models allows us to investigate the role of possible targets and their related pathways in the development and progression of disease. Numerous studies have been conducted where genes identified in previously mentioned transcriptomics studies have been explored[40,78–80]. Moreover, animal models provide a platform to target novel pathways with medications, which can provide a better understanding of their mechanisms of action in different CKD settings[81,82]. Further, verifications of identified biomarkers should be performed in independent patient cohorts, including kidney biopsies, urine or blood samples collected from different disease stages, to confirm the correlation between the biomarker and the stage before implementing them to clinical practice.

1.5 Novel targets and their role in glomerular diseases

Comprehensive transcriptomic studies in kidney tissue have provided essential information on the molecular players that maintain kidney health and that contribute to the development of disease. The studies have given the chance to identify and explore novel molecular targets that might play an essential role in glomerular diseases and CKD.

From the previously mentioned studies two genes caught our attention, Retinoic acid receptor responder 1 (Rarres1) and Natriuretic peptide receptor 3 (NPR3).

1.5.1 Rarres1

Rarres1 gene or tazarotene induced gene 1 (TIG1) was initially identified as a highly upregulated gene in retinoid treated skin raft cultures, with a suggested mechanism-of-action of retinoid treatment in psoriasis[83]. Rarres1 is described as a transmembrane protein with a short N-terminal cytosolic domain, a transmembrane domain, and a large C-terminal domain. Studies have shown the presence of a secreted or released form of Rarres1, which might be secreted as a splice isoform or proteolytically cleaved from the cell surface[84].

Rarres1 was shown to be expressed in a variety of tissue including prostate, lung, liver and kidney[85]. The role of Rarres1 in disease has been studied in different cancer tissues and cell lines. Its expression was significantly decreased in prostate cancer samples when compared to normal prostate tissue[85]. Rarres1 overexpression in prostate cancer cell lines reduced invasiveness and tumorigenicity, making it a potential tumour suppressor gene in human prostate cancer. Moreover, downregulation of Rarres1 was seen in endometrial cancer, head and neck cancer and nasopharyngeal carcinoma[86]. However, the potential effect of Rarres1 as a tumour suppressor gene was shown to be cancer type specific. Rarres1 showed to be upregulated and correlated with molecular phenotype in glioblastoma and was suggested to be involved in the immune process of the disease[87]. Similar observations were seen in invasive breast carcinoma where Rarres1 contributed to malignant processes[86].

Analysis of fibrosis-related microarray data sets generated from liver, lung and kidney tissues showed Rarres1 as one of the two common upregulated genes. Further studies on liver fibrosis showed a significant increase in Rarres1 expression during the induction and progression of fibrosis in both *in vitro* and *in vivo* settings. In line with this, a significantly increased expression was observed in patient samples of liver fibrosis[88].

Differential expression of Rarres1 was also detected in metabolic diseases, where it was linked to biological hallmarks that require metabolic reprogramming. Rarres1 was among one of the most upregulated genes in subcutaneous adipose tissue of obese individuals, and among the most downregulated genes in adipose tissue during weight maintenance[89]. In studies on adipocytes differentiation, Rarres1 was induced during the dedifferentiation and decreased during the differentiation process, where the metabolic programming is crucial. Moreover, Rarres1 manipulation demonstrated alterations in global fatty acid metabolism, with an important role in diseases where lipid metabolism is a hallmark of disease progression[90]. Animal models have shown differential expression of Rarres1 in hepatic steatosis and cholestatic liver disease, and a decreased expression in heart disease like hypertrophic dilated cardiomyopathy[90].

Mechanistically, studies have linked Rarres1 and Axl signalling pathway. Stabilization and activation of Axl tyrosine kinase by Rarres1 leads to the activation of the downstream pathway molecules including NF-kB. The activation of Axl pathway has been shown to contribute to cell proliferation, survival, apoptosis, and inflammatory cytokine release in different diseases settings including kidney disease experimental models of kidney diseases[91,92].

Only few studies have explored the role of Rarres1 in kidney tissue. Transcriptomic studies have shown Rarres1 as one of the most upregulated genes in the glomerular fraction of DN patients[38]. Available disease datasets such as Nephroseq (www.nephroseq.org) and nephrotic syndrome study network consortium (NEPTUNE) shows upregulation of Rarres1 expression in both glomerular and tubular compartments. Specifically, a positive correlation between Rarres1 expression and decline in renal function in human glomerular diseases, such as focal segmental sclerosis (FSGS) and DN, was observed. Rarres1 seems to be expressed solely by podocytes in healthy conditions, where its overexpression induce podocyte apoptosis[93]. These pro-apoptotic effects were suggested to be mediated by autocrine and paracrine actions of Rarres1[94].

1.5.2 NPR3

Natriuretic peptides (NPs) are a family of hormones/paracrine factors consisting of atrial Natriuretic Peptide (ANP), B-type Natriuretic Peptide (BNP) and C-type Natriuretic Peptide (CNP). ANP is highly expressed and released by heart tissue (specifically the atria), though high expression is also observed in the ventricles along with expression in brain, adipose and kidney tissue. BNP is, on the other hand, produced in the ventricles alongside with the atria and brain tissue. CNP is found mainly in the brain and the endothelium[95,96].

There are three NP receptors. All three members contain a relatively large extracellular domain and a single membrane spanning region. Natriuretic Peptide Receptor 1 (NPR1/NPRA) and Natriuretic Peptide Receptor 2 (NPR2/NPRB) contain a large intracellular domain consisting of guanylyl cyclase domain, which mediate their signalling effect by catalysing the synthesis of intracellular cyclic GMP (cGMP). In contrast, Natriuretic Peptide Receptor 3 (NPR3/NPRC) has only a small intracellular domain that lacks the guanylyl cyclase activity, and functions primarily as a clearance receptor[96]. NPR1 is the principal receptor for ANP and BNP, while NPR2 is the principal receptor for CNP. NPR3 has, on the other hand, a high affinity for all NPs. The interaction between the NPs and their receptors is a major player in the regulation of blood pressure. Their blood pressure lowering effects have been studied extensively, NPs interactions with NPR1/NPR2 resulting in natriuretic and kaliuretic properties, decrease in the sympathetic flow and increase in the endothelial permeability. Aside from the blood pressure effect, their involvement has been reported in an array of physiological processes with effects reported on the immune

system, bone growth, fibrosis, and thyroid function. These biological effects are mainly attributed to the intracellular activation of cGMP pathway and downstream signaling[95,96].

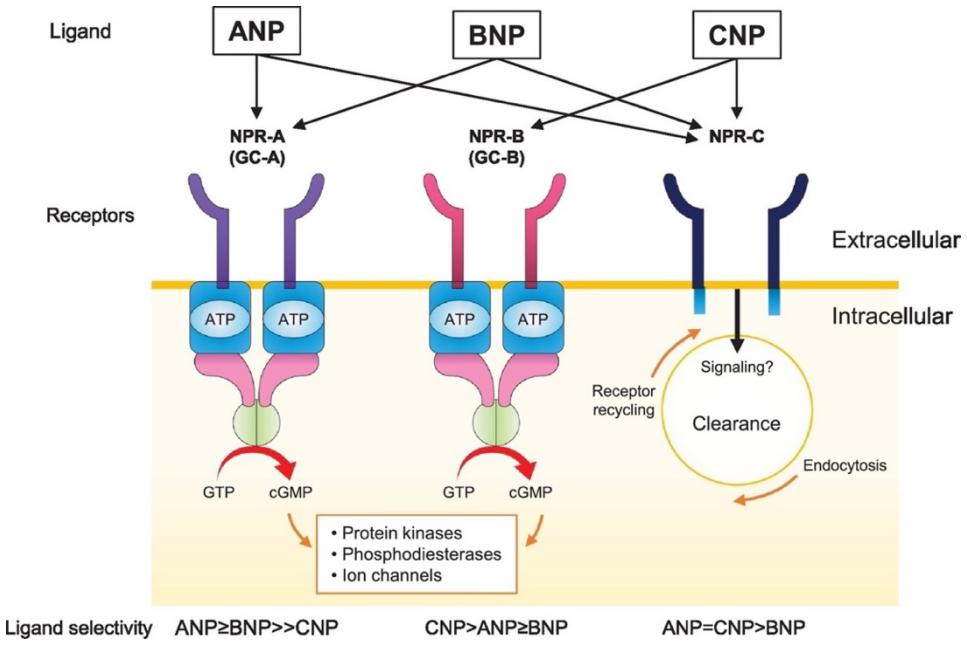

Figure 6 Natriuretic peptide receptors and their ligands

An overview of the natriuretic peptides and their receptors. Image adapted from Kuwahara, Koichiro, *Pharmacology & Therapeutics*, 2021, published under CC BY license.

NPR3 is playing an important role in removing NPs from the circulation through receptor-mediated internalization and degradation, and therefore modulates the levels of NPs available to interact with NPR1/2[97]. This was confirmed by generating mice lacking NPR3 that showed an increased half-life of ANP in circulation, hypotension, diuresis and blood volume depletion[98]. In addition to its role as a clearance receptor, NPR3 has been reported to be involved in several intracellular signalling mechanisms. An inhibitory effect of NPR3 on adenylyl cyclase – cyclic AMP (cAMP) signal transduction system has been detected in thyroid tissue, vascular smooth muscle cells, neonatal cardiomyocytes and platelets[97,99]. Moreover, it has been reported that some analogues of ANP peptides bind selectively to NPR3 and stimulate the phosphatidyl inositol (PI) turnover. Few studies have shown the contribution of NPR3 in an increased endothelial nitric oxide synthase (eNOS) activity, and an attenuation in mitogen activated protein (MAP) kinase activity[97].

NPR3 is expressed abundantly in many tissues and cells, including the kidney, vascular smooth muscle cells, fibroblasts, adipose and lung tissue[96,97]. In the kidney, NPR3 have been shown to be enriched in the glomerular fraction, where it is believed to play a major role in the regulation of blood pressure[98].

Global NPR3 KO mice show cardiovascular-kidney related phenotypes mentioned above and additionally, these mice demonstrate serious skeletal deformities associated with increased bone turnover[98]. Targeted KO in adipose tissue shows that NPR3 contributes to

energy expenditure, inflammation, and the distribution of lipid storage between the liver and visceral fat[100]. These findings have concluded that NPR3 locally modulates the availability of NPs in their target organs, allowing the activity to be tailored to specific physiological needs.

Several studies have analysed the role of NP system in the kidney through global and targeted KO models in mice and patient derived material. Global KO of NPR1 gene in mouse result in hypertension, cardiac hypertrophy and congestive heart failure, along with a drastic reduction of GFR and renal plasma flow, as well as an increase in renal fibrosis and tubular damage[101]. When NPR1 was specifically knocked out in podocytes, no difference in the blood pressure or GFR was observed. However, upon induction of hypertension and kidney damage using the mineralocorticoid deoxycorticosterone-acetate (DOCA) treatment in combination with high salt intake, podocyte KO mice showed an increase in albuminuria levels, ROS production and renal fibrosis[102]. Thus, NPR1 signalling contributes to the renal protection by maintaining podocytes integrity and function in pathological conditions. In addition, a significant downregulation of NPR1 and NPR3 in glomeruli of DN patients has been reported using transcriptomic approach[38]. However, no studies with kidney-specific KO mouse models for NPR3 have been performed. There is obviously a need to explore the role of NPR3 in the physiology and diseases of the kidney.

NP/NPR system has been investigated as a therapeutical target in heart failure. Synthetic ANP and BNP infusion in patients with heart failure showed beneficial effects and has been used as a treatment approach, however, the short *in vivo* half-life and the adverse effects (especially hypotension) limits the use of these agents[96,103]. Previous studies have shown that the effect of NPs on guanylyl cyclase cGMP activation depends on the ratio of functional receptor NPR1/NPR2 vs the clearance rate. Therefore, an alternative approach to manipulate the system by decreasing NP clearance has been explored. Neprilysin is a naturally occurring enzyme that breaks down circulating NPs. Studies have shown that the inhibition of neprilysin alone leads to vasodilation as expected, but also to increased levels of AgtII and (ET)-1, which counteracts the vasodilatory effect, and thus neutralizes anti-hypertensive properties [104]. This led to the development of a combination therapy with neprilysin inhibitor (NEPi) and ARB, which proved to be effective in hypertensive and heart failure patients[104,105].

The use of NEPi/ARB combination in pre-clinical models have shown a reno-protective effect in diabetic settings, through a reduction in inflammation, apoptosis and an improved podocyte integrity[106,107]. Kidney fibrosis models have also shown an elevation in cGMP levels and reduced myofibroblast formation upon administering NEPi/ARB combination[108]. Currently, there are no clinical studies assessing NEPi/ARB combination with a sole focus on kidney function. The data available on renal function is from studies in patients with heart failure or with a main focus on blood pressure management.

Another approach would be to target NPR3 with ligands that block peptide clearance. Osteocrin is a soluble peptide identified in osteoblasts that binds with high affinity to NPR3. A study conducted on adriamycin induced kidney injury showed a protective effect on podocytes in mice with high levels of circulating osteocrin, while the deletion of circulating osteocrin exacerbated adriamycin induced injury. Moreover, the study showed that the effect seen was mainly mediated by increase in NP signalling via NPR3 inhibition[109]. Treatment with NPR3 inhibitor ANP (4-23) in a murine model of FSGS showed a significant decrease in albuminuria and an improved expression of podocyte marker nephrin. However, only a trend towards better histopathological features and fibrosis was observed. This was attributed to the rapid degradation of ANP (4-23) in mouse serum[110]. Finally, two studies have reported the development of selective potent NPR3 inhibitors, with an increased intracellular cGMP level in cultured cells, and a substantial increase in plasma cGMP upon continuous administration in mice[103,111]. Yet, no experimental studies have been conducted to explore the role of these inhibitors in kidneys. Taken together, potential renoprotective effects of targeting the clearance of NPs locally in the kidney environment is looking promising, yet clearly more studies are needed to define the specific role of NPR3 and the clearing of NPs in kidney tissue.

1.6 Conclusion

CKD is a massive cause of morbidity and mortality. As the incidence of CKD keeps increasing worldwide, it is of critical importance to find new solutions to diagnose and treat renal disorders. The field of nephrology has been left behind in the progression of big data analyses due to several reasons, including complex (variable) phenotypes and morphologies, limited access to patient material and the kidney being a highly heterogenous organ. Adapting an integrative approach of target discovery and validation through the dynamic use of transcriptomics analysis, animal models and screening of patient materials can provide a platform to generate critical data on the disease pathogenesis and help us to take steps towards developing new tools and interventions to diagnose/treat CKD

2 Research aims

The overall aims of this thesis were: (i) To identify genes and pathways involved in the development and progression of glomerular disease, through transcriptomic approach using single cell RNA-sequencing. (ii) To analyse key genes of interest in animal models in health and disease.

The specific aims of the different papers are:

Paper I

Title: Single-cell RNA sequencing reveals the mesangial identity and species diversity of glomerular cell transcriptomes

Aim: To gain an insight to the molecular signature of the glomerular cells in mouse and human using scRNA-seq.

Paper II

Title: Profiling glomeruloprotective mechanisms of angiotensin converting enzyme inhibitors (ACEi) using single cell RNA-sequencing

Aim: To define the transcriptomic changes of the glomerulus induced by ACEi treatment in a mouse model of glomerulonephritis.

Paper III

Title: Retinoic acid receptor responder1 promotes development of glomerular diseases via the Nuclear Factor-kB signalling pathway

Aim: To identify the role of Rarres1 in the pathogenesis of glomerular disease

Paper IV

Title: Unraveling the role of natriuretic peptide clearance receptor (NPR3) in glomerular diseases

Aim: To identify the role of NPR3 in the pathogenesis of glomerular disease

3 Materials and methods

3.1 Ethical Consideration

3.1.1 Human Samples

The conducted studies complied with all relevant regulations regarding the use of human study participants and was conducted in accordance with the criteria set by the Declaration of Helsinki. The use of human material for this study was approved by the Ethical Review Board in Stockholm, Sweden, archive numbers 2010/579-31 and 2016/615-32. The study design and conduct of experimental procedures handling human material were carried out in accordance with relevant guidelines and regulations defined in the ethical permits. Renal biopsies were obtained from Karolinska University Hospital (Stockholm, Sweden) along with control samples from the healthy kidney poles of individuals who underwent tumor nephrectomies.

3.1.2 Animal work

For mouse studies all experimental protocols were approved by The Ethical Committee for Research Animals, Linköping, Sweden (archive numbers DNR 41-15 and 14071-2019). For rat studies, the experimental protocol was approved by The Ethical Committee for Research Animals, Gothenburg, Sweden (archive number 002327). All methods used in mouse/rat experiments were carried out in accordance with relevant guidelines and regulations defined in the ethical permits. All animals were housed in standard, single ventilated cages with 12 h light–12 h dark cycle and had ad libitum access to water and chow. The house temperature was maintained at 20 ± 2 °C and the relative humidity was kept at 50 ± 5%.

3.2 Animal Studies

3.2.1 Transgenic Mouse lines

Rarres1

Floxed Rarres1 mouse line was generated (C57Bl/6J background, Rarres1fl/fl; Cyagen). The mice were crossed with podocin (B6.Cg-Tg [NPHS2-cre] 295Lbh/J; Jackson Laboratory) or Tie2 cre mice (B6.Cg-Tg(Tek-cre)12Flv/J) to generate cell specific knockout mice. A conditional knock-in mouse line for Rarres1 was generated (C57Bl/6J background, Rarres1 cKI; Cyagen) by targeting a mouse Rarres1 gene into ROSA26 locus and by placing a floxed stop codon in front of the gene. The line was crossed with Tie2 cre mice to produce a mouse line overexpressing Rarres1 in endothelial cells. The Gt(ROSA)26Sortm1(CAG-Rarres1-T2AEGFP)/J mice were crossed with a Tie2-cre line to activate Rarres1 expression specifically in ECs.

Npr3

Floxed Npr3 mouse line was generated (C57Bl/6J background, Npr3fl/fl; Cyagen) in which exon 3 was targeted. The line was crossed with podocin cre (B6.Cg-Tg [NPHS2-cre] 295Lbh/J; Jackson Laboratory) to generate a cell-specific knockout mice.

Podocyte td-Tomato

Podocin cre mice (B6.Cg-Tg [NPHS2-cre] 295Lbh/J; Jackson Laboratory) were crossed with floxed STOP td-Tomato mice (B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J; Jackson Laboratory) to activate the td-Tomato expression specifically in podocytes.

Pdgfrb-EGFP

Pdgfrb-EGFP transgenic reporter animals were a kind gift from Christer Betsholtz [112].

Breeding and genotyping were done according to standard procedures. All animal studies were carried out in Preclinical Laboratory (Karolinska Institutet) according to the relevant guidelines and regulations.

3.2.2 Mouse perfusion for glomerular isolation

To isolate mouse glomeruli, anesthetized mice first were perfused with 1×HBSS to remove circulating blood, followed by perfusion of Dynabead (14013, Thermo Fisher). Kidneys were collected and kept in cold HBSS until handling.

3.2.3 Disease models and treatment

Anti-GBM glomerulonephritis model in mice

Animals were pre-immunized subcutaneously with a 50 μl sheep IgG Freund's complete adjuvant (F5881, Sigma-Aldrich). Four days later, 130μl of nephrotoxic serum (NTS) (PTX-001S, Probetex) was administered by an intravenous injection to induce nephrotoxic nephritis (NTN). Urine was collected and body weight recorded according to the study design. Animals were sacrificed 7 or 14 days after the induction of disease, and urine, blood and organs collected.

Treatment of glomerulonephritis model with ACEi inhibitor

After the induction of glomerulonephritis mice were randomly divided into ACEi- (200mg/kg of enalapril in drinking water, Sigma Aldrich) or vehicle-treated groups. Treatment continued throughout the length of the study.

Treatment of glomerulonephritis model with NPR3 inhibitor

NPR3 selective inhibitor (AZ7657, AstraZeneca) (9 mM) or vehicle (water/DMSO 50/50) was delivered continuously throughout the treatment period via Alzet micro-osmotic pumps (Model 1002, Agnthos AB, Sweden). The osmotic pumps were implanted subcutaneously in anesthetized mice one day prior to the induction of glomerulonephritis. The implantation was done according to manufacturer's instructions and following the guidelines of the ethical permit.

Rat diabetic kidney injury model and treatment with NPR3 inhibitor

Male ZSF rats were purchased at 10-week of age from Charles River (Charles River, USA). Two groups of obese (ZSF1-Lepr^{fa}Lepr^{cp}/Crl 378), and lean (ZSF1-Lepr^{fa}Lepr^{cp}/Crl 379) animals were used in the following experiments. After 2 weeks of acclimation period obese ZSF rats were uninephrectomised (UNx) by removal of the right kidney. The animals underwent a two-week recovery period, followed by randomization into different treatment groups. Lean animals were used as a control group throughout the experiment. The animals were followed for a total of 10-weeks.

3.3 Glomerular Isolation

3.3.1 Glomerular isolation from mouse tissue

Mouse kidney perfused with Dynabeads

Collected kidneys were minced and digested with collagenase IV (1 mg/ml, Thermo Fisher), pronase E (1mg/ml) (Sigma Aldrich) plus DNase I (50 U/ml, Thermo Fisher) at 37°C for 30 min, and passed through two 100-µm strainers and pelleted by centrifugation. The glomerular pellet was resuspended in HBSS, and beads-containing glomeruli were gathered by placing on a magnet holder. Purity of isolated glomeruli was validated under the microscope[113].

Mouse kidney adhesion method

Collected mouse kidneys were minced and digested with collagenase IV (1 mg/ml) at 37°C for 15 min and passed through two sieves from 100- to 75-µm strainers. The filtrate containing glomeruli and tubular fragments were collected using a 40-µm strainer. The enriched glomeruli were obtained after settling the mixture on a 10-cm culture dish for 1–2 min allowing adherence of tubules to the dish bottom[114].

3.3.2 Glomerular isolation from human tissue

Human glomerular isolation from nephrectomies

Kidney cortex was minced and passed through serial sieving (500 μm, 250 μm, 100μm) including multiple washing steps with cold PBS. The retaining glomerular fraction on the 100 μm was collected after multiple washes and pelleted by centrifugation[115].

Human glomerular isolation from biopsies

Minced kidney biopsies were first incubated with collagenase IV (1mg/ml) plus DNase I (50 U/ml) at 37 °C for 15 min, followed by a single sieving using a 300-μm strainer. The filtrate containing glomeruli and tubular fragments was passed through 100-μm strainer. Glomeruli retained on 100-μm strainers were collected by followed by multiple washes and pelleted by centrifugation.

3.4 Single-cell dissociation

The glomerular fractions were dissociated using digestion buffer (collagenase IV (1mg/ml)), pronase E (1mg/ml), and DNase I (50U/ml)) for 15 minutes, at 37°C, 300rpm. Single cells were collected, and non-digested glomeruli went through a second round of dissociation. The cell pellet was collected through centrifugation, washed, and passed through 50-μm filters (Filcon cup-type, BD Biosciences).

3.5 FACS sorting

To purify the cell suspension viable cells positive for CellTracker (CMFDA-Green, Thermo Fisher) were sorted into collection tube (1.5 ml Eppendorf) using FACSAriaTM (BD Bioscience) or into 384-well plate using FACSMelody (BD Bioscience).

To enrich certain cell populations, we excluded immune cells (CD45+) and endothelial cells (CD31+). Cells positive for CMFDA-Green and negative for CD45 and CD31 were sorted.

Cells isolated from reporter mice (Pdgfrb-EGFP, Podocyte tdTomato) were used in combination with DRAQ5 (Thermo fisher) staining for sorting.

3.6 Single Cell RNA Sequencing (ScRNA-seq)

3.6.1 SmartSeq2

Sorted single cells were lysed and reversely transcribed into cDNA using oligo(dT) primer and SuperScript II reverse transcriptase (Thermo Fisher). The synthesized cDNA was amplified and purified. cDNA was tagmented using Tn5 transposase followed by a standard index PCR using Illumina Nextera XT index kits (set A-D)[116]. cDNA/library quality

was validated using Bioanalyzer High Sensitivity DNA Kit (Agilent). Sequencing was performed using HISEQ 3000 (Illumina). Sequencing was performed by the single-cell core facility at ICMC, Karolinska Institute.

3.6.2 10x Genomics Chromium

scRNA-seq was performed using Chromium Next GEM Single Cell 3′ Reagent Kits v3.1 (10XGenomics). Volume of single-cell suspension used were calculated from the total cell density, aiming to achieve a minimum of 8000 cells per sample. cDNA synthesis and indexed libraries were prepared according to manufacturer's instructions, with 12 and 15 PCR-cycles used respectively. cDNA/library quality was validated using Bioanalyzer High Sensitivity DNA Kit (Agilent). Libraries were sequenced using Nextseq 2000 (Illumina). Sequencing of samples was performed by BEA core facility, Karolinska Institute.

3.7 Bioinformatic Analysis

3.7.1 SmartSeq2 data analysis

Raw sequencing data was de-multiplexed and aligned to mouse genome mm10 and human genome hg38, respectively, using the STAR aligner. Cells were filtered according to the recommended criteria and cells passing the quality control were included in the downstream analysis. Cell clusters were identified and assigned to a corresponding cell type based on known cell type marker genes. To identify genes differentially expressed between defined cell/sample groups, Kruskal–Wallis test with Benjamini–Hochberg multiple testing correction were performed on the log2-transformed expression data. For differential expression analysis between more than two groups, post-hoc tests were performed on all possible pairwise group comparisons. Genes that were significantly upregulated in one group vs the other groups (adjusted $P < 0.01$ and minimum fold change of 2) were considered as group-specific upregulated genes. Batch effects from different datasets were adjusted using the empirical Bayes method ComBat. Detailed information on the bioinformatic analysis performed for mesangial like cell phenotype prediction and trajectory analysis, identification of conserved species-specific genes, and crosstalk analysis can be found in paper I.

3.7.2 10x Genomics data analysis

The 10x raw sequence data were processed in Cell Ranger software (v6.0.1) (10X Genomics) and the reads were aligned to the mouse reference genome mm10 to generate a gene-cell counts matrix for each sample. The raw counts matrix was then loaded in R (version 3.6.1) for downstream processing. Seurat package (version 3.1.1) was used for quality control, filtering, normalization, clustering, and further analysis[117]. The cells which have less than 500 genes or mitochondrial genes greater than 10% were filtered out. For normalization, a scale factor of 10,000 was applied to the qualified cells. The UMAP

dimension reduction and Seurat clustering were performed using the first 30 principal components and with other default parameters in Seurat. After clustering, FindAllMarkers function was applied to identify the marker genes for each cluster. The cluster markers were manually examined in the literature and also canonical kidney cell type markers were checked in the clusters before the final annotation. For differential expression analysis, the two cell groups in each cell cluster were compared using the FindMarkers in Seurat. It identifies differentially expressed genes between the two groups using a Wilcoxon Rank Sum test. To visualize and compare the differential expression result, the volcano plot was applied to the different cell types using the EnhancedVolcano package (version 1.2.0) in R software. For pathway analysis, differentially expressed genes were analyzed by EnrichR (https://maayanlab.cloud/Enrichr/). For MCs, the criteria for gene selection were the default lists of differentially expressed genes (DEGs) from Seurat FindMarkers function. For other cell types the criteria for gene selection was: Individual p-value of <0.05 fold, fold change (log) between the two groups of >0.1 and gene expressed in more than 10% of the cells.

3.8 Sanger Sequencing

cDNA from wildtype (WT) and conditional knockout glomeruli ($NPR3^{PodKO}$) was amplified using PCR. The amplification product was isolated using Zymoclean Gel DNA Recovery Kit (ZymoResearch) according to the manufacturer's instructions. Sanger sequencing was performed at KIGene facility at Karolinska Institutet, Solna following standard protocols.

3.9 Cell Culture

Human podocytes were cultured as described previously[118]. JetPEI transfection reagent (Polyplus-transfection SA) was used for stable transfection of pRP[Exp] CMV>hRARRES1[ORF011242]:T2A:Bsd – plasmid (VectorBuilder Inc.). Clones were expanded under puromycin (Merck KGaA) selection. Transfection of short interfering RNA (siRNA) for Axl/Rarres1 was performed using siRNA from Origene. Cells were transfected with 30 nM siRNA followed by complexation with JetPEI transfection reagent (Polyplus-transfection SA) with or without TGF-β stimuli (10 ng/ml in culture medium). The Axl-inhibitor R428 (Selleck Chemicals) was added 1 hour before treatment (3 mmol/l).

3.10 Immunostaining, Histology and Imaging

3.10.1 Immunofluorescence

Immunofluorescence of mouse or human cryosections

Mouse and human frozen kidney sections (5-8 μm) were fixed in ice-cold acetone and blocked using 5% or 10% normal goat serum solution for 1hr at room temperature. Sections were incubated with primary antibodies at 37 °C for 1 h or 4 °C overnight, followed by

incubation with corresponding Alexa Fluor conjugated secondary antibodies (Invitrogen) and Hoechst 33342 (Thermo Fisher) staining for nuclei. Sections were mounted in Dako fluorescent medium (S3023, Agilent).

Immunofluorescence of mouse or human paraffin sections

Mouse or human kidneys were fixed with 4% paraformaldehyde (PFA) and subsequently embedded with paraffin. Paraffin-embedded kidney sections (4μm) were deparaffinized and microwave-treated in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0), blocked using 10% goat serum + 0,1% Tween20 or Dako universal blocking solution (Agilent) for 1hr at room temperature. Sections were incubated with primary antibodies at 4 °C overnight. Followed by incubation with corresponding Alexa Fluor conjugated secondary antibodies (Invitrogen) and Hoechst 33342 (Thermo Fisher) staining for nuclei. Final step of incubation with sudan black B (0,1% in 70% Ethanol, #199664, Sigma-Aldrich) was performed followed by mounting in Dako fluorescent medium (S3023, Agilent).

3.10.2 Immunohistochemistry

Immunohistochemistry of mouse or human paraffin sections

Mouse or human kidneys were fixed with 4% PFA and subsequently embedded with paraffin. Paraffin-embedded kidney sections (4-6μm) were deparaffinized and microwave-treated in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0), or sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Followed by permeabilization with 0.3% Triton X-100 and blocked with 10% normal goat serum, 3%hydrogen peroxidase, and the Vector Blocking Kit (Vector Laboratories).

Slides were developed using the DAB peroxidase (horseradish peroxidase) substrate kit, 3,30-diaminobenzidine (VectorLaboratories). Incubation with primary and secondary antibodies were performed as mentioned above. Nuclear counterstaining was performed using Mayer hematoxylin solution.

All images were observed on a Zeiss Observer Z1, confocal Leica TCS SP8 or confocal Zeiss LSM 710.

3.10.3 Histopathology

Collected tissue were fixed in 4% PFA followed by embedding into paraffin and sectioning (4μm). Histopathology of animals was evaluated using hematoxylin/eosin, trichrome and periodic acid–Schiff-stained sections by scoring at least 30 glomeruli/mice as “normal” or “abnormal” (including segmental sclerosis, crescents, mesangial expansion, and necrosis). To assess the extent of tubular cast formation, the whole cross-section was

evaluated (score was determined as follow: 0, no casts; 1, >0-10%; 2, 11-30%, 3, >30% of the tubules with casts).

3.10.4 RNA *in situ* Hybridization

The RNAscope analyses were performed in paraffin-embedded human kidneys according to the manufacturer's protocol (ACD). The probes used were NPHS1 - Cat No. 416071-C2, PECAM1 - Cat No. 487381-C3, and RARRES1 - customized probe targeting nucleotides 291-1515.

3.10.5 Transmission Electron Microscopy

Kidney samples were fixed in 2.5% glutaraldehyde, TEM was carried out according to the standard protocols by the electron microscopy unit (Emil) at Karolinska Institutet, Huddinge University hospital.

3.11 RT-PCR and quantitative PCR

Total RNA was extracted using RNeasy mini kit (Qiagen). First-strand cDNA synthesis was carried out using the iScript cDNA Synthesis Kit (BioRad).

For RT-PCR human glomeruli and other animal glomeruli were used (WT mouse, WT rat, minipig and cynomolgus monkey). Mouse and rat multiple tissue cDNA panels (#636745 and #636751, TakaraBio) were used as positive controls. The PCR reaction was performed onto a PCR cycler (Bio Rad) and the size of the PCR amplicons was determined by running 1% agarose gel electrophoresis with GeneRuler 1 Kb plus DNA ladder (ThermoFisher).

For real-time qPCR analysis, the CFX96Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad) were used. The relative gene expression was calculated with the $2^{-\Delta\Delta Cq}$ method normalizing the gene of interest to housekeeping gene in the same sample. Data are presented as relative fold-change.

3.12 Western Blot

Cells and kidney fractions were were lysed with RIPA lysis buffer (ThermoFisher) supplemented by protease/phosphatase inhibition cocktails (Roche). The protein content of the samples was determined using Pierce™ bicinchoninic acid assay kit (ThermoFisher Scientific). Equal amounts of protein were separated on sodium dodecylsulfate–polyacrylamide gels (Invitrogen), blotted onto polyvinylidene fluoride membranes and blocked in 5% nonfat dry milk or 5% bovine serum albumin in triethanolamine-buffered saline with 0.1% Tween-20. Western blots were incubated with primary antibodies overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies and Clarity Western ECL Substrate (Bio-Rad) were used to detect signals.

3.13 Biochemistries

The urinary albumin/creatinine ratio was measured by using the commercial kits Albuwell (#1011, Exocell) and Quantichrome creatinine assay kit (#DICT-500, Bioassay systems). Serum and urine cGMP were measured using enzyme immunoassay kit (#CG201-1KT, Sigma-Aldrich).

3.14 Phagocytosis Assays

3.14.1 Phagocytosis in *ex vivo* mouse kidney

EGFP+ glomerular cells from Pdfrb-EGFP reporter mice were used to collect mesangial cells. pH-sensitive fluorescent latex beads (Protonex Red 600-Latex Bead Conjugate, AAT Bioquest), were used for phagocytosis assay. Isolated cells were mixed with the beads and incubated for 30 minutes at 37 °C with mild shaking. Phagocytosis was terminated using cold HBSS. To determine the baseline of bead-positive cells, tubular cells isolated from WT mouse were incubated with the beads for 30 min at 4°C. Cells were analysed on FACSMelody and gated for viable cells (DRAQ5+) and bead fluorescence Red 600. To visualize bead+, EGFP+ cells, isolated glomerular cells from Pdgfrb-EGFP mice were treated with beads at 4 °C and 37 °C, respectively. Visualization was performed on Zeiss confocal microscope (SLM 710).

3.14.2 Phagocytosis *in vitro* human glomerular cells

Isolated glomeruli were resuspended with cell culture media (RPMI 1640 media containing 10% FBS, 1% penicillin/streptomycin and 1% insulin transferring selenium A, ThermoFisher). Glomeruli were seeded onto fibronectin (SigmaAldrich) coated glass coverslips and at 33 °C and 5% of CO2 in cell culture media. On day 14, phagocytosis assay was performed using Protonex Red 600-Latex Bead Conjugate (AAT Bioquest, 21209) according to manufacturer's instructions. Cells were fixed using 4% PFA-PBS and stained with primary antibody overnight at 4 °C, followed by Alexa fluor secondary antibody (ThermoFisher). Imaging was performed with Leica SP8 confocal microscope.

3.14.3 Albumin uptake by mesangial cells *in vivo*

WT mice were intraperitoneally injected with 400 µl of 0.5 mg/ml BSA or BSA-fluorescein isothiocyanate conjugate (Merck KgaA). After 1 h, the mice were sacrificed, and kidneys were fixed in 10% neutral buffered formalin solution at 4 °C overnight. Slides were deparaffinized and stained according to the standard procedure mentioned above. Prior to imaging, samples were immersed in 80.2% (w/w) fructose with 0.25% 1-thioglycerol and mounted in a MatTek dish (MatTek P35G-1.5-14-C). A Leica SP83X STED system was used for imaging.

3.15 Statistical Analysis

Statistical analysis was performed with GraphPad Prism software (La Jolla, CA). In paper I The Chi-square test with Yates correction or Fisher's exact test was used to estimate statistical significance. In paper II and IV *in vitro* and *ex vivo* experiments, data were analysed using Student's t-test or one-way ANOVA followed by Tukey's multiple comparisons test when appropriate. For multiple treatment groups comparisons mixed model ANOVA was used. In paper III the data were first examined for normality (Shapiro-Wilk test). When the distribution was Gaussian, the data were analysed using a parametric test (Student's t test for 2 groups, and analysis of variance with Tukey's post-test for more than 2 groups). When the distribution was not Gaussian, nonparametric tests (Friedman test with Dunn's post-test for more than 2 groups and the Mann-Whitney U test for 2 groups) were used. The differences were considered significant when $P < 0.05$.

4 Results and Discussion

4.1 Paper I. Single-cell RNA sequencing reveals the mesangial identity and species diversity of glomerular cell transcriptomes

In this study, we used Smart-seq2 platform to identify the transcriptomic profile of different glomerulus-associated cells. We managed to capture and annotate all the main glomerular cell types: podocytes (*Nphs1*$^{+}$/*NPHS1*$^{+}$), endothelial (*Pecam1*$^{+}$/*PECAM1*$^{+}$) and mesangial like cells (*Pdgfrb*$^{+}$/*PDGFRB*$^{+}$), along with tubular cells and immune cells. Additionally, this was the first study in which distinct population of parietal epithelial cells (PECs) and macula densa cells (MDC) were described.

Since Pdgfrb is known as a mural cell marker and not limited to the MCs, we investigated in detail the mesangial like cell (MLC) population. Four distinct subpopulations were identified with clearly different transcriptional profiles. Two populations were vascular smooth muscle cells (vSMCs) from the afferent and efferent arteriole (and some of them renin producing cells). Through validation studies using a reporter mouse line and immunostainings the third population was confirmed to be true glomerular MCs, and the following combination of cell markers were assigned to it (*Pdgfrb*$^{+}$, *Gata3* $^{+}$, *Pdgfra*$^{+}$). Interestingly, the fourth population was a unique mural cell type found in the stalk of the glomerular tuft, corresponding to the previously described extra glomerular MCs/ Lacis cells. Not only were we able to distinguish this cell group from other mural cell populations, but we also captured their distinct transcriptional profiles.

After the identification of MCs, we aimed to get more information on their molecular machinery through pathway analysis and ligand-receptor analysis with endothelial cells and podocytes. MCs showed to possess pericyte and fibroblast-like molecular profiles, supporting their role in the regulation of glomerular filtration and matrix production. Interestingly, we also identified the presence of a prominent phagocytic machinery in MCs. Moreover, we identified potential crosstalk pathways related to phagocytosis, for example the highly expressed multifunctional scavenger protein (LPR1) that was expressed by MCs and its ligands on endothelial cells. We further validated the phagocytic activity through two *in vitro* assays in which a robust phagocytosis was detected in isolated mouse and human Pdgfrb^{+} glomerulus-associated cells. Moreover, in an *in vivo* assay we detected the accumulation of FITC-labelled bovine serum albumin (BSA) inside MCs shortly after introducing them to the circulation.

Since we profiled both mouse and human glomeruli, we were able to analyse and compare transcriptomes between the species. As expected, most genes were expressed similarly in both human and mouse glomerular cells, such as genes essential for their function (such as in podocytes NPHS1, NPHS2 and SYNPO). Nevertheless, 47 human specific and 33 mouse specific podocyte-expressed genes were identified. MCs showed 75 genes with

species-specific patterns between mouse and human, such as mouse specific *Des* and human specific *COL6A1*. For endothelial cells, only six human specific and no mouse-specific genes were identified. Especially of interest was *RXFP1* gene which is a receptor for relaxin and is being evaluated as a possible therapeutical target for kidney diseases. Our data highlights the translational challenges in the field as even in healthy state glomerular cell types demonstrate significantly different molecular signatures between mouse and man.

MCs play a major role in maintaining glomerular structure and function, and have been described as specialized microvascular pericytes of the glomerulus[119]. Pdgfrb has been used to identify these cells, yet it is not very specific. In our study, we identified the subclusters of glomerulus-associated Pdgfrb^{+} cells and reported their distinct transcriptomes. The resolution of transcriptional profiling using scRNA-seq allowed us to determine the set of genes needed to identify specific subpopulations. Additionally, we gained an insight into the different functions of these cell populations. In the current study we used Smart-seq2 platform, the only platform that provides a full length read coverage of the transcriptome on a single cell level[120]. Regardless of the relatively small number of cells profiled compared to other published studies, we provided a higher gene detection rate and better data quality. This improved the downstream analysis significantly and allowed us to get many novel insights into the biology of glomerulus-associated cells.

In conclusion, we have shown the power of scRNA-seq as a platform to determine the transcriptional molecular profiles of glomerulus-associated cells. We have demonstrated the ability of this platform not only in identification of cell populations but also in defining the precise cellular identity of cells, capturing rare cell types, and exploring the cross-species differences. Our findings provide an insight into the glomerular mesangium and highlight the species diversity which provides a guide into future translational studies.

4.2 Paper II. Profiling glomeruloprotective mechanisms of angiotensin converting enzyme inhibitors (ACEi) using single cell RNA-sequencing

In this study, we utilized scRNA-seq to gain a deeper understanding on the glomeruloprotective molecular mechanisms of ACEi. We induced glomerulonephritis and massive albuminuria in mice by a single injection of nephrotoxic serum. Treatment with ACEi ameliorated albuminuria by the 7th day and histologically, less glomerular damage was observed as demonstrated by a significant decrease in mesangial expansion, fibrinoid necrosis and tubular casts.

Sequenced cells from glomerular fractions of ACEi- and vehicle-treated animals resulted in a total of 43,582 good quality sequenced cells to be included in subsequent analysis. 14 distinct cell clusters were identified including principal glomerular cells (podocytes, glomerular endothelial cells, MCs), glomerulus-associated cells (vSMCs of afferent and efferent arteriole, PECs, extra glomerular endothelial cells, renin cells) and immune cells (B-cells, T-cells, neutrophils, natural killer cells, macrophages, mast cells).

Next, we compared the transcriptomes between ACEi- and vehicle-treated groups across all cell types. MCs showed the highest number of DEGs with 50 up and 119 downregulated genes. When molecular pathways and biological processes were analysed, we detected a downregulation of pathways related to ECM, cytoskeletal regulation and cell adhesion. ECM production and accumulation are directly related to fibrosis in later stages; therefore, we measured glomerular fibrosis in the different treatment groups. ACEi-treated mice showed a significant decrease in fibronectin levels in both glomeruli and whole cortex. For the upregulated genes, several processes were related to smooth muscle-like phenotype. These findings are in line with a previously reported role of MCs in contractility and regulation of glomerular filtration area. Moreover, upregulation of pathways related to cell proliferation could be an explanation for the decreased mesangial proliferation seen histologically. When we analysed the expression of AngII receptor, it was only detected in MCs and vSMCs, which could be an explanation to the observed effect.

Somewhat surprisingly, only few molecular changes were observed in podocytes regardless of the strong anti-albuminuric effect of ACEi-therapy. This was in line with our electron microscopic findings as no difference in foot effacement or GBM thickness was observed. Similarly, no difference was seen in the number of podocytes per glomerulus.

Endothelial cells were one of the largest cell populations sequenced, yet, despite the large number of cells only 28 up and 10 downregulated DEGs were detected. These included the downregulation of Cyp26b, Dhrs3 and Lpl that are connected to retinoic acid metabolism. Retinoic acid has been shown to have reno-protective mechanisms in different animal models [121]. Similarly, a downregulation of endoglin (Eng) was observed,

which is known to be upregulated during injury and thought to interact with TGF-β receptors and activating the pathway [122,123]. On the other hand, we found an upregulation of several members of kruppel like factors family (Klf), that are known to play a role in endothelial biology and homeostasis through their anti-inflammatory and anti-thrombotic actions[124].

We were able to capture distinct transcriptional profiles for the vSMCs of the afferent and efferent arterioles, that are believed to be the main responders to ACEi treatment. However, we were not able to detect any DEGs in the initial comparison between the treatment groups. This could be secondary to the low number of cells captured for each population.

Finally, we took a closer look into PECs that are speculated to act as progenitor cells for podocytes in disease [125]. Upon further analysis 4 subclusters were identified, of which one expressed several podocyte genes along with known PEC marker Cldn1 and progenitor marker Ncam1. To avoid the possibility of contamination we compared the subcluster to podocytes. Several podocyte specific genes overlapped, yet certain genes were only expressed in only one of the populations (podocytes or PEC subpopulation), speaking strongly against the possibility of a contamination. This finding suggests that there is a subtype of PECs that is differentiating into podocytes, supporting the idea of PECs as podocyte progenitor cells. However, when we compared the transcriptome changes between ACEi and vehicle treated animals, no DEGs were detected PECs.

These results suggest that MCs have an important role in the response to ACEi treatment, especially in the early stages of the disease. Obviously, this can be attributed to the fact that AngII was highly expressed by these cells. Maintenance of glomerular homeostasis and function is a result of a dynamic interaction between different cell types [6], where signalling from one cell to the others plays an important role. We can speculate that the minimal involvement of other cell types could be a result of choosing an early time point for profiling. It is important to note that we cannot exclude that the transcriptional changes seen are secondary to the systemic hemodynamic effect and the regulation of glomerular pressure driven by ACEi. Further profiling of ACEi treatment in different disease models (with blood pressure monitoring) and in human kidney tissue is needed to conclude the extent and type of involvement of different cell types.

In conclusion we have shown the power of using scRNA-seq approach in generating a comprehensive view on the molecular responses of different glomerular cell populations to ACEi treatment in an animal model of glomerular injury.

4.3 Paper III Retinoic acid receptor responder1 promotes development of glomerular diseases via the Nuclear Factor-kB signalling pathway

One of the targets identified in previous transcriptomic studies that caught our attention was Rarres1. When we assessed the expression pattern of Rarres1 we found it to be upregulated in the glomerular fraction in both mouse and human. Immunofluorescent staining in human kidney tissue showed Rarres1 to be highly expressed in podocytes.

To elucidate the role of Rarres1 in podocytes, we performed a series of *in vitro* experiments on cultured podocytes where we over-expressed (stable transfection) and downregulated Rarres1 (siRNA). After the induction of NFκB signalling pathway through treatment with TGF-β, Rarres1 overexpressing cell lines showed a significant increase in NFκB activation. As Rarres1 has been linked to activation of tyrosine kinase receptor Axl, we went further to investigate whether Axl is a potential functional partner of Rarres1 in kidney tissue. A positive correlation between Rarres1 over-expression and constitutive activation of Axl was detected. Moreover, treatment with selective Axl inhibitor (R428) in TGF-β treated cells inhibited NFκB activation. However, only the inhibition of Axl and not downregulation (using siRNA) showed an effect on EMT genes. Therefore, we concluded that Rarres1 promotes NFκB signalling in cultured podocytes via Axl activation.

To analyse the role of Rarres1 in podocytes *in vivo*, we generated a transgenic mouse line with a specific KO of Rarres1 in podocyte ($Cre^{+}/Rarres^{fl/fl}$). $Cre^{+}/Rarres^{fl/fl}$ were viable, fertile and no abnormalities in kidney function or structure were observed. In response to injury (NTS-induced glomerulopathy model), no differences were seen in albuminuria levels, histological and electron microscopy readouts, or expression of podocyte, fibrosis, and inflammatory genes between $Cre^{+}/Rarres^{fl/fl}$ and controls.

Moving on, to explore the role of Rarres1 in CKD we analysed publicly available RNA datasets. A significant elevation of Rarres1 was detected in glomeruli isolated from patients with DN, IgAN and ANCA-associated vasculitis. We confirmed the upregulation using qPCR analysis on a glomerular fractions isolated from DN patients. Additionally, we evaluated immunohistochemically renal biopsies from patients with DN, IgA and ANCA-associated vasculitis. In these diseases, Rarres1 staining seemed localise to endothelial cells of the glomerulus and peritubular capillaries. Double validation of this observation was done using immunofluorescence and RNAscope in which Rarres1 colocalised with endothelial markers in patient samples.

To elucidate the role of Rarres1 in endothelial cells in both health and disease, we generated two transgenic mouse lines: one with an overexpression of Rarres1 in endothelial cells (Rarres1_Tie2_KI) and the other with inactivation of Rarres1 in endothelial cells (Rarres1_Tie2_KO). Both lines showed no kidney abnormalities under healthy conditions. Next, to know whether Rarres1 expression in endothelial cells modulates the

disease progression, we challenged the mice with NTS serum injection and followed them up for 14 days. Rarres1_Tie2_KI developed significantly higher albuminuria after 3 days, an effect that was not detected at later stages (day 7,10 and 14). At the end of the experiment, electron microscopic evaluation showed a significant increase in GBM thickness and podocyte foot effacement. Moreover, profound reduction in podocyte markers (nephrin, synaptopodin, WT1) were seen along with a lower number of WT1 positive podocytes per glomerulus. Additionally, an upregulation in genes related to inflammation and fibrosis was seen. Importantly, an opposite effect was seen in Rarres1_Tie2_KO with less glomerular alterations, including GBM thickness, foot processes effacement and a higher number of WT1 positive podocytes per glomerulus.

To determine the connection between Rarres1 and Axl *in vivo,* we analysed kidney tissues collected from Rarres1_Tie2_KI and Rarres1_Tie2_KO animals. Mice challenged with NTS showed an activation of Axl pathway as measured by its phosphorylation. Importantly, the activation was significantly higher in Rarres1_Tie2_KI mice compared to controls. In line with this, we observed an increase in a number of pP65 positive nuclei in Rarres1_Tie2_KI glomeruli showing an increased activation of the NFκB pathway.

Rarres1 raised our interest first by being a highly podocyte-associated gene, and studies in cultured podocytes showed its role in promoting Axl-mediated NFκB pathway. However, no differences were observed in podocyte knockout models neither in health nor disease. We speculate that this could be due to the compensatory mechanisms as NFκB signalling pathway can be modulated by multiple pathways/molecules. Rarres1 showed to be upregulated in endothelial cells in patient biopsies of different kidney diseases. We showed that the induction of Rarres1 in endothelial cells *in vivo* exacerbated the renal injury while the inactivation ameliorated it. Yet, the direct link to Axl activation of NFκB needs to be confirmed in endothelial cells *in vitro*. Based on these results we speculate that Rarrres1 could act as a new biomarker for diabetic microvascular disease and other glomerulopathies. More studies are needed to analyse the levels of Rarres1 in urine and plasma of these patients to determine the possibility of using it as a non-invasive biomarker. Finally, targeting Axl and NFκB as a strategy to preserve kidney function has been studied as a therapeutic option. However, due to the global activity of these pathways, they can be a challenging target in CKD. Therefore, Rarres1 can be a good candidate as a more kidney-associated target to modulate Axl and NFκB pathways.

In conclusion, we have demonstrated the upregulation of Rarres1 in common human glomerulopathies. In disease, Rarres1 is induced in endothelial cells promoting inflammation, fibrosis, and podocyte injury. The effects are potentially mediated by the activation of NFκB pathways via receptor tyrosine kinase Axl.

4.4 Paper IV Unraveling the role of natriuretic peptide clearance receptor (NPR3) in glomerular diseases

NPR3 was one of the genes reported in previous transcriptomic studies that caught our attention. Expression of NPR3 was higher in the glomerular fraction compared to rest of kidney tissue (in both mouse and human). Additionally, we found it to be highly upregulated in mouse podocytes by qPCR and in previously reported scRNA-seq studies. In human tissue, we confirmed the findings as NPR3 immunostaining co-localised with podocyte marker synaptopodin.

To analyse the role of NPR3 in podocytes *in vivo*, we generated a transgenic mouse line with a specific KO of NPR3 in podocyte ($NPR3^{PodKO}$). $NPR3^{PodKO}$ were viable, fertile, and developed normally, with no differences observed in them compared to their littermate controls. Next, we wanted to assess whether $NPR3^{PodKO}$ contributes to the development or the progression of glomerular disease. Therefore, we induced glomerulonephritis in mice by injecting a single dose of NTS serum. No differences were seen in albuminuria levels, histological and electron microscopic evaluations, or in a number of WT1 positive podocytes between $NPR3^{PodKO}$ and WT controls.

Then, we wanted to test whether pharmacological inhibition of NPR3 can affect the outcomes of NTS-induced glomerular damage. We treated NTS-challenged WT mice with a selective NPR3 inhibitor that was delivered continuously over the treatment period (14 days) using subcutaneous osmotic pumps. When we compared challenged mice treated with NPR3i to vehicle-treated controls, we found less histological changes by evaluating a number of affected glomeruli and presence of fibrinoid necrosis. Additionally, we observed higher WT1-positive podocyte numbers per glomerulus in NPR3i-treated animals, suggesting a partial protection of podocytes by the inhibitor. Fibrosis marker α-SMA showed a trend towards less fibrosis in NPR3i-treated mice, but the result was not significant. No differences were seen on albuminuria levels, GBM thickness or foot process effacement.

Next, we wanted to test pharmacologic inhibition in a model of DN. Uninephrectomised obese ZSF rats (Unx-ZSF) were treated with NPR3i through subcutaneous injections, and animals were followed for a total of 10 weeks. In this experiment, we also combined NPRi with ARB as targeting natriuretic peptide clearance through neprilysin inhibition had shown previously a beneficial effect in DN. We started by confirming target engagement of NPR3i, and measured cGMP levels in urine and serum after treatment. We could detect an increase in urinary cGMP levels after increasing the dose of NPR3i (from 3 times per week to once daily, subcutaneous injections). Following the adjustment, a significant increase in urinary cGMP was observed in NPR3i and NPR3i+ARB groups. Of note, plasma cGMP levels were only increased in NPR3i treatment alone.

Unx-ZSF rats developed progressive albuminuria over the duration of treatment. NPR3i alone did not impact albuminuria levels, GBM thickness, foot process effacement, or a number of WT-1 positive podocytes. In histological evaluation, a slightly lower percentage of affected glomeruli was detected but this was not statistically significant. ARB treatment, on the other hand, significantly reduced albuminuria, and the combination of NPR3i+ARB showed even further decrease in albuminuria levels. Histological changes and fibrosis measured by α-SMA levels were slightly lower in NPR3i+ARB compared to ARB alone, yet not significant. Similar to NPR3i treatment alone, no changes were seen in GBM thickness, foot process effacement or in a number of WT1 positive podocytes per glomerulus between ARB and NPR3i+ARB treatment groups.

NP system is known to be a major player in the regulation of cardiovascular and renal homeostasis. Additionally, several studies have highlighted its role in the glomerular environment [102,126,127]. In our study, we explored the possibility of manipulating local NP levels in the glomerulus through NPR3 inactivation and inhibition. Genetic inactivation of NPR3 in podocytes did not affect kidney function or structure, neither in health nor disease. This could potentially be explained by the systemic or extra-glomerular compensation of NP system [128,129]. Pharmacological inhibition of NPR3 showed variable reno-protective results ranging from only effects on albuminuria to few histological readouts. The discrepancies seen between NPR3 inactivation and inhibition could be explained by the short-term vs long-term manipulation of the system and the compensatory mechanisms present. Alternatively, the beneficial effects of pharmacological inhibition could be attributed to the extraglomerular inhibition of NPR3. Nevertheless, NPR3 inhibition showed variability in reno-protective effects depending on the disease model, dose/route of inhibitor delivered and the duration of treatment. We speculate that this is partially due to the fast degradation of peptides, especially as improved target engagement was observed with more frequent dosing (in the rat diabetic model). This is in line with a previous report in FSGS model treated with NPR3 inhibitor[110]. On the other hand, we cannot exclude that the reno-protective effects of NPR3 inhibition are partially through the global regulation of blood pressure.

In conclusion, the pharmacological inhibition of NPR3 could be a possible strategy to manipulate NP system in the renal environment. Further studies are needed to determine the dose, route of delivery and the right animal model to conclude whether NPR3 targeting can be beneficial in kidney disease.

5 Conclusions

Glomerular diseases are one of the leading causes of CKD, a major health burden affecting about 10% the global population. In this thesis we aimed to get a better insight into genes and pathways involved in the development and progression of glomerular diseases.

In the first part of the thesis (**Paper I and II**), we utilized the transcriptomic approach using scRNA-seq to get an insight into the kidney glomerulus in various conditions: health, disease, drug treatment and in mouse vs human.

In **paper I** We successfully identified the transcriptomic profile of various glomerular cells, in both mouse and human. We highlighted the advantage of scRNA-seq in defining the real transcriptomic signatures of specific cell types ($Pdgfrb^{+}$) and gained a better insight into the functionality of those cells. We were also able to capture and report some rare cell-types along with their detailed transcriptional profiles. Additionally, we explored the cross-species gene expression differences in several glomerular cells, which provides the knowledge needed for future translational studies.

In **paper II** we profiled the commonly used ACEi inhibitors in a model of glomerular injury in mice. We reported all the main glomerular and glomerular-associated cell types along with their transcriptomic profiles in both vehicle-treated and ACEi-treated mice. scRNA-seq gave us a clear insight into the cell populations mainly affected by the treatment. We identified MCs as the main and early responder to ACEi treatment, with downregulation in genes and pathways involved in ECM production and accumulation which were in line with a decrease in fibrosis levels. Despite the clear anti-albuminuric effect, neither podocytes nor endothelial cells showed big transcriptomic changes after treatment. However, we were able to pinpoint several genes in these cells linked to different anti-inflammatory and anti-fibrotic pathways.

These two studies show the advantage of scRNA-seq as a tool to gain insight into the molecular signatures of heterogenous and complex tissue like the kidney.

In the second part of the thesis (**Paper III and IV**), we verified some of the transcriptomic findings (i.e., genes of interest) in animal models in both healthy and disease conditions. From previous studies we had identified two genes that were highly upregulated in the glomerulus and altered expression in disease states: Rarres1 and NPR3.

In **paper III** we investigated the role of Rarres1 in the glomerulus. Although highly expressed by podocytes. Its inactivation in mouse podocytes did not result in renal abnormalities or affect the outcome of NTS-induced glomerulopathy. On the other hand, we demonstrated the upregulation of Rarres1 in endothelial cells in patients with various glomerular diseases. The overexpression of Rarres1 in endothelial cells, promoted the progression of glomerular and kidney damage in a mouse model of glomerulonephritis,

through inflammation, fibrosis, and podocyte injury. The effects observed were potentially mediated through increased activation of NFκB pathway via receptor tyrosine kinase Axl. Thus, we propose Rarres1 as a promising therapeutic target and a possible injury biomarker.

In **paper IV** we looked into the role of natriuretic clearance receptor NPR3 in the glomerulus, and especially into the possibility of manipulating glomerular NP system through NPR3. Although highly expressed by podocytes, the inactivation of NPR3 in podocytes did not affect kidney development nor the progression of NTS-induced disease. However, pharmacological inhibition of NPR3 showed variable reno-protective results in different animal models. The protective effects seemed to be dependent on the dose, route of delivery and the animal model used, all of which need further optimisations to determine the best practice. Therefore, we concluded that manipulation of NP system through NPR3 inhibition not through NPR3 inactivation in podocytes could be a promising strategy to treat glomerulopathies. yet further studies are needed.

In conclusion, in this thesis we demonstrated the power of transcriptomic approach in determining new targets of interest. As well as the importance of further validation of those targets or other findings in both *in vitro* and *in vivo*.

6 Points of perspective

Incidence of CKD is rising, with the disease threatening not only high-income countries but spreading all over the world. There is a great need to find new interventions to stop the progression of CKD or even reverse the damage [3,5]. This is challenging especially with the complex nature of the kidney as an organ and the existence of many comorbidities[30]. The goal of this thesis work was to contribute to the knowledge on the pathogenesis of CKD by focusing on the glomerulus, a micro-organ of the kidney that plays a key role in the development of most CKD cases.

We chose transcriptomics as an approach to study the molecular mechanisms contributing to the pathogenesis. The use of scRNA-seq as a tool has gained popularity in the recent years. Yet, with the increased interest and the vast amount of data generated comes several challenges. One of the topics we have touched upon earlier is the need of a standardized bioinformatics approach. In the era of big data, we are facing new challenges, starting from having suitable storage units, data analysis tools and ending with having a proper expertise to analyze the data and put it in the perspective of biological events [130]. Another challenge is that a lot of the studies are only "scratching the surface", with lots of researchers opting to provide an atlas for a specific organ or disease. These cell atlases provide a great resource for researchers to mine the data from different materials of different species. However, it is of high importance to provide the right tools for data visualization, easy access, and comparison for the data sets [130]. For the data generated in **paper I,** we have developed an open access platform that can be used by other researchers (https://patrakkalab.se/kidney), with the aim to develop a similar platform for **paper II**. In **paper II,** we used a lot of information generated in **paper I** to identify baseline expression in WT mice and human tissue. Additionally, we have used the published data from Chung, Jun-Jae, et al.[58] to compare the data generated from WT mice and a NTS- induced glomerulopathy model.

CKD is often handled as a single entity regardless of different etiologies contributing to it. This can be attributed to the poor understanding of the molecular mechanisms of the underlying causes or the limited treatment options available. Present treatments are widely initiated in different populations, mostly due to their inexpensive nature and low potential risk[5,30]. Treatments like ACEi, ARBs and recently introduced SGLT2i have managed to slow the progression and improve the outcomes. Better understanding on the molecular mechanisms of these agents will not only contribute to a better utilization of those agents but also potentially can help in the development of novel therapies and interventions. That was our goal in **paper II,** to gain a better insight into the cellular players, genes and pathways involved in ACEi intervention. While this thesis was in preparation, Wu, Haojia, et al.[68] , published a study with a similar approach, with treatments targeting

DN. This shows that mapping the molecular profiles under therapy is of interest, and hopefully will results in improved therapies and interventions.

Translation from animal models to human has been a challenge in the field of nephrology. As we have shown in **paper I**, significant differences of gene expression were observed in the healthy principal glomerular cells between mouse and human. Accordingly, the findings of **paper II** need to be verified in human material. Ultimately, the best would be to profile patient biopsies from different glomerulopathies with and without reno-protective therapy. Several scRNA-seq studies on human kidney material have been published, mostly using the healthy portions of nephrectomies [51,59,67,131], and a few studies using patient biopsies [61–63]. The limited amount of material available, along with the different preservation methods used (frozen or paraffin embedding), makes it a challenge to profile human renal biopsies. However, this can be overcome with the use of snRNA-seq in a combination with a robust adjusted method of processing and single nuclei isolation. Another alternative would be the use of *ex vivo* systems, such as precision cut kidney slices [132]. This method has been used in kidney tissue to screen anti-fibrotic therapies [133] or to study the role of different fibrotic genes and pathways [133,134].

After generating and analyzing the transcriptomic data comes the next challenge of validating functionally the findings. The road from a gene expression to a protein and an activated pathway is affected by multiple layers of regulation and translation. We opted to validate a couple of genes we found of interest in both *in vivo* and *in vitro* settings. This included the generation of cell-specific KO or KI models and testing the gene involvement in healthy kidney tissues and in different disease settings. The studies in **paper III** and **paper IV** proved the complexity of the matter.

In case of Rarres1 in **paper III** we generated a lot of promising data, and we speculate that it can potentially be used as a biomarker and a therapeutic target. To validate Rarres1 as a biomarker, future studies on urine and plasma of patients with different glomerulopathies needs to be performed, and the results should be correlated to Rarres1 expression in kidney biopsies. Around the same time of publication of **paper III,** Chen et al.[93] published a study where they observed over-expression of Rarres1 in disease by podocytes and not endothelial cells. The discrepancy could be explained for instance due to detection methods used as both membrane bound and soluble forms of Rarres1 were measured [94]. Thus, further expressional profiling of Rarres1 in different kidney diseases is needed, including the use of more precise techniques (such as scRNA-seq). Finally, the connection of Rarres1 to NFκB activation via Axl was proved in podocytes, yet similar approach needs to be tested in endothelial cells.

As for NPR3 in **paper IV**, we cannot make any strong conclusions on the matter. The global activity of NP system and the involvement of various players contributing to its regulation makes it a complicated target to study[95,96]. The nature of peptides (including for instance

rapid degradation) makes it a rather tricky treatment strategy. Determining the right dose and route of delivery is of high importance[135]. Yet, the absence of NPR3 activity for a long time seems to be compensated by other mechanisms. Further studies are clearly needed to understand the role of NP system and NPR3 in renal and glomerular biology. Another major contributor to NP system is blood pressure regulation. In the current study we chose a disease model in which no apparent changes in blood pressure have been reported. Nevertheless, it is of a high importance to include blood pressure measurements in future studies. Moreover, it would be of interest to profile NPR3 inactivation and inhibition in a disease model of hypertension. Previous study of NPR1 using a podocyte-specific KO mice showed its role in maintaining podocyte integrity and function after the induction of hypertension and kidney damage, but not in a healthy condition [102]. Thus, a similar disease challenge would be of interest in both NPR3 podocyte KO animals and under NPR3i treatment.

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