Molecular perspectives on glomerular cell physiology in chronic kidney disease

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ABSTRACT

Glomerulonephritis is one of the most common causes of chronic kidney disease (CKD) in the world. Recent establishment of guidelines for classification of CKD in five stages has led to increased awareness of the risks of comorbidity and mortality, also in patients with early stages of disease, and the need to further advance our understanding of their pathogenic mechanisms. Many glomerular diseases are complex and curative treatment options are currently lacking, in part due to the difficulties to identify new molecular targets for treatment. The work included in this thesis focuses on physiological and pathophysiological mechanisms in glomerular mesangial cells and podocytes, especially in the disease IgA nephropathy (IgAN). Through analysis of mesangial cells derived from IgAN patient biopsies we found that patient cells proliferated more than healthy control cells in response to pathogenic IgA or PDGF-BB. They also released more PDGF-BB and IL-6 into the growth medium than control cells in response to the same stimuli, suggesting an increased sensitivity and thereby susceptibility for disease in the patient cells. A subsequent study of the glomerular transcriptome from patients with IgAN and healthy kidney donors was done by microarray and bioinformatic analysis. It demonstrated that differential expression of mesangial cell specific standard genes was prominent in IgAN, while podocyte standard genes were less significant in this context. The mesangial cell standard genes also correlated to patients’ clinical parameters after z-score transformation. Finally, we identified potential functions for the protein CKAP4 in glomerular cells, where it appears to be involved in regulation of proliferative signaling in mesangial cells through association with the PDGF pathway, and in maintenance of the podocyte structural stability through effects on the actin cytoskeleton.

In conclusion, our findings support previous knowledge about the central role of mesangial cells in IgAN, as well as suggest that these cells can have an altered susceptibility to disease. We also identified glomerular transcriptomic and mesangial cell proteomic pathways relevant for further research into development and progression of IgAN. We also found that CKAP4 is involved in disease mechanisms of mesangial cells and podocytes, warranting further investigation into the functions of the protein in health as well as in different forms of glomerular disease.

Keywords: Chronic kidney disease, IgA nephropathy, mesangial cell, podocyte, CKAP4

SAMMANFATTNING PÅ SVENSKA

Kroniska sjukdomar som drabbar de kärlnystan i njurarna över vilka blodet filtreras kan leda till läckage av proteiner och röda blodkroppar från blodbanan till urinen, och på så sikt även till terminal njursvikt. Trots en intensiv forskningsinsats återstår fortfarande många frågeställningar kring sjukdomarnas uppkomst och utveckling, och även om många framsteg har gjorts inom njurforskningen så saknas idag botande behandling för kroniska njursjukdomar. Ungefär en fjärdedel av de patienter i Sverige som genomgår dialys eller som är transplantera har njursjukdomar som uppstått i de kärlnystan där filtrationen av blod till urin sker, de så kallade glomeruli. Ett samlingsnamn för dessa sjukdomar är glomerulonefriter. Den vanligaste glomerulonefriten bland patienter som behandlas med dialys eller transplantation är IgA nefrit. Studierna som ingår i den här avhandlingen handlar till största delen om IgA nefrit, men vissa fynd är också relevanta för andra sjukdomar som påverkar cellerna i njurens filtrationsnystan.

De två första studierna i avhandlingen handlar om de mesangiala cellernas roll i IgA nefrit. Dessa celler finns mellan blodkärlen i njurens filtrationsnystan och har flera funktioner, bland annat att upprätthålla filtrationsenhetens struktur och rensa bort stora molekyler som exempelvis antikroppar så att de inte ansamlas där. Vid IgA nefrit sker just en ansamling av antikroppar av IgA-typ kring mesangiecellerna, och det blir en inflammatorisk respons som drabbar alla celler i filtrationsbarriären. Resultaten från de två första studierna bekräftar och förtydligar vad tidigare forskning visat om att mesangiecellerna har en avgörande roll för utvecklingen av IgA nefrit, och föreslår dessutom att en ökad mottaglighet för sjukdom hos mesangiecellerna läggs till den nuvarande teorin om vilka faktorer som är av betydelse för sjukdomens utveckling.

De två senare studierna undersöker funktionen av ett protein som tidigare inte studerats i njurens filtrationsnystan. Proteinet, vars förkortning är CKAP4, verkar ha olika funktioner i de två celltyper där det har störst uttryck; mesangieceller och podocytter. Podocytterna är specialiserade celler som utgör den yttersta delen av filtrationsbarriären, och sitter på utsidan av njurens filtrerande blodkärl. I mesangieceller observerades ett tydligt minskat uttryck av en receptor som reglerar celltillväxt, PDGFR, i celler där uttrycket av CKAP4 tystats ned med gentekniska metoder. I podocytter verkar CKAP4 istället vara viktig för att upprätthålla cellernas skelettstruktur, vilken är nödvändig för att filtrationsbarriären ska fungera korrekt. Felaktigt reglering av mesangiecells tillväxt och podocyters cellskelett är båda kända faktorer
som bidrar till glomerulära njursjukdomar och läckage av proteiner till urinen.

Sammantaget bekräftar resultaten i den här avhandlingen mesangiecellernas betydande roll i utvecklingen av IgA nefrit, och föreslår fortsatt forskning kring det intressanta proteinet CKAP4 för att ytterligare klargöra dess roll i glomerulära sjukdomar.
LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Mesangial cells from patients with IgA nephropathy have increased susceptibility to galactose-deficient IgA1


*BMC Nephrology (2016) 17:40*

II. Transcriptomic and proteomic profiling provides insight into mesangial cell function in IgA nephropathy


III. Cytoskeleton-associated protein 4 (CKAP4), a new player in the regulation of mesangial cell proliferation


*Manuscript*

IV. Cytoskeleton-associated protein 4 (CKAP4) is essential for podocyte integrity


*Manuscript*
CONTENT

ABBREVIATIONS........................................................................................................V

1 INTRODUCTION ........................................................................................................1

1.1 The kidneys and nephrons .............................................................................. 2

1.2 The glomerulus ............................................................................................... 4

1.2.1 Mesangial cells ...................................................................................... 5

1.2.2 Podocytes ............................................................................................... 6

1.2.3 Glomerular endothelial cells .................................................................... 6

1.3 Chronic kidney disease .................................................................................. 7

1.3.1 Glomerulonephritis ................................................................................ 7

1.3.2 IgA nephropathy ................................................................................... 8

1.3.3 Diabetic nephropathy ........................................................................... 11

1.3.4 Mesangial PDGF signaling in CKD .................................................... 11

1.3.5 Cytoskeleton-associated protein 4 (CKAP4) ........................................ 12

2 AIMS ........................................................................................................................14

3 METHODOLOGICAL CONSIDERATIONS ............................................................ 15

3.1 Ethics ............................................................................................................. 15

3.2 Patients and biopsies ..................................................................................... 15

3.2.1 Purification of IgA ............................................................................... 16

3.3 Cell culture ................................................................................................... 16

3.3.1 Healthy and diseased mesangial cells ................................................. 17

3.3.2 Podocytes .............................................................................................. 18

3.3.3 Other cell types ................................................................................... 18

3.4 Animal experiments ....................................................................................... 18

3.4.1 BTBR ob/ob mice ................................................................................. 19

3.4.2 Zebrafish ............................................................................................ 19

3.5 Gene expression analysis ....................................................................... 20

3.5.1 Microarray .......................................................................................... 20

3.5.2 RNA sequencing ................................................................................ 21
3.5.3 TaqMan™ PCR ................................................................. 21
3.5.4 Bioinformatic analysis of gene expression ..................... 22
3.6 Protein expression analysis ................................................ 23
  3.6.1 Immunofluorescence ...................................................... 23
  3.6.2 Immuno-EM ................................................................. 23
  3.6.3 Western blotting ............................................................ 24
  3.6.4 Mass spectrometry ......................................................... 25
  3.6.5 Biotinylation of cell surface proteins ............................. 25
  3.6.6 Bio-plex immunoassay .................................................. 26
  3.6.7 Bioinformatic analysis of protein expression ................. 26
3.7 Co-immunoprecipitation .................................................... 27
3.8 Cell proliferation and viability assays ............................... 27
  3.8.1 BrdU cell proliferation assay ......................................... 28
  3.8.2 Alamar Blue cell viability assay ..................................... 28
3.9 Gene silencing and overexpression .................................... 29
  3.9.1 Gene silencing by shRNA .............................................. 30
  3.9.2 Gene overexpression .................................................... 30
3.10 The PAN nephrotoxic model in vitro .................................. 30
3.11 Statistical analysis ............................................................ 31
4 RESULTS AND DISCUSSION ......................................................... 32
  4.1 Paper I: Mesangial cells from patients with IgA nephropathy have increased sensitivity to galactose-deficient IgA1 ................................................................. 33
  4.2 Paper II: Transcriptomic and proteomic profiling provides insight into mesangial cell function in IgA nephropathy ................................. 36
  4.3 Paper III: Cytoskeleton-associated protein 4 (CKAP4), a new player in the regulation of mesangial cell proliferation ......................... 42
  4.4 Paper IV: Cytoskeleton-associated protein 4 (CKAP4) is essential for podocyte integrity ................................................................. 46
5 CONCLUDING REMARKS .......................................................... 52
6 FUTURE PERSPECTIVES .......................................................... 54
ACKNOWLEDGEMENTS ............................................................. 56
**ABBREVIATIONS**

αSMA  Alpha-Smooth Muscle Actin  
BrdU  5-Bromo-2’-deoxyuridine  
CCL5  Chemokine (C-C motif) ligand 5  
CKAP4  Cytoskeleton-Associated Protein 4  
CKD  Chronic Kidney Disease  
cIgA  Control IgA  
DN  Diabetic Nephropathy  
EGFP  Enhanced Green Fluorescent Protein  
EGFR  Endothelial Growth Factor Receptor  
eGFR  Estimated Glomerular Filtration Rate  
ERK  Extracellular-signal Regulated Kinase  
ESL  Endothelial Surface Layer  
ESRD  End Stage Renal Disease  
FSGS  Focal Segmental Glomerulosclerosis  
GBM  Glomerular Basement Membrane  
gd-IgA  Galactose-deficient IgA  
GN  Glomerulonephritis  
IgA  Immunoglobulin A  
IgAN  Immunoglobulin A Nephropathy
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>IgAV</td>
<td>Immunoglobulin A Vasculitis</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
</tr>
<tr>
<td>JNK</td>
<td>cJun N-terminal Kinase</td>
</tr>
<tr>
<td>LN</td>
<td>Lupus Nephritis</td>
</tr>
<tr>
<td>MCD</td>
<td>Minimal Change Disease</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>PAN</td>
<td>Puromycin Aminonucleoside</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived Growth Factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-Kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>cRNA</td>
<td>Coding RNA</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Ribosomal RNA</td>
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<td>Micro RNA</td>
</tr>
<tr>
<td>SAM</td>
<td>Significant Analysis of Microarray</td>
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<tr>
<td>shRNA</td>
<td>Short-hairpin RNA</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TMT</td>
<td>Tandem Mass Tag</td>
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<tr>
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<td>Wilm’s Tumor 1</td>
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1 INTRODUCTION

Chronic kidney disease, abbreviated CKD, is a term used for a collection of heterogeneous diseases having long term effects on kidney structure and function. According to guidelines established in 2002, CKD is classified and categorized in five stages based on measurements of the glomerular filtration rate (GFR) and amount of protein in the urine, also called albuminuria or proteinuria (1, 2). Causes of CKD are commonly hypertension or diabetes, although there are alternative etiologies, and genetic and environmental factors together contribute to disease development in many cases (3). The worldwide prevalence of CKD is reportedly around 10% (3-5), although there is considerable variation between countries (6). As several risk factors for development of CKD are increasingly common, such as obesity, diabetes and hypertension, the global burden of CKD is increasing (7). The early stages of CKD can have few or no symptoms, but are associated with a heightened risk of cardiovascular disease (8), highlighting the importance of an early diagnosis and management of risk factors and comorbidities.

In addition to diabetes and hypertension, glomerulonephritis is another common cause of CKD. There are several types of glomerulonephritis, which are all characterized by damage to the filtration units of the kidneys, the glomeruli. The damage is often caused by inflammation and can lead to fibrosis if there is sustained injury to the glomerular cells, resulting in progressively worsened kidney function (3). Among the Swedish patients receiving dialysis or transplantation due to end stage renal disease (ESRD), corresponding to CKD stage five, glomerulonephritis was the most common disease etiology (9). The complex origins of different forms of glomerulonephritis are being intensely researched, since there are no curative treatment options currently available.

The focus in this thesis is on how the physiology of the glomerular cells is altered in glomerulonephritis, specifically the mesangial cells and the podocytes. The first two papers consider specifically IgA nephropathy (IgAN), which is the most common specified glomerulonephritis among the patients receiving dialysis or transplantation described in the Swedish Renal Registry (9). In these studies, we investigate cells and patient glomeruli affected by the disease or exposed to disease-like conditions to elucidate the differences in function or gene and protein expression between healthy and diseased cells in order to understand how IgAN develops. The two following papers are slightly different in their objective, as they include analyses of the function of a particular protein in mesangial cells and podocytes. The protein
has to our knowledge not previously been studied in these cells, and is seemingly involved in cellular functions important both in healthy cells and in disease pathogenesis.

This introductory section aims to provide a short background relevant to the research context of the thesis. From the broad field of the kidney and its function, the focus is on diseases affecting the glomerulus and its resident cell types, especially IgA nephropathy and diabetic nephropathy.

1.1 The kidneys and nephrons

The kidneys are essential organs for maintaining body homeostasis. Apart from their familiar function of filtering blood, the kidneys also maintain bodily water and salt balance, manage acid-base regulation and produce enzymes and hormones. The involvement of the kidneys in whole body function is summarized in Figure 1.

![Figure 1. Homeostatic processes involving the kidneys. The image was obtained with permission from Elsevier](image)

1 From original publication in The Lancet 382:9887, Eckardt KU et al., Evolving importance of kidney disease: from subspeciality to global health burden, pp. 158-169, Copyright Elsevier (2013)
Filtration of the blood takes place in the functional units of the kidneys, the nephrons (Figure 2). Each kidney contains about 800 000 to 1 million of these units and the number decreases with age, since there is no regeneration of new nephrons (10). The first part of the nephron is the glomerulus, where blood is filtered over capillaries with specialized endothelial and epithelial cells further described in the following section. The glomerular filtration rate of an average young adult is about 125 ml/min, which adds up to 180 L of filtrate produced per day, and decreases with age (10, 11). The glomerular barrier is permeable to water, small solutes and proteins of low molecular weight, while it retains proteins larger than about 60-70 kDa (such as albumin) and red blood cells (5). In diseases affecting the glomerular filtration barrier, albumin and red blood cells can sometimes be detected in urine, where they should otherwise be absent.

Figure 2. Schematic drawing of a nephron.

Following the glomerulus as the second part of the nephron is an intricate tubular system, where the filtrate is modified by reabsorption and secretion to produce the final urine excreted from the body. Most of the water and small ions such as Na⁺, K⁺ and HCO₃⁻ are readily reabsorbed, while end products from the body’s metabolic processes such as urea and creatinine typically end up in the final urine. Some substances, e.g. drug metabolites, can be secreted into urine from a capillary system closely intertwined with the tubules. This
system is also involved in reintroduction of reabsorbed water and solutes into the circulation. Reabsorption and secretion in the tubular system is tuned to the needs of the body, which is important for maintenance of homeostasis, as for instance the bodily concentration of electrolytes needs to be kept within narrow intervals (10).

1.2 The glomerulus

The glomerulus is the filtering part of the nephron, and consists of a capillary bed surrounded by a structure called Bowman’s capsule, which constitutes the first part of the tubular system. The hydrostatic pressure inside the glomerular capillaries is high relative to other capillaries, which is necessary for the kidneys to maintain a high filtration capacity, and not allow for accumulation of metabolic waste products in the circulation (10). The filtration barrier is upheld by both specialized glomerular cells and non-cellular structures, which are produced by the cells. The first such structure encountered by the blood in the glomerular capillaries is the glycocalyx and endothelial surface layer, which consist of glycoproteins and proteoglycans attached to the endothelial cells and adsorbing plasma proteins (12). The glycoproteins and proteoglycans give this layer a negative charge, providing charge selectivity to the filtration barrier. The endothelial cells and epithelial cells (podocytes) together produce the contents of the second non-cellular structure, the glomerular basement membrane (GBM), which is mainly made up of extracellular matrix glycoproteins such as laminin, collagen and nidogen (13). The GBM both provides anchorage for the endothelial cells and podocytes, and restricts filtration of plasma proteins, which makes it an essential part of the filtration barrier.

The three glomerular cell types include the previously mentioned specialized endothelial cells and podocytes, as well as glomerular mesangial cells (Figure 3). Mesangial cells have similarities with both smooth muscle cells and macrophages, and are localized between the glomerular capillaries. Since this thesis focuses mainly on the mesangial cells and podocytes, they will be described in more detail than the endothelial cells.
Figure 3. Schematic illustration of a glomerular capillary and the three cell types involved in glomerular filtration; endothelial cells, mesangial cells and podocytes. All three cell types are attached to the glomerular basement membrane (black line). Attached to the endothelium is the glycocalyx and endothelial surface layer (light blue). The image is not drawn to scale.

1.2.1 Mesangial cells

The mesangial cells have several functions in the glomerulus; they provide support to the glomerular capillaries, produce their own extracellular matrix and can both send out and receive signaling molecules from the neighboring endothelial cells and podocytes through glomerular crosstalk, which is essential for normal development and function of the glomerulus (14). Mesangial cells also have contractile properties, by which they can regulate capillary surface area and increase filtration pressure to change the glomerular filtration rate (15). Since the cells are in direct contact with the glomerular endothelium without separation by the basement membrane, some larger proteins and protein complexes may cross the endothelial cell barrier and end up in the mesangium (14). The mesangial cells however are under normal conditions able to clear such macromolecules from their surroundings through endocytosis (16), preventing accumulation and potential injury to the cells.
The important functions carried out by mesangial cells can make injuries to the cells detrimental for the whole glomerular system. The mesangial cells are also involved in many glomerular diseases, often through release of inflammatory cytokines and chemokines, increased cell proliferation and expansion of the mesangial extracellular matrix (17). Common glomerular diseases such as IgAN and diabetic nephropathy (DN) both involve pathological changes to mesangial proliferation and matrix expansion, and trigger inflammatory and pro-fibrotic reactions eventually leading to glomerulosclerosis and loss of filtration area (18, 19).

1.2.2 Podocytes

The podocytes are specialized epithelial cells enveloping the glomerular capillaries and constituting the last part of the filtration barrier. The cells can be divided into three parts; the cell body, major extending processes and foot processes (20). The foot processes from different cells interdigitate in a manner specific for the podocytes to cover the entire surface of the capillaries. In the space between the foot processes, they form a specific cell-cell junction called the slit diaphragm. This structure makes up the final part of the filtration barrier and include proteins such as nephrin and podocin, which have been found to be involved in both genetic and acquired glomerular diseases (21). Both proteins included in the slit diaphragm and proteins related to podocyte adhesion to the glomerular basement membrane, e.g. integrins, are linked to the actin cytoskeleton of the podocytes (22). Actin is the main cytoskeletal component of the foot processes, while the cell body and major processes have a microtubule-based cytoskeleton (23). Dysregulation of actin dynamics can lead to loss of the intricate structure of the foot processes, called foot process effacement, and ultimately proteinuria (24). The presence of proteinuria is generally indicative of structural damage to the glomerular filtration barrier, causing proteins normally retained in the blood to leak into urine. In diseases such as focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD), the podocytes are the main glomerular cells affected, and both diseases are associated with proteinuria, which can be in the nephrotic range of more than 3.5 g of protein loss per day (25, 26). Also in diseases such as IgAN that mainly affects mesangial cells, podocytes can be damaged and have foot processes effacement, probably as an effect of glomerular crosstalk (27).

1.2.3 Glomerular endothelial cells

The endothelial cells lining the insides of the glomerular capillaries have characteristic pores called fenestrae. The cells are also lined with a glycocalyx and endothelial surface layer, as described in the introduction to
this section. The fenestrated endothelium allows for filtration of large fluid volumes, while the glycocalyx provides and initial obstacle for large plasma proteins, which would otherwise accumulate near the podocytes or leak through the glomerular filtration barrier (22). Dysfunction of the glomerular endothelial cells, e.g. due to atherosclerosis, hypertension or hyperglycemia, is thought to contribute to the increased risk of cardiovascular complications in chronic kidney disease (28).

Crosstalk between all the three glomerular cell types enables the glomerulus to work as a unit and react to its dynamic environment, though it also permits transmission of disease related signals, and can make injury to one cell type affect the function of others. One example of essential crosstalk is the production of platelet-derived growth factor (PDGF) by endothelial cells, which has been shown to be necessary for mesangial cell development (29). Another example is vascular endothelial growth factor A (VEGF-A), mainly produced by podocytes and acting on endothelial cells, which under normal conditions maintains glomerular function but in case of dysregulation is associated with several glomerular diseases (30, 31).

1.3 Chronic kidney disease

As described earlier on, chronic kidney disease (CKD) is a group of heterogeneous diseases affecting both the structure and function of the kidneys. CKD can develop secondary to a systemic disease such as diabetes, or the kidneys may be the primary organ affected. In some CKD, a single genetic component or multiple susceptibility genes have been identified, and genetic predisposition is a risk factor for disease development (32, 33). Other risk factors include hypertension, dyslipidemia and occurrence of acute kidney injury (32). CKD can differ in severity and progression, as well as be asymptomatic or have few symptoms in its early stages. The relatively recent establishment of generally accepted guidelines for classification of CKD stages have reportedly shifted the view on the impact of CKD on global health, especially since all stages of CKD are associated with increased risk of comorbidities, mainly cardiovascular disease (5, 34). Among the patients with ESRD (CKD stage 5), the majority have CKD affecting the glomerulus (35).

1.3.1 Glomerulonephritis

Immune-mediated glomerular diseases are collected under the term glomerulonephritis (GN), and can be either chronic or acute (36). The immunological component can stem from e.g. autoimmunity, infection, or
malignant or metabolic diseases, and affect the glomerular cells and glomerular function differently (36). Among the chronic GN is IgAN, which is the most common GN in Swedish patients receiving renal replacement therapy for ESRD (9). Other examples of GN are focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD), which are more often associated with high levels of proteinuria and occurrence of the nephrotic syndrome than IgAN. To distinguish between the different forms of GN it is in many cases necessary to do a histological evaluation of a renal biopsy. The treatment options for GN target the disease symptoms, such as glomerular hypertension and over-activity of the immune system, while curative treatments are not yet available. Research of the underlying pathogenesis both on individual cell level and systemically in the glomerulus and higher structures of the kidney and circulation can hopefully contribute to development of new and more precise treatments in the future.

### 1.3.2 IgA nephropathy

The GN mainly investigated in this thesis is IgAN. It is the most common GN in the world, and a leading cause of CKD and ESRD (19). There are however geographical differences in disease prevalence, as it is most common in Asia, less prevalent in Europe and rare in populations of African ancestry (37). The disease has a higher incidence in children and young adults than in elderly, although elderly IgAN patients are more likely to have a further progressed and severe form of the disease, which is reflected in the average age of patients in renal replacement therapy in Sweden of about 60 years (9). Over a follow-up period of 20-25 years, about 30-50% of IgAN patients develop ESRD (38).

The pathogenesis of the disease has been described through a four-hit hypothesis (Figure 4), where hits 1 and 2 are required for hit 3 and 4 (39).

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**Figure 4. The four-hit hypothesis of IgAN pathogenesis.**
The first hit describes how patients with IgAN have elevated levels of galactose-deficient IgA1 in the circulation. IgA is an immunoglobulin mainly produced in mucosal tissues and in bone marrow, and present in external secretions as well as in the circulation. The circulatory IgA is mainly of the IgA1 isotype, while the IgA in external secretions contain different distributions of IgA1 and IgA2 depending on the mucosal tissue where they are produced (40). Another difference between circulatory and secretory IgA is the content of polymeric or monomeric forms. Most circulatory IgA is monomeric, while polymeric IgA is more common in external secretions, where IgA molecules are linked together by a joining chain (J-chain) (41). IgA1 has a hinge region of O-linked glycans, which can be galactosylated at serines or threonines, and which is the region aberrantly galactosylated in IgAN (40). IgA2 does not have serines or threonines in the hinge region and cannot be galactosylated (Figure 5).

The galactose-deficient IgA1 (gd-IgA1) is recognized by specific anti-glycan autoantibodies, hit 2 in disease development, and they interact to form pathogenic immune complexes as hit 3 (42). The immune complexes deposit
in the glomerular mesangium and activate the mesangial cells as hit 4, leading to increased mesangial cell proliferation, extracellular matrix expansion and production of cytokines and chemokines (43). The immune complexes deposited in the mesangium are mainly containing polymeric IgA1, suggesting a mucosal origin of the gd-IgA1. The fourth hit of mesangial cell activation is exaggerated by the ability of IgA to activate the alternative and lectin pathways of the complement system (44).

IgAN is similar to the disease IgA vasculitis (IgAV, previously known as Henoch-Schönlein purpura nephritis) regarding histopathological findings in the kidney, where both e.g. have depositions of gd-IgA1 (45, 46). Patients with IgAV however also have deposition of immune complexes in other capillaries, for instance in the skin and gut, and have characteristic purpura, mainly on the lower extremities.

The mechanisms regulating onset and progression of IgAN are still being elucidated. Recent genome wide association studies (GWAS) have identified susceptibility loci suggesting defects in adaptive and innate immunity and the alternative complement pathway in patients with IgAN (47). Aberrant galactosylation of IgA might also be an inherited trait, suggested by studies where high levels of gd-IgA1 was detected in serum from asymptomatic relatives, but not in serum from unrelated in-laws (48, 49). There is a known familial form of IgAN, although it accounts for only about 5-10% of cases, while the remaining 90-95% occur as sporadic disease (43). These findings together support the hypothesis that several factors contribute to disease development. Also, about 60% of patients with IgAN receiving a renal transplant have recurrence of glomerular IgA deposits in the kidney graft, although fewer of the transplanted patients, between 8-53% in a recent summary of current literature, develop de novo clinical IgAN (50).

In 2009, a working group from the IgAN Network and the Renal Pathology Society collaborated on development of a scoring system for histological findings in IgAN, each independently associated with disease progression (51, 52). The Oxford MEST classification system includes scoring of renal glomerular biopsies based on mesangial hypercellularity (M), endocapillary hypercellularity (E), segmental sclerosis (S) and interstitial fibrosis/tubular atrophy (T). Later a score for crescents (C) was also added to the system (53).

The extensive and collaborative efforts to standardize evaluation of risk factors for disease progression helps determine which IgAN patients are at risk of progressing towards ESRD, and which patients might have a slower disease course. In addition to the Oxford MEST scores, common risk factors
for CKD, such as hypertension, lower glomerular filtration rate (GFR) and proteinuria, are also risk factors for progression of IgAN (54). The molecular disease mechanisms are also being intensely studied, focusing both on the production of gd-IgA1, as well as the involvement of cells and the whole glomerulus in disease onset and progression. A strong incentive for continued molecular biological research into IgAN is the possibility of finding a more precise target for treatment of the disease than is currently available.

1.3.3 Diabetic nephropathy

CKD as a complication in diabetes mellitus type 1 or 2, also called diabetic nephropathy (DN), occurs in about one third of patients with diabetes type 1 and about half of patients with diabetes type 2 (55, 56). It is however difficult to establish if the patients develop CKD because of their diabetes or due to other causes. Many risk factors for CKD development can be present at the same time, such as hypertension, dyslipidemia and glomerular atherosclerosis (56). Patients with simultaneous diabetes type 2 and CKD, irrespective of the cause, have increased mortality risk compared to type 2 diabetes patients without CKD (57).

All three glomerular cell types and the glomerular basement membrane are affected in DN. One of the pathological disease features is glomerulosclerosis, caused by mesangial matrix expansion, which is also strongly associated with disease progression (58, 59). Results from a study by Weigert et al. show that upregulation of the glucose transporter GLUT1 is not enough to upregulate expression of the pro-fibrotic factor TGFβ in mesangial cells, suggesting that multiple mechanisms are responsible for TGFβ upregulation in mesangial cells in DN (60). One probability is glomerular crosstalk, as also podocyte damage and loss has been linked to development of glomerulosclerosis in DN (61). Persistent hyperglycemia is also known to damage the endothelial cells, leading to increased production of reactive oxygen species (ROS) through mitochondrial dysregulation, which has been seen to be essential for podocyte loss in a model of FSGS; another disease involving glomerulosclerosis (62).

1.3.4 Mesangial PDGF signaling in CKD

Signaling through the PDGF pathway induces proliferation of mesangial cells both in vivo and in vitro (63, 64). The mesangial cells express both PDGF receptors (PDGFRα and β) and are thereby responsive to all isoforms of PDGF (PDGF-A, -B, -C and -D), while expression in podocytes and glomerular endothelial cells is less comprehensive and not as well characterized (64). There have however been studies demonstrating the
importance of crosstalk between mesangial cells and endothelial cells through PDGF during development of the glomerulus (29, 65). Upregulation of PDGF receptors and ligands, especially PDGFRβ and PDGF-B, is present in many glomerular diseases such as IgAN and DN, where mesangial proliferation is known to be dysregulated (66, 67). PDGF-B, -D and PDGFRβ are also reported to be involved in fibrotic signaling through a mechanism downstream or independent from TGFβ, which is relevant in both IgAN and DN as both diseases result in extracellular matrix expansion that can eventually lead to fibrosis and glomerulosclerosis (68).

1.3.5 Cytoskeleton-associated protein 4 (CKAP4)

Two of the papers included in this thesis concern the function of cytoskeleton-associated protein 4 (CKAP4) in glomerular cells. CKAP4, also called CLIMP-63 or sometimes p63, was identified independently by two research groups in the early 1990’s. Mundy and Warren identified it as a highly palmitoylated protein during mitosis (69), while Schweizer et al. discovered it while exploring proteins expressed in the ER-Golgi intermediate compartment in Vero cells, which are kidney epithelial cells obtained from an African green monkey (70). The subcellular localization was re-evaluated two years later to the rough ER (71). Later research has shown that CKAP4 participates in several important cellular functions, such as maintaining ER structure (72), linking ER to the microtubules (73), and more recently it was found to function as a receptor for various ligands in different cell types. The linkage between CKAP4 and microtubules was found to be negatively regulated by phosphorylation, leading to disruption of the interaction during mitosis (74). Several publications also account for interactions between CKAP4 and various intracellular proteins, such as Dicer (75), Myc target 1 (76) and the SNARE protein Syntaxin 5 (77).

CKAP4 is a type II transmembrane protein, meaning it faces the ER lumen with its C-terminal segment, and only has one transmembrane domain. Palmitoylation of the protein, which is the reversible addition of a 16-carbon residue to a cysteine, seems to be necessary for its localization to the plasma membrane. The palmitoyl post-translational modification is mediated by the palmitoyl transferase DHHC2 to cysteine 100 on the cytosolic tail of the protein (78). CKAP4 is very stably expressed in different cell lines, with a reported half-life of between 20 h (HEK cells) and 156 h (C2C12 cells) (79).

For the past two decades, CKAP4 has gained most of its research attention due to its function as a cell surface receptor. It has been found to bind to surfactant protein A (SP-A) in type II pneumocytes and thereby mediate
Emelie Lassén

surfactant protein turnover (80), as well as to bind tissue plasminogen activator (tPA) in vascular smooth muscle cells (81). Studies on bladder epithelial cells have shown that CKAP4 is a receptor for antiproliferative factor (APF), a small peptide propagating antiproliferative signaling in these cells and found in elevated levels in urine of interstitial cystitis patients (82), and that CKAP4 upon APF binding could translocate to the nucleus and initiate transcription of the gene CCN2, coding for connective tissue growth factor (CTGF) (83).

In a comprehensive study published in 2016, CKAP4 was found to be a receptor for dickkopf-1 (DKK1), a Wnt signaling inhibitor, in lung and pancreatic cancer cells and a mediator of proliferative signaling through the PI3K/AKT pathway (84). Simultaneous upregulation of CKAP4 and DKK1 in cancer cells was there found to be associated with poor prognosis. Similar results were shown by the same research group for CKAP4 and dickkopf-3 (DKK3) in esophageal cancer two years later (85). There is however also a report of increased CKAP4 expression associated with a decrease in proliferation and invasion potential of hepatocellular carcinoma, which was there explained by a suppressive interaction between CKAP4 and EGFR (86). The expression and function of CKAP4 in glomerular cells has to our knowledge not previously been investigated. One study has however studied CKAP4 in clear cell renal cell carcinoma, which commonly originates from proximal tubular cells, and found upregulation of the protein associated with poor prognosis. The study also suggested that upregulation of CKAP4 induced cell proliferation in the cancer cells through a mechanism involving Cyclin B signaling (87).
The overall aim of this thesis was to elucidate physiological and pathophysiological mechanisms in human glomerular cells contributing to development of glomerular diseases such as IgAN.

Specific aims were:

**Paper I** To test the hypothesis that mesangial cells from patients with IgAN have an altered susceptibility to disease-associated gd-IgA1 compared to healthy cells

**Paper II** To identify differentially regulated genes, proteins and signaling pathways in IgAN in order to gain understanding about disease development

**Paper III** To study the function of the protein CKAP4 in mesangial cells and its possible role in regulation of mesangial cell proliferation

**Paper IV** To explore the function of CKAP4 in podocytes and its relation to podocyte actin cytoskeletal stability
3 METHODOLOGICAL CONSIDERATIONS

This section aims to discuss the methods used in the research included in this thesis. Further information about the experimental protocols are found in the respective papers where they were used.

3.1 Ethics

The studies using human material from patients (Paper I and II) were conducted in accordance with the declaration of Helsinki. Ethical permission for collection of human biopsies or blood samples for Paper I and II was obtained from the regional ethical review board in Gothenburg (#432-09 for blood samples, #413-09 for biopsies), and patients included in the study signed an informed consent before participating. The experiments and analyses involving mice performed in co-operation with Astra Zeneca were approved by the same review board (#109-2012). The experiments performed using zebrafish in Paper IV were approved by the Institutional Animal Care and Use Committee (IACUC) in the United States (#17-03).

3.2 Patients and biopsies

The patients donating biopsy material for the research included in Paper I all had glomerular IgA1 deposits, though were either diagnosed with IgAN or IgA vasculitis (IgAV). Renal involvement in IgAV, which was found to be present in 45-85% of the patients in cohorts included in a recent review of the disease in adults (88), have histological profiles that can be identical to those of IgAN. While the diseases are similar in their manifestation in the kidneys, there are differences for instance in clinical symptoms and disease incidence. IgAV is more common in children than in adults (89) and renal involvement is mainly characterized by more acute inflammation than seen in IgAN, and can resolve without intervention or develop into chronic disease (90), while IgAN usually has a more indolent but persistent course (43). IgAN patients are commonly children or young adults at the time of biopsy (43), and they were found to be older on average than IgAV patients when diagnosed in the ongoing study CureGN, which includes children and adults with IgAN or IgAV, among other GN (45).

The glomeruli used for culture of mesangial cells in Paper I were obtained by dipping newly taken biopsies in ice-cold PBS before preserving them for diagnosis, further described in section 3.3.1. Glomeruli from patients with
IgA1 deposits were used in the study, though the number of included patients was limited to the ones whose glomeruli yielded viable mesangial cells in vitro. Three patients with IgAN and three healthy individuals each donated 50 ml blood for the purification of IgA1, described in section 3.2.1.

Collection of the material used for microarray analysis in Paper II started as a collaboration with Sahlgrenska University Hospital in 2004. The patients consenting to participate in the study donated material not needed for diagnosis to be included in our research. During a routine biopsy procedure, one of the biopsies taken were first refrigerated in RNAlater and then stored in -80 °C for use in the study unless needed for diagnosis. Only patients diagnosed with IgAN were included in the study. Biopsies from living healthy kidney donors were collected under the premises just described, after transplantation into the graft recipient and reperfusion. Serum creatinine concentrations from the IgAN patients measured at the time of biopsy and during follow-up tests for monitoring of disease progression up to 4 years after diagnosis were acquired and used for analysis. Classification of the biopsies according to the Oxford MEST criteria for IgAN was done by renal pathologist Dr. Johan Mölne at the Sahlgrenska University Hospital. In total, 25 IgAN patient biopsies and 26 living donor biopsies were used for microarray analysis in Paper II.

### 3.2.1 Purification of IgA

Serum IgA1 was purified for use in Paper I and II from blood samples donated from three IgAN patients and three healthy controls. The method for purification with jacalin agarose has been described in a previous publication by Dr. Min Jeong Kim and colleagues (91). Jacalin is a lectin found in seeds of the jackfruit and was found to be binding specifically to IgA and not to other immunoglobulins in 1985 (92), and also found to separate IgA1 from IgA2 (93). Following purification, determination of the IgA1 concentration was done by nephelometry at the Immunology laboratories at Sahlgrenska University Hospital. The amount of gd-IgA1 in serum from IgAN patients compared to controls was assessed by a sandwich ELISA using an N-acetyl galactosamine specific lectin from the garden snail Helix aspersa. When treating cells with purified IgA1, the samples from all three IgAN patients or controls were pooled prior to treatment.

### 3.3 Cell culture

The four papers included in this thesis rely to a large extent on experiments performed in vitro using primary or immortalized glomerular cells. Working
with monocultures of cells such as mesangial cells or podocytes can yield information about the specific cell types and their response to stimuli, such as growth factors or gd-IgA, exemplified in Paper I. It is also easy to obtain enough material for protein or gene expression analysis, as well as to genetically modify the cells, and experiments are usually easy to reproduce. One obvious drawback is the absence of cross-communication with other cell types, which occurs in the glomerulus in vivo. The cells in culture may also differ in morphology, which is evident in the cultured human podocytes that do not form foot processes in vitro. There is also a risk of contamination by other glomerular cells than the ones intended for culture. This can be managed by testing the cells for expression of cell-specific proteins. Despite its drawbacks, cell culture is a fundamental method for life science research, and new techniques allow for more complex cell culture environments, closer mimicking the cells’ surroundings in vivo.

3.3.1 Healthy and diseased mesangial cells

The aim of the first study was to determine if mesangial cells from patients with IgAN are more susceptible to PDGF-BB and gd-IgA than mesangial cells from healthy individuals. Culturing cells from patients with disease presents many challenges, but can also yield specific information about the effect of disease on the cells that might not be obtained through the more common use of commercially available, healthy cells and a mimicked disease setting. The challenges of using patient cells include a possible selection bias as cells from all patients are not viable for culture, as well as the risk that some cells may have a changed phenotype due to ongoing inflammation. The latter might be mitigated through culture of the cells in standard, non-inflammatory conditions. Another aspect to consider is the economic viability of culturing patient cells, as the time and material requirements before the cells can be used in experiments are higher than for commercially available cells. All primary cells also senesce after being sub-cultured a number of times, and thereby have a limited life span.

The mesangial cells from patients included in the first study were obtained by a new method, where biopsies intended for diagnosis were dipped in phosphate buffered saline before fixation in an RNA preserving solution. Glomeruli detached into the saline solution were transferred to the cell culture onto dishes coated with attachment factor, and cultured in medium supplemented with human serum and antibiotics. As the mesangial cells are the fastest growing glomerular cell type, these cells grew out of the glomerular periphery within 10-20 days and could then be sub-cultured for further experiments. They were determined to be mesangial cells through
observation of their typical stellate morphology, as well as positive immunofluorescence staining of smooth muscle actin, while no binding was observed to the lectin *Ulex Europaeus Agglutinin I* (endothelial cell marker) or anti-synaptopodin (podocyte marker).

Commercially available human mesangial cells were used in Paper I-III. Cells were purchased from Lonza (Basel, Switzerland) or Cell Systems (Kirkland, WA, USA).

### 3.3.2 Podocytes

The culture of human podocytes is challenging due to the difficulty to preserve their complex architecture and differentiation *in vitro*. There is however a need for an *in vitro* model to study cellular events and signaling pathways specifically in podocytes. In 2002, Professor Moin Saleem at the University of Bristol developed a conditionally immortalized cell line of human podocytes expressing cell-specific markers such as nephrin and podocin after differentiation (94). The podocytes were immortalized through transduction by retroviruses carrying the SV40 large T antigen gene, which can be inactivated through thermo-switching from the growth permissive temperature 33°C to the non-permissive temperature 37°C, at which point the cells start to differentiate. After about 14 days of differentiation the cells are ready for experiments. Professor Saleem kindly provided these human podocytes for our experiments in Paper IV.

### 3.3.3 Other cell types

Commercially available human glomerular endothelial cells from Cell Systems were used for evaluation of their expression of CKAP4 in Paper III. Specific culture conditions are detailed in the manuscript. For production of lentiviral particles, we used HEK293 cells stably expressing the SV40 large T antigen gene (HEK293T), which enables a high expression of constructs containing the SV40 origin of replication after transfection (95).

### 3.4 Animal experiments

When studying the function of a protein in health and disease such as in Paper III and IV, it is sometimes necessary to include animal models. Although primary cells isolated from patients or healthy kidney donors can provide much information about cellular function, the absence of some factors from the cells’ natural environment, such as signaling molecules from neighboring cells and effects of capillary flow, limits how much information can be gleaned from *in vitro* experiments. Animal models are however also
models of disease states in humans, and differences between species can make results difficult to translate. One example is the challenge of finding a suitable animal model for IgAN, which is difficult due to that the IgA1 subclass of IgA is only found in humanoid primates (96).

The animal experiments in this thesis were done in collaboration with Mount Desert Island Biological Laboratories in Maine, USA (zebrafish), or were performed by researchers at Astra Zeneca in Mölndal, Sweden (mice) who kindly shared their data from RNA sequencing analysis. Both analyses were done aiming to further understand the role of CKAP4 in glomerular disease.

3.4.1 BTBR ob/ob mice
The BTBR ob/ob mice lack the hormone leptin, are insulin resistant, and develop severe diabetes. They also develop progressive DN, detectable by albuminuria and glomerular histological changes such as podocyte loss and mesangial matrix expansion appearing after about 8 weeks (97). In Paper III, the RNA expression of CKAP4 was assessed in glomerular sections from BTBR ob/ob mice at 8, 14 or 20 weeks of age, and compared to expression in lean mice 20 weeks old. Immunostainings of mouse glomeruli was also done to investigate CKAP4 protein expression during progression of DN in the mouse model.

3.4.2 Zebrafish
The use of zebrafish (Danio rerio) as an animal model to study renal diseases is new compared to the use of rodent models. Improvements in gene editing techniques have made zebrafish increasingly popular as a model organism, especially for studying genetic diseases or the roles of specific genes in disease development, and about 70% of human genes have a zebrafish orthologue (98). Zebrafish are advantageous over rodents as model organisms due to the large number of offspring produced (up to 300 eggs per week), enabling high throughput studies, and because the fertilized eggs are transparent and therefore can be easily monitored through development (99). Zebrafish embryos develop a pronephros consisting of two nephrons and one glomerulus, which functions about 48 hours post-fertilization (hpf) (100). The pronephros has similarities to human nephrons, despite being the first stage of human kidney development, and all three cell types involved in the glomerular filtration barrier are present (endothelial cells, mesangial cells and podocytes) (101).

In Paper IV, we aimed to investigate if transient knock-down of the zebrafish homolog of CKAP4 would affect the glomerulus in the zebrafish pronephros.
Knock-down of CKAP4 was done with morpholinos; oligonucleotides 18-30 bp in length which block the translation of a specific mRNA into the target protein (99). The morpholinos were injected into one- to four-cell stage zebrafish embryos, which were analyzed 96 hpf or 120 hpf for proteinuria and edema, as further described in the method of the manuscript. Glomerular sections were also preserved for analysis of the filtration barrier by electron microscopy.

When using morpholinos for transient gene silencing there is a risk for off-target effects, which can challenge the assumption that an eventual phenotypic change seen is the result of a knock-down of the targeted gene (102). One way to test the specificity of a knock-down is to introduce a coding RNA (cRNA) to overexpress mRNA for the silenced gene, and thereby attempt to rescue the phenotype associated with the knock-down (99). The cRNA can be constructed from mRNA originating from other species than zebrafish (e.g. mouse or human) to test if the gene function in zebrafish can be translated to other species relevant to the research question.

### 3.5 Gene expression analysis

Analysis of gene expression is included in all four papers in this thesis. The method is useful for analysis of global mRNA expression (transcriptomics) as well as for studies of specific genes, making it a valuable tool in both top-down and bottom-up approaches to study cellular function. The RNA molecule is however easily degraded by RNases, and this presents a challenge in the preparation of samples before analysis. The RNA is therefore first reverse transcribed into complementary DNA (cDNA), which is more stable. Additionally, the PCR, the microarray and the RNA sequencing reactions all require a cDNA template and DNA-polymerase, which is less error prone than RNA polymerase by about 3 orders of magnitude (103).

In Paper II, the glomerular transcriptome from IgAN patients and healthy living kidney donors was evaluated by microarray, and in Paper III the expression of CKAP4 in glomeruli from diabetic or lean mice was assessed based on RNA sequencing results kindly shared by Dr. Anna Granqvist at Astra Zeneca. TaqMan™ PCR gene expression assays were used in all four papers.

#### 3.5.1 Microarray

In Paper II, the global gene expression in microdissected glomeruli from biopsies of IgAN patients was compared to the expression in glomeruli from
healthy living kidney donors. From the original 25 IgAN patient biopsies and 26 biopsies from healthy kidney donors, 19 respectively 22 passed the quality check and were used for further bioinformatic evaluation.

The initial gene expression analysis was done using an Affymetrix microarray platform. The microarray technique relies on hybridization between cDNA and oligomers of synthesized DNA representing the whole transcribed genome or a select number of genes. Through labeling of the input cDNA, the expression levels can be compared between samples for the genes included on the array card, and a comparison is possible between healthy and diseased or treated and untreated cells. Limitations to the technique include the need for a priori knowledge of the gene sequences whose expression is evaluated, and a limited ability for quantification of lowly or very highly expressed genes (104). Since microarray analysis is a high throughput method the data needs to be processed through bioinformatics, as described in section 3.5.4.

### 3.5.2 RNA sequencing

RNA sequencing (RNA seq) provides a method for high throughput analysis of gene expression, similar to RNA microarrays, but can also enable better resolution and better coverage of the transcriptome. One example of the increased resolution of RNA seq is the possibility to investigate expression of other types of RNA than protein coding mRNA, such as pre-mRNA and microRNA (104). All RNA types cannot be investigated simultaneously however, as the abundance of rRNA (ribosomal RNA, reportedly accounting for >95% of total cellular RNA in most cells) would limit the range of detection of less expressed RNAs. Therefore, an enriching step is required before sequencing starts (104).

In Paper III, the expression of the homolog of CKAP4 was determined in mouse glomeruli based on results from RNA seq performed at Astra Zeneca. Glomeruli from diabetic mice at 8, 14 and 20 weeks were sequenced and compared to glomeruli from diabetic mice fed a high protein diet at 14 and 20 weeks, as well as lean controls (n=6/group).

### 3.5.3 TaqMan™ PCR

Gene expression analysis by TaqMan™ PCR was included in all four papers of the thesis. In addition to a forward and reverse primer required in a regular PCR, TaqMan™ makes use of gene specific probes carrying fluorescent labels for signal detection. In addition to the fluorescent label, the probes also contain a quencher, inhibiting emission of light from the fluorophore by
fluorescence resonance energy transfer (FRET). As the TaqMan™ probe is cleaved by Taq-polymerase in an amplification cycle of the PCR, the separation of the fluorophore and the quencher enables a fluorescent signal to be detected after excitation of the fluorophore. The number of PCR cycles required for the fluorescence to reach a threshold value is the read-out from a TaqMan™ PCR assay and labeled the $C_T$ value. This value is inversely proportional to the amount of the target cDNA available in the input sample, and can be used to calculate the relative difference in expression of a gene between samples.

TaqMan™ PCR is a specific, sensitive technique for evaluation of relative gene expression, though one limitation is the need for a specific probe for each sequence to be analyzed. That makes it difficult to take splice variants of the same gene into consideration for example.

In Paper I, TaqMan™ PCR was used for investigation of gene expression of the PDGFB and PDGFRB genes in healthy and diseased mesangial cells, as well as expression of other genes related to IgAN development. In Paper II, the method was used for validation of the microarray results and in Paper III and IV it was used for investigation of growth factor receptor expression and verification of silencing or overexpression of CKAP4.

### 3.5.4 Bioinformatic analysis of gene expression

The microarray analysis in Paper II required bioinformatic and statistical analysis to elucidate differential gene expression between glomeruli from IgAN patients and healthy controls. Since the arrays were run at 5 different time points, the data needed to be normalized within each batch and the batch-effect removed. After data processing to identify and remove outliers described in detail in the paper, as well as clustering the data to assess if patient samples separated from controls, significant differential gene expression was evaluated by the Significant Analysis of Microarray (SAM) method. Cutoffs for up- or down-regulation were SAM q-value <0.01 and 1.5 < fold change < 0.67. The differential expression of genes related to specific cellular signaling pathways was analyzed by Ingenuity pathway analysis (IPA) and the Genomatix pathway tool. Additionally, the expression of cell-specific standard genes identified by Dr. Wenjun Ju and colleagues (105) was assessed in our data set for mesangial cells and podocytes, and compared to clinical parameters from the participating patients after $z$-score transformation.
3.6 Protein expression analysis

The analysis of protein expression through Western blotting, immunocytochemistry and mass spectrometry are ubiquitous in molecular biology research, and included in each of the four articles in this thesis. Each method is associated with advantages and disadvantages further detailed in this section and in the methods of the papers.

3.6.1 Immunofluorescence

The method of using immunolabeled antibodies for detection of proteins by confocal or fluorescence microscopy was used in Paper I, III and IV. It is useful for assessment of localization of proteins, and eventual changes associated with treatments or genetic manipulation of the cells. In Paper III and IV it was also used to stain entire glomeruli in an analysis of CKAP4 expression. Several fluorophores can be used simultaneously for labeling of different antibodies, which enables investigations of co-localization of proteins in cells or tissue. The method can also be used for discrimination between glomerular cell types by detection of cell specific proteins such as α-smooth muscle actin (mesangial cells), synaptopodin or Wilm’s tumor 1 protein (podocytes), or binding of the lectin *Ulex Europaeus Agglutinin I* (endothelial cells).

While immunofluorescence is useful for determining protein localization and abundance, there are some caveats. One is the challenge of quantifying protein expression based on fluorescence intensity, which requires a standardized procedure for obtaining and processing each image. In the case of manual assessment, the researcher performing it should be unaware of which image belongs to which treatment group. Another challenge can be cross-reactivity of the primary or secondary antibodies, leading to background fluorescence or other unspecific binding to other proteins than the protein of interest. Due to these caveats, immunofluorescence analysis of protein expression is often used together with other methods to determine the localization and quantity of a protein in cells or tissue.

3.6.2 Immuno-EM

One method used only in Paper III was immunogold labeling of CKAP4 in human glomerular tissue and subsequent analysis by electron microscopy (EM), which was done in a collaboration with Dr. Kjell Hultenby at the Karolinska Institute in Stockholm. Colloidal gold particles attached to secondary antibodies, which were bound to primary antibodies detecting CKAP4 in human glomerular tissue. The high electron density of the gold
particles makes them distinguishable from the surrounding tissue in the EM analysis, although the signal is slightly separated from the binding site of the primary antibody due to the size of the antibodies. In Paper III the method was used as a complement to immunofluorescence for determination of the localization of CKAP4 within the glomerular cells.

3.6.3 Western blotting

Protein expression analysis by Western blotting is a standard in molecular biology. It is a semi-quantitative method which enables comparison of average amounts of protein in cell or tissue samples. In this thesis, Western blotting was performed on cell samples only, and the method was used in Paper III to evaluate expression of CKAP4, PDGF receptors or downstream effectors of PDGF signaling after PDGF-BB treatment, and in Paper IV to investigate the expression of proteins associated with podocyte injury.

Prior to analysis, the protein concentrations in cell lysates were determined by bicinchoninic acid assay (BCA), so equal amounts of protein could be used as input in the analysis. The proteins in each sample were prepared by addition of a reducing agent (dithiothreitol, DTT) and an SDS-containing sample buffer to the equalized cell lysates, and were subsequently exposed to high temperature (95 °C) for 5 min, which would linearize and add a uniform negative charge of the proteins. The complex protein samples were resolved by SDS-PAGE and blotted onto a PVDF membrane, which were probed using a primary antibody against the protein of interest. A secondary antibody coupled to horse-radish peroxidase was used for detection after addition of substrate for the enzyme, emitting chemiluminescent light.

Although Western blotting is a useful method for semi-quantitative comparison of protein content between samples, there are some limitations to be considered. One is the difficulty to compare results between blots, as background noise and exposure times during development in the camera equipment may vary between runs. There is also a need for normalization of the output signal to a loading control protein or the total protein content in each sample, which is necessary before any further comparison is made, but can be a cause of variation between runs. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control in Paper III and IV. GAPDH catalyzes the sixth step of glycolysis, and is used for normalization because of its stable expression in many cell types. When using the expression of a so called housekeeping protein for normalization it is important to consider the possibility that it may be differentially expressed in treated or genetically manipulated cells compared to their untreated controls.
An alternative to the use of housekeeping proteins is to normalize against the total protein content of each sample run in the Western blot. This method is new relative to the use of housekeeping proteins, and the main reason for choosing to use GAPDH for normalization was to keep consistent between analyses performed over a longer period of time.

### 3.6.4 Mass spectrometry

Analysis of the proteome by mass spectrometry was included in Paper II, III and IV. In the papers, the total protein content in samples from cultured mesangial cells or podocytes was investigated and a semi-quantitative analysis of relative expression was performed to glean information on up or down regulation after genetic manipulation or treatment with gd-IgA1. Comparison of the relative protein abundance was enabled by 10-plex tandem mass tagging (TMT) (106), with a detailed method description in each respective paper. Sample preparations and mass spectrometry analyses were done by the Proteomics Core Facility at the University of Gothenburg.

### 3.6.5 Biotinylation of cell surface proteins

In Paper III, we were interested in if CKAP4 was present at the mesangial cell surface, as one hypothesis regarding its function was that it could interact with the PDGFRβ in the plasma membrane. One method for isolation of cell surface proteins is addition of sulfo-NHS-SS-biotin to intact cells. This compound is used to label the proteins on the cell membrane, which allows for subsequent precipitation of biotinylated proteins by NeutrAvidin. In the study a ready-to-use kit from ThermoFisher was used (Pierce™ Cell Surface Protein Isolation Kit, #89881) for labeling of commercially obtained human mesangial cells (Cell Systems). The input sample, flow-through after precipitation and the eluate were analyzed by Western blotting and the membrane probed with anti-CKAP4, anti-PDGFRβ (cell surface protein) or anti-ERK 1/2 (intracellular protein).

One limitation to the biotinylation assay is the effect of steric hindrance on the binding of sulfo-NHS-SS-biotin, as some proteins on the cell surface might be shielded by others. There is also a risk of biotinylating intracellular proteins if cell integrity is compromised during handling, or if the biotinylation reaction is not properly quenched before cell lysis. The analysis of a known cell surface protein and an intracellular protein during Western blotting are indicators of if cellular integrity was maintained during the experiment.
3.6.6 Bio-plex immunoassay

The Bio-plex 200 multiplex immunoassay (BioRad) was used in Paper I for investigation of cytokines and growth factors released into the culture medium by mesangial cells exposed to IgA from IgAN patients or healthy individuals. The cells originated from IgAN patient biopsies or a TGBM patient biopsy, or were commercially purchased (Lonza). The assay enables quantitative analysis of several proteins and peptides simultaneously, based on a system of reagents (e.g. antibodies) conjugated to beads, each colored with different concentrations of two fluorescent dyes. Detection of the specific fluorescent signature of each bead is coupled with quantification of the fluorescence emitted by a reporter dye on a second antibody targeting the same antigen as the bead-conjugated first antibody. Laser of two different wavelengths (green 525 nm, red 635 nm) is used to excite the fluorescent label of the beads and reporter dyes. The emission of fluorescent light from the bead label and the reporter dye are then detected and used for quantification of protein concentration for each type of protein included in the assay. Usually, the proteins included are involved in the same cellular disease process, such as inflammation or cancer. The use of multiple antibodies in each sample enables quantification of several proteins simultaneously, which provides an advantage compared to enzyme-linked immunosorbent assays (ELISA) where only expression of one protein can be determined per experiment.

3.6.7 Bioinformatic analysis of protein expression

Bioinformatic analysis was required to identify differential expression of proteins after mass spectrometry analysis. In Paper II, the effect of 48 h incubation with IgA purified from serum obtained from patients with IgAN on the protein expression in commercially available human mesangial cells (Lonza) was investigated. The differences between treated cells and controls cells were assessed by t test statistics with Benjamin-Hochberg multivariable adjustment, and proteins with a fold difference <0.85 or >1.15 together with a p-value <0.05 were considered significantly down or up regulated, respectively, after considering the technical variance of 10% introduced by the use of tandem mass tags during analysis. The proteins with significantly different expression were analyzed by Ingenuity Pathway Analysis (IPA) and the Genomatix Genome Analyzer for association with cellular pathways.

In Paper III and IV, there were 3 groups of cells included in the mass spectrometry analysis; WT, virus control and cells with either silenced or overexpressed CKAP4. Expression of each detected protein was compared to the averaged expression in the WT samples, and comparison between cells
with silenced or overexpressed CKAP4 and their respective virus controls was done by Student $t$ test with Benjamin-Hochberg multivariable adjustment. Proteins with a fold difference <0.8 or >1.2 were considered down or up regulated, respectively, and included in pathway analysis by IPA to identify significant differentially regulated pathways.

### 3.7 Co-immunoprecipitation

In Paper III, one hypothesis regarding the function of CKAP4 in mesangial cells was that the protein might interact directly or indirectly with the PDGFRβ. The method of endogenous co-immunoprecipitation (co-IP) was used to test whether such an interaction could exist. Cell lysates from human mesangial cells were first incubated with an antibody either against CKAP4 or PDGFRβ, and the antibody-protein complexes were precipitated using magnetic beads coated with Protein G. The inputs and eluates were analyzed for presence of CKAP4 and PDGFRβ by Western blotting.

One advantage with co-IP performed on primary cell lysates is the increased probability of a true physiological interaction between the proteins, compared to co-IP performed using cells (e.g. HEK-cells) genetically modified to overexpress both proteins, also called exogenous co-IP. Both methods however risk detecting non-physiological interactions due to mixing of all cellular proteins in a lysate, where the inherent separation of proteins in different cellular compartments is lost. An endogenous co-IP can also be disadvantageous when compared to the exogenous version due to limited endogenous expression of one or both proteins included in the experiment. In order to increase the likelihood of detecting a native interaction in the co-IP, a control experiment is performed where the experimental setup is reversed with respect to the precipitating antibody.

### 3.8 Cell proliferation and viability assays

Cell proliferation was assessed in Paper I and III, and viability in Paper III and IV. As Paper I and III included investigations into the effects of treatments or genetic manipulation on the PDGF signaling pathway, which is associated with mitogenic effects on mesangial cells, proliferation was used as a functional readout. Assessment on viability was done with the intention to evaluate if silencing or overexpression of CKAP4 would affect this parameter. Since detachment was observed for both cultured mesangial cells and podocytes after CKAP4 silencing but not overexpression, it was
important to elucidate whether this was due to a decrease in viability or might be attributable to other factors.

### 3.8.1 BrdU cell proliferation assay

Assessment of cell proliferation was done using the BrdU Cell Proliferation ELISA (Roche/Sigma Aldrich), measuring chemiluminescence. Mesangial cells in 96-well plates (5000 cells/well) were starved overnight (0.5% FBS) and exposed to treatments such as IgA purified from serum of IgAN patients or recombinant PDGF-BB for 20 h before analysis, as detailed in the methods of the respective papers. The thymidine analogue 5-bromo-2’-deoxyuridine (BrdU) was then added to the growth medium and incorporated into newly synthesized DNA. An antibody against BrdU coupled to peroxidase was used to enable chemiluminescent detection of BrdU incorporation, after addition of substrate for the enzyme. The obtained chemiluminescent signal in relative light units per second (rlu/s) directly correlates with the amount of newly synthesized DNA, and thereby with the number of dividing cells during the time of exposure to BrdU, assuming there were the same number of cells at the start of the analysis.

### 3.8.2 Alamar Blue cell viability assay

The viability of cultured human mesangial cells and podocytes after lentiviral mediated silencing or overexpression of CKAP4 was assessed by the Alamar Blue cell viability assay (ThermoFisher). The reducing ability of the cells is used in the assay as an indicator of cell viability, as viable cells are able to reduce the active compound of Alamar blue, resazurin, to the highly fluorescent compound resorufin. The shift in color from blue (resazurin) to pink (resorufin) is observable by eye and quantifiable by measurement of fluorescence. An equal number of cells seeded before addition of Alamar blue is important to enable comparisons between different treatments.

In Paper III, the viability of mesangial cells seeded in a 96-well plate (5000 cells/well) was assessed directly in the plate by addition of 10% Alamar blue reagent to the growth medium and incubating for 2 h at 37°C before detection of fluorescence. Measurements were done before lentiviral transduction, as well as 24, 72 and 144 h after. In Paper IV, human podocytes were cultured in a 12-well plate and Alamar blue was used to measure viability before infection as well as 45 and 117 h after, by transferring medium from cells incubated with 10% Alamar blue for 2 h at 37°C to wells of a 96-well plate before measuring fluorescence. The culture of podocytes directly in the same collagen coated 96-well plate used for fluorescence readout was attempted
unsuccessfully, as cells belonging to both control and lentiviral treated groups started to detach from the bottom of the wells.

3.9 Gene silencing and overexpression

In Paper III and IV, gene silencing or overexpression of CKAP4 in mesangial cells and podocytes were used with the aim to elucidate its function in these cell types. There are several methods for gene silencing or overexpression in vitro, with varying mechanisms of action and stability. In both papers, we used lentiviral mediated shRNA silencing or overexpression, which alters the genome of the cells so they express either shRNA against the mRNA of the protein, or the protein itself. Other methods can induce transient instead of a stable gene expression (transfection instead of transduction), or in the case of gene silencing, a completely removed gene expression (knock out) instead of a reduced expression (knock down).

Lentiviruses were used as vectors for the transfer of shRNA into the cellular genome. They are a subtype of retroviruses able to transduce both dividing and non-dividing cells. The viruses used for genetic manipulation are derivatives of the HIV-1 virus, modified for increased safety by e.g. dividing the genetic material needed for assembly of the virus on several plasmids, and deleting regions needed for viral replication after integration into the host genome (107). The viruses were produced by HEK293T cells transfected with two separate plasmids for production of the lentiviral capsid and envelope, as well as a third transfer plasmid containing the shRNA or overexpression gene. Assembled viral particles were released into the growth medium of the HEK293T cells, which was aliquoted and stored at -80 °C before use. An assessment of the percentage of virus-containing medium to be added to the growth medium of mesangial cells or podocytes for optimal effect of knock down or overexpression was done for each new batch of viruses, with reduction or overexpression of the target mRNA and protein analyzed by TaqMan™ PCR and Western blotting.

When considering gene silencing, it may be advantageous to reduce expression instead of removing it if the gene is important for normal cell function. Previous publications investigating the function of CKAP4 in non-renal cell types have used the method of siRNA transfection, silencing CKAP4 expression only transiently (84, 108). To my knowledge, there are no publications today where CKAP4 expression has been knocked out in vitro.
3.9.1 Gene silencing by shRNA
An shRNA targeting the sequence GCAGGATTTGAAAGCCTTAAA was used in Paper III and IV for silencing of CKAP4 in mesangial cells and podocytes. The shRNA was purchased from Sigma-Aldrich already inserted into the transfer vector pLKO.1, from the first version of The RNAi Consortium (TRC) shRNA library (109). After virus production and titration as previously described, mesangial cells or podocytes were transduced with virus-containing medium in the presence of 4 μg/ml polybrene (hexadimethrine bromide) to enhance transduction efficiency. Mesangial cells were transduced after reaching about 40% confluence, while podocytes were transduced 5-7 days before completing differentiation. The virus medium was kept on the cells overnight (16 h) and the cells were thereafter handled normally. Eventual treatments and harvest of the cells were done 5-7 days post transduction.

3.9.2 Gene overexpression
Ectopic expression of CKAP4 in mesangial cells and podocytes was achieved by transduction of cells with the CKAP4 gene incorporated into the VVPW-EGFP vector backbone (kindly provided by Dr. Anna Greka at Massachusetts General Hospital, Boston, USA). The CKAP4 gene was amplified for use in molecular cloning using the gene inserted in a pCMV-AC-GFP vector obtained from Origene (#RG210270) as a template. The gene was cloned into the VVPW-EGFP vector upstream of EGFP, in order to produce a fusion protein with EGFP at the cytosolic N-terminus of CKAP4. The gene sequence of CKAP4 in the new vector was verified by Sanger sequencing, done by Eurofins laboratories in Ebersberg, Germany. Once verified, the VVPW-CKAP4-EGFP vector was used as transfer plasmid in lentivirus production as previously described. The procedure for the subsequent transduction of mesangial cells and podocytes was similar for overexpression as for shRNA silencing. The fusion protein CKAP4-EGFP weighed approximately 96 kDa (CKAP4 63 kDa + EGFP 33 kDa) and was fluorescent, making it distinguishable from endogenous CKAP4 in Western blotting and visible by fluorescence or confocal microscopy.

3.10 The PAN nephrootoxic model in vitro
The human podocytes in Paper IV were exposed to puromycin aminonucleoside (PAN) as a method of simulating chronic injury to the cells. Administration of PAN to rats induces a condition similar to minimal change disease (MCD), with loss of structure in the podocyte foot processes and proteinuria (110). PAN has also been shown to induce disruption of the
cytoskeleton of murine podocytes, as well as increase oxidative stress and
decrease cell viability (111). A drawback to the use of PAN in vitro is the
lack of available crosstalk with other glomerular cells, which is of great
importance in the cells’ response to injury in their physiological setting.

3.11 Statistical analysis

The statistical analyses were performed in GraphPad Prism versions 6 to 8,
unless otherwise stated. Error bars are consistently represented as SEM in
Paper I, while in Paper III they are represented as SD, and Paper IV both SD
and SEM are used, as indicated in the figure legends.

For statistical comparisons between when there were only two groups, the
Student’s t-test was used, while comparisons between multiple groups were
done by parametric ANOVA followed by Bonferroni-Sidak multiple
comparisons test. When comparing gene expression of connexin 43 between
groups in Paper IV, the Kruskal-Wallis non-parametric test in combination
with the Mann-Whitney U post hoc test was used for statistical analysis. A p-
value <0.05 was considered statistically significant, and *p<0.05, **p<0.01,
***p<0.001 in the graphical representations of the data.
4 RESULTS AND DISCUSSION

The four papers constituting this thesis investigate the differential molecular profiles of healthy glomerular cells and glomerular cells affected by chronic kidney disease. The first paper considers the hypothesis that mesangial cells from patients with IgAN are more sensitive to stimuli associated with the disease than healthy mesangial cells, and suggests that mesangial cell susceptibility to these stimuli could be an additional “hit” required for development of IgAN.

In the second paper the difference between healthy and diseased cells in the context of IgAN is further explored, both from a glomerular perspective and from the more specific perspective of mesangial cells exposed to gd-IgA in vitro. The transcriptomic and proteomic analyses included in Paper II highlight the effect of the disease primarily on mesangial cells, and finds signaling pathways including differentially expressed genes and proteins that could be of interest for further research into IgAN development and progression.

The third paper focuses on the regulation of mesangial cell proliferation, which is relevant both in the context of IgAN and other diseases affecting the mesangial cells, such as DN. The function of the protein CKAP4 is investigated in healthy human mesangial cells, as it has been associated with proliferative signaling in other cell types and in cancer, but has not previously been studied in the glomerulus. When silencing CKAP4 expression in the mesangial cells the expression of the PDGF receptors was significantly downregulated. The downstream signaling pathway was also less activated after PGDF-BB stimulation, which suggests a role of CKAP4 in mesangial proliferative signaling that warrants further investigation.

The fourth paper concerns the function of CKAP4 in podocytes, where preliminary results indicate that the protein has an essential role in maintenance of the actin cytoskeleton structure. As some chronic kidney diseases involve loss of podocyte cytoskeletal integrity, leading to effacement and consequently impaired renal filtration, there are incentives to further investigate the role of CKAP4 in these cells as well as in the mesangial cells.

In this section, the results from each study are discussed in more detail.
4.1 Paper I: Mesangial cells from patients with IgA nephropathy have increased sensitivity to galactose-deficient IgA1

The multi-hit hypothesis describing the pathogenesis of IgAN highlights the contribution of several factors to disease development. In addition to increased levels of gd-IgA1 in the circulation, patients with IgAN also have IgA or IgG autoantibodies against the gd-IgA1, which when bound to gd-IgA1 forms circulating immune complexes that deposit in the mesangium of the kidney, activate the mesangial cells and initiate glomerular injury (39). The exact details around how the gd-IgA1 containing immune complexes activate the mesangial cells and trigger proliferation and extracellular matrix expansion still remain to be elucidated. There are however strong indications that IgAN is a systemic disease, as 60% of transplanted IgAN patients get recurring IgA1 depositions in the new graft (112), although the risk of recurring disease varied between 12 and 61% (mean 33%) according to an overview of several studies done by Dr. Claudio Ponticelli and colleagues (113). In the familial form of IgAN, making up 5-10% of patients, the levels of gd-IgA1 in serum is higher than in patients with sporadic IgAN (90-95% of patients) in both the affected patient and in relatives without renal symptoms (49). These findings motivate studies of potential differences between mesangial cells from IgAN patients and healthy individuals.

In Paper I, our hypothesis was that mesangial cells may be more susceptible to disease in patients with IgAN than in healthy individuals, which could be an additional hit required for disease development. As only humanoid primates have IgA1 with the type of hinge region susceptible to galactosylation and therefore formation of gd-IgA1, there are limitations to the use of small-animal models of IgAN. Cell culture of human mesangial cells has then been described to be a convenient alternative to study the effect of gd-IgA1 containing immune complexes on this cell type (40). In this paper, we designed a new technique to obtain mesangial cells from routine biopsies used for diagnosis of IgAN, described in detail in the method section of the paper, in order to compare them to cells from healthy kidney donors or cells from a patient with thin glomerular basement membrane disease (TGBM) and normal renal function. The TGBM patient was included as a disease control without an inflammatory component. In total, mesangial cells from six patients with either IgAN (n=3) or IgA vasculitis (n=3) were included in the study. In addition, three patients with IgAN and three healthy volunteers donated blood for purification of IgA1. An ELISA using lectin from the garden snail *Helix aspersa* was used to determine levels of gd-IgA1.
in the blood samples, and showed that the IgAN patient samples had a higher ratio of gd-IgA1 in the IgA1 fraction compared to the healthy volunteers.

PDGF is known as the most potent growth factor for mesangial cells (65), and PDGF isoforms are upregulated in mesangioproliferative diseases (67, 114). Mesangial cells from IgAN patients or controls were therefore treated with IgA from IgAN patients (gd-IgA) or IgA from healthy controls (clgA) purified from serum, and were subsequently analyzed for mRNA expression of PDGFB (the gene coding for PDGF subunit B) and PDGFRB (the gene coding for PDGFR receptor subunit β). The release of PDGF-BB into the cell medium by IgA treated cells was also determined. Mesangial cells from IgAN patients had a higher mRNA expression of the PDGFB gene after treatment with either clgA or gd-IgA, while healthy mesangial cells did not significantly increase mRNA expression in response to either treatment. The expression of PDGFRB was unchanged for both healthy and diseased cells after clgA or gd-IgA exposure, though the baseline expression was notably lower in IgAN patient cells compared to control cells. Both healthy and IgAN patient cells released more PDGF-BB into the culture medium after being treated with gd-IgA than untreated cells or cells treated with clgA. Additional analysis of proliferation after exposure to PGDF-BB showed that the cells from IgAN patients responded to the growth factor by increasing proliferation more than control cells did. Cells from IgAN patients thereby seemed to be more responsive to PGDF-BB, and also to release more PDGF-BB than healthy mesangial cells when exposed to gd-IgA. A similar result was seen in a previous study from Dr. MJ Kim et al., who observed an increased production of PDGF-BB after healthy human mesangial cells were treated with gd-IgA (91).

TGFβ1 is one of the cytokines released from mesangial cells as a consequence of IgA immune complex deposition in IgAN (43, 115). Stimulation of cultured rat mesangial cells with TGFβ has been shown to induce upregulation of mRNA coding for PDGFB and PDGFRB (116), and stimulation of human mesangial cells with PDGF-BB has been observed to induce upregulation of mRNA for TGFβ1 (117). In our hands, treatment with gd-IgA significantly increased mRNA expression of TGFβ1 in the cells from IgAN patients, while the same trend was seen for healthy mesangial cells, although not sufficiently for statistical significance. The cells from IgAN patients also released more TGFβ1 into the cell culture medium when treated with clgA or gd-IgA when compared to untreated cells, as measured by a Bio-Plex multiplex immunoassay (BioRad). These results are in line with the previous findings from Lai et al. (115).
Both mesangial cells from IgAN patients and from healthy donors increased production and release of the cytokine IL-6 in response to cIgA or gd-IgA, although the release of IL-6 into the culture medium was exaggerated in the diseased cells compared to the healthy controls. Previous studies have indicated that IL-6 is upregulated in glomerular tissue in IgAN (118), and that mesangial cells in culture produce and release IL-6 in response to exposure to IgA purified from IgAN patient serum (119). The same response as for IL-6 was seen for the chemokine CCL5, also called RANTES, however only for cells treated with gd-IgA and not cIgA. In contrast to these results, the release of IL-8 and MCP-1 by mesangial cells from IgAN patients were unaffected by exposure to either cIgA or gd-IgA, while control cells increased secretion of both chemokines when exposed to gd-IgA, and IL-8 also when treated with cIgA. In the previously referenced study by Dr. MJ Kim et al., production of all the above mentioned cytokines and chemokines were elevated in the human mesangial cells after exposure to gd-IgA from IgAN patient serums (91). The effect of cIgA was however only investigated for MCP-1 in that study, and the mesangial cells originated from healthy individuals and not IgAN patients.

Based on the resulting increases in production and secretion of TGFβ1, IL-6 and CCL5 in both mesangial cells from IgAN patients and from healthy individuals included in this study, we hypothesized that these cytokines were released as a general response to exposure of the cells to gd-IgA, and not as part of an IgAN specific phenotype.

Since a consequence of immune complex deposition in the mesangium in IgAN is the expansion of the mesangial cell matrix, we investigated the expression of matrix-associated genes in healthy mesangial cells and IgAN patient cells before and after treatment with cIgA or gd-IgA. Among the six genes included in the analysis, only decorin expression was significantly increased in IgAN cells compared to healthy control cells at basal level. There was however a trend of increased expression of all the genes, except for biglycan. Treatment with cIgA or gd-IgA did not seem to alter the expression of matrix proteins in either group of cells.

There were limitations to this study. One was the risk of selection bias due to the exclusion of some patients whose cells we were not able to culture. This draw-back was discussed in the paper, as well as the risk of patient cells having an altered phenotype due to active inflammation in the glomerulus of the IgAN patients included in the study. Continuous culture under standard, non-inflammatory conditions could possibly have reduced this risk and allowed for cells obtained from an inflammatory environment to recover.
In conclusion, the results of this study show that mesangial cells from patients with IgAN have increased sensitivity to stimulation with PDGF and gd-IgA compared to cells from healthy individuals or from a patient with non-inflammatory renal disease and normal renal function. The results were less conclusive regarding release of cytokines and chemokines in response to gd-IgA. From these results we suggest that mesangial cells with a proliferative phenotype could be an additional “hit” required for development of IgAN.

4.2 Paper II: Transcriptomic and proteomic profiling provides insight into mesangial cell function in IgA nephropathy

The fourth “hit” in the hypothesis on the pathogenesis of IgAN is the deposition of immune complexes in the glomerular mesangium and activation of the mesangial cells. Through mechanisms not yet fully elucidated, the mesangial cells start to proliferate and produce more extracellular matrix, cytokines and chemokines, which also have deleterious effects on the neighboring podocytes and on the tubulointerstitium (43). Activation of the complement system via the alternative or the lectin pathway systemically by the immune complexes, or in situ in the mesangium is also associated with disease development (120). Although the mesangial cells are greatly affected by the disease, studying them in the context of IgAN can be challenging due to the difficulty of developing a suitable animal model (121) and the lack of mesangial cells specific markers. To further understand the molecular mechanisms underlying disease development it is important to study the cellular responses of the isolated mesangial cells, such as in Paper I, as well as to profile more general changes in transcription and translation in more complex cell systems, comparing healthy and diseased tissues.

In Paper II, we aimed to investigate the role of the mesangium in IgAN by an integrated transcriptomic and proteomic analysis. The transcriptomic analysis was performed on biopsy specimens collected from IgAN patients or healthy living kidney donors after reperfusion in the graft recipient, with the consent of the patients to collect the material as part of a routine renal biopsy. Clinical data was obtained from the participating patients at the time of biopsy and up to 4 years later. The proteomic analysis was performed on cultured commercially available mesangial cells treated with IgA1 isolated from serum of IgAN patients.
The transcriptomic analysis included glomeruli from 25 patients with IgAN and 26 healthy living kidney donors. The IgAN patients were determined to be in CKD stage 1 to 3 out of 5, based on estimated GFR (eGFR) at the time of biopsy. Since collection of biopsies was ongoing from 2004 and forward, microarray analyses were run at five time points in total during that time period. Two analyses contained the same sample from an IgAN patient as a control for variation between runs. After quality control within each batch, 20 IgAN patient samples and 22 control samples were used in further statistical analysis. Results from the batches were merged and differentially expressed genes were identified by significance analysis of microarrays (SAM) (122).

Building on the excellent work of Dr. Wenjun Ju and colleagues at the University of Michigan, the transcriptomic data was analyzed for expression of cell-type specific standard genes, in a collaboration with Dr. Ju and her research team. In a publication from 2013, they introduced an iterative machine learning algorithm for identification of cell-type specific genes called “in silico nanodissection”, which considers microarray data from whole tissue samples (105). The genes in our microarray data set identified by in silico nanodissection as specific for mesangial cells or podocytes were assessed for differential expression, and the results were evaluated for correlation with clinical parameters (serum creatinine and eGFR). Out of 35 positive standard genes identified for mesangial cells through in silico nanodissection, 27 (77%) were found in our microarray data set. Among the 27 genes, 21 (78%) were differentially expressed in IgAN patients compared to healthy controls. For podocyte positive standard genes, there were 50 genes identified by in silico nanodissection, out of which expression of 35 genes (70%) were found in our microarray data. There were however only 15 of the 35 genes (43%) with significant up or down regulation between diseased and control samples. Statistically significant differential expression equaled SAM q-value <0.01. The findings are summarized in Table 2, which is an adaptation of the Supplementary Table 2 in the published article (123).
Table 1. Mesangial cell and podocyte standard genes from in silico nanodissection identified in our microarray analysis and evaluated for differential expression. Genes detected in the microarray data set and found to be significantly up or down regulated are under “Significantly up/down regulated”, while genes found in the data set but not differentially regulated are in the column “Found in data set, not up/down regulated”. The genes last column represents genes identified as cell-specific standard genes through in silico nanodissection, but not found in our data set.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Significantly up/down regulated</th>
<th>Found in data set, not up/down regulated</th>
<th>Not found in data set</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mesangial cell</strong></td>
<td>ACTA2, AGTR1, AKR1C3, BMP4, CAD, CALD1, CD34, ETS1, F2R, FBN1, IGFBP5, ITGAV, KLF6, Nox4, SERPINE1, PCDH12, TAGLN, TNS1, TNS3, TXNIP, USP2</td>
<td>F5, GAS6, ITGA8, NES, SERPINE2, THY1</td>
<td>ANGPT2, ITGB1, PDGFRA, PDGFRB, PTGS1/COX1, SERPINB7, SOX9, TG2</td>
</tr>
<tr>
<td><strong>Podocyte</strong></td>
<td>ACTN4, TCF21, MAGI2, KIRREL, CASK, TJP1, CLIC5, MME, UCHL1, PALLD, FYN, PLAUR, DAG1, EZR, MAFB</td>
<td>NPHS1, NPHS2, SYNPO, PTPRO, NES, DDN, WT1, KIRREL2, TRPC6, NCK2, PDPN, LMX1B, FAT1, PLCE1, AGRN, EFNB1, UTRN, BASP1, SULF1, CX3CL1</td>
<td>LRRC7, MAGI1, RAB3A, KIRREL3, CD2AP, IQGAP1, DES, PODXL, CDH3, CDH13, CD80, Sv2b, FOXC2, MYOC, SCEL</td>
</tr>
</tbody>
</table>

The 27 mesangial cell standard genes identified in our microarray data set were able to cluster IgAN patient samples and the healthy donor controls separately in both hierarchical clustering with the Ward averaging method (124) and by principal coordinate analysis. The 35 identified podocyte standard genes did not separate the groups using either method. This
indicates that the mesangial cell standard genes are relevant in the development of IgAN, while the podocyte standard genes might be less important in this context. Considering the early stages of CKD affecting the patients included in the study, the results suggest that the mesangial cell standard genes identified through in silico nanodissection are relevant in early pathogenesis of IgAN. However, when considering the mesangial cell hypercellularity criteria in the MEST scoring system, only 3 out of 19 IgAN patients scored 1 instead of 0, where a score of 1 is given if >50% of glomeruli show mesangial hypercellularity (51). The mesangial cells might still be affected in patients scoring 0, and the response of the cells to IgA1 exposure with increased production and release of cytokines such as TGFβ (115) could be speculated to contribute to the segmental sclerosis score of 1 observed in 14 out of the 19 patients. The low presence of mesangial cell hypercellularity among the patient samples is also indicative of that the results seen in the microarray analysis are not due to a higher number of mesangial cells in the patients’ glomeruli.

Information on serum creatinine level for each patient included in the study were collected with the patient’s consent at the time of biopsy and during follow up meetings up to 4 years after diagnosis when monitoring disease progression. In total between 8 and 22 serum creatinine values per patient were obtained and used for analysis. Each patient’s GFR were estimated by the CKD-EPI creatinine equation (125) using the measured serum creatinine concentrations. To enable an analysis of possible correlation between the mesangial cell or podocyte standard genes and serum creatinine or eGFR, the standard gene expression in each patient sample was compared to the standard gene expression in all patient samples, yielding a Z-score as described by the equation below.

\[
Z = \frac{(AveStd_{\text{patient}} - AveStd_{\text{all}})}{StdevStd_{\text{patient}}/\sqrt{n}}
\]

Where \( AveStd_{\text{patient}} \) is the average value of the positive standard gene expression in each patient, \( AveStd_{\text{all}} \) is the average value of the positive standard gene expression among all the patients included in the study, \( StdevStd_{\text{patient}} \) is the SD of the positive standard genes in each patient, and \( n \) is the number of positive standard genes. The higher absolute value of the Z-score, the more the average expression of the cell-specific positive standard genes deviates for the individual patient compared to the average of the entire group of patients in the study.
When using the Z-scores calculated for mesangial cell standard genes there was a statistically significant Pearson correlation with serum creatinine concentrations at the time of biopsy ($r^2=0.25$, p-value=0.024) as well as with the calculated eGFR ($r^2=0.25$, p-value=0.025), while there was no significant correlation between patient Z-scores for podocyte specific standard genes and either of the clinical parameters. When grouping patients according to each parameter in the Oxford MEST score and comparing with the Z-scores for mesangial cell standard gene expression there was a significant difference between patients scoring 0 or 1 for segmental glomerulosclerosis (S), which was not present when comparing to Z-scores for podocyte standard gene expression, while no other MEST parameter were significantly correlated with expression of either set of standard genes. These results provide further indications that the mesangial cell specific standard genes are important for development of IgAN.

To further focus on changes in mesangial cells during IgAN development we used the same method as in Paper I to mimic conditions of IgA1 deposition. IgA1 was purified from serum of three IgAN patients and three healthy controls and the content of gd-IgA1 assessed before treating the cells. Only purified IgA1 from serum of IgAN patients was used for cell treatment, and compared to untreated cells as control. Analysis by LC-MS/MS was followed by statistical evaluation of protein expression, where ±15% difference in fold change between IgA-treated cells and controls was applied as cutoff for differential expression together with p-value<0.05 in a t-test. The differentially expressed proteins were analyzed by Ingenuity Pathway Analysis (IPA, Qiagen), and compared to the pathways with changed glomerular transcriptomic expression in IgAN patients compared to healthy controls. Among the top ranked pathways overlapping between the analyses, most were related to inflammation. One of them with a known role in IgAN is the complement system, as the disease is associated with activation of the alternative and the lectin pathway, and pathway components such as properdin, factor H and mannan-binding lectin can be found in mesangial immune deposits (120).

The results from Paper II point to the mesangial cells as primarily affected by IgAN, and suggest that the inflammatory response elicited by the cells in response to gd-IgA1 may have a more prominent role in disease development than mesangial cell hyperproliferation in the cohort studied. This is based on the low number of patients scoring M1 by the MEST scoring system (16%), while many patients had a score of S1 (74%), and the finding of mainly inflammatory differentially regulated pathways in common between the glomerular transcriptomic analysis and the gd-IgA1 treated cell proteomic...
analysis. In a study of a European IgAN cohort aiming to validate the Oxford MEST scores as predictors of disease progression, there were also more patients scoring S1 than M1 (70% and 28% respectively), although both parameters were independently found to be associated with proteinuria, mean arterial pressure and eGFR at the time of biopsy (126).

Although the podocyte standard genes identified in our microarray analysis did not cluster patient samples from healthy controls and did not show a significant correlation with serum creatinine or eGFR, they have been found to be affected in IgAN through crosstalk with the mesangial cells (43, 127). A study from 2017 also suggests a subclassification of the S-score in MEST considering podocyte injury (128). As the effect on podocytes could at least in part be a consequence of altered mesangial cell signaling in IgAN, it can be speculated that the early CKD stages of the patients included in this study might not have reached the stage of deleterious effects on podocytes possibly emerging with progression of disease. However, the results concerning patient clinical parameters in this study were based on correlations between Z-scores of standard gene expressions and measurements of serum creatinine, while it would have been interesting from the perspective of podocyte injury to also assess correlation with proteinuria. Another limitation was the absence of analysis of endothelial cells standard genes in the glomerular transcriptomic data. This was not done since the endothelial cell standard genes had not yet been verified by the time our analysis was done.

In conclusion, this study suggests that the standard genes for mesangial cells determined by in silico nanodissection are relevant for further research into development of IgAN, as many of them were differentially expressed in glomeruli from IgAN patients compared to healthy kidney donors in our cohort. The mesangial cell standard genes also had a stronger predictive value than the podocyte standard genes in this study, further supporting their prominent role in IgAN. The mesangial standard gene expression Z-scores from the IgAN patients could be correlated to the patients’ serum creatinine concentrations and eGFR, supporting the assumption that expression of these genes is relevant for disease development. The signaling pathways found to have significant differential expression in the glomerular transcriptome of IgAN patients compared to healthy kidney donors, and that were also differentially expressed in the proteome of mesangial cells treated or untreated with gd-IgA1, highlights the role of inflammation in the early disease stages and could be further investigated in the context of IgAN development.
4.3 Paper III: Cytoskeleton-associated protein 4 (CKAP4), a new player in the regulation of mesangial cell proliferation

Increased mesangial cell proliferation following deposition of immune complexes containing gd-IgA1 in the mesangium is one of the pathological events in IgAN (129). Presence of mesangial cell hyperproliferation at the time of biopsy is, as previously mentioned, independently associated with lower renal survival and part of the Oxford MEST scoring system (126). Other glomerular diseases, such as DN, also affect mesangial proliferative signaling, although DN is mainly associated with hypertrophy of the mesangial cells and increased extracellular matrix production in the glomerular mesangium (130, 131). Glomerulonephritis associated with systemic lupus erythematosus, called lupus nephritis (LN), can in some instances cause mesangial cell hyperproliferation due to deposition of immune complexes (132). The complexes however usually have dominant or co-dominant IgG instead of IgA, and do not contain aberrantly glycosylated IgA as do the complexes in IgAN (46). IgAN, DN and LN are distinct diseases and differ in etiology, but all three involve dysregulated mesangial cell proliferation. There have been reports of upregulation of the important mesangial mitogens platelet-derived growth factors (PDGFs) and their receptors PDGFRα and β in all three diseases (65, 66, 133, 134), while it remains to be elucidated if PDGF signaling might have different roles in the different diseases.

In Paper III, we aimed to study the function of a protein which as far as we know has not previously been studied in human glomerular cells. The protein, cytoskeleton-associated protein 4 (CKAP4), has known functions in other cell types, and has attracted attention recently for its involvement in cancer cell proliferation as a receptor for dickkopf-1 and -3 (DKK1 and 3) (84, 85). Our starting hypothesis was however not directly linked to proliferation, as initial data suggested a possible interaction between CKAP4 and IgA1 purified from IgAN patients.

In an attempt by our research group in 2012 to find proteins interacting with pathogenic IgA1, cell lysates from IgAN patient mesangial cells or healthy control cells were resolved by SDS-PAGE and exposed to IgA1 purified from IgAN patients as primary antibody. A band of approximately 60 kDa in size was detected through Western blotting, and found to differ in intensity between the patient and control sample. It was therefore further analyzed by mass spectrometry to determine included proteins. Out of the proteins
identified in the 60 kDa band, CKAP4 emerged through literature curation as an interesting candidate for further analysis, considering its previously known function as a receptor for various ligands, such as surfactant protein A (80) and tissue plasminogen activator (81).

The effect of shRNA silencing of CKAP4 on mesangial cell proliferation was assessed after treatment with patient serum derived IgA1, based on the hypothesis that silenced CKAP4 expression would diminish the cells’ proliferative response to gd-IgA1. Aggregated results from five proliferation assays failed to confirm the hypothesis, but another interesting effect of CKAP4 silencing was observed. The mesangial cells with silenced CKAP4 seemed unable to achieve the same proliferative response as virus control cells to their main growth factor, PDGF-BB, used in the proliferation assays as a positive control. This finding prompted a further investigation into an eventual link between CKAP4 and PDGF-signaling. Previous studies have also found that CKAP4 is involved in regulation of cell proliferation in different cell types. One study suggested that CKAP4 interacts with the epidermal growth factor receptor (EGFR) in hepatocellular carcinoma cells and deters proliferation (86), and CKAP4 has been suggested to mediate the anti-proliferative effect of anti-proliferative factor (APF) in bladder epithelial cells (82). Its recently discovered role as a receptor for DKK1 and DKK3 has however been associated with increased proliferative signaling in lung-, pancreatic and esophageal cancer cells. The shifted focus to regulation of PDGF-signaling expanded the scope of the study to consider general proliferative signaling via the PDGF pathway in mesangial cells. The implications could then also be extended to diseases such as DN and LN, where both PDGF and overall proliferative signaling are dysregulated.

Since CKAP4 to our knowledge had not previously been studied in the glomerulus, we first assessed its expression in human glomerular tissue through immunofluorescence analysis. Co-localization was observed between CKAP4 and the mesangial cell marker α-smooth muscle actin (α-SMA) in several areas, while the endothelial cell marker Ulex Europaeus Agglutinin I showed little overlap with CKAP4 expression. When staining the nuclei of podocytes using an antibody against Wilm’s tumor 1 (WT-1), simultaneous staining of CKAP4 appeared to surround the podocyte nuclei, indicating expression of CKAP4 in the podocyte cell body. Western blotting of cell lysates from each of the three glomerular cell types supported the finding of relatively low expression of CKAP4 protein in endothelial cells compared to in mesangial cells and podocytes.
As we were considering a potential function of CKAP4 on the mesangial plasma membrane, its presence among cell surface proteins was assessed through surface biotin labeling of intact cells and isolation of labeled proteins through NeutAvidin pulldown. CKAP4 was detected in the flow-through as well as the eluate by Western blot analysis, indicating that the protein is localized both intracellularly and at the plasma membrane of human mesangial cells.

To follow up on the possible link between CKAP4 and PDGF-signaling in mesangial cells, the expression of CKAP4 was either silenced through lentiviral mediated shRNA transduction, or ectopically expressed by the same delivery method as a fusion protein tagged with enhanced green fluorescent protein (EGFP). Analysis of gene and protein expression of the PDGFRα and -β after CKAP4 silencing showed decreased expression of both receptors on both gene and protein level, suggesting that the diminished proliferative response to PDGF-BB seen in the previous proliferation assays might be due to decreased PDGF-receptor expression.

The phosphorylation and expression of proteins known to be activated by ligand binding to the PDGFRs were studied in mesangial cells treated with 25 ng/ml PDGF-BB for 2, 5 or 10 minutes. Autophosphorylation of PDGFRβ at tyrosine 1009, which is necessary for association of phospholipase C-gamma (PLCγ) with the receptor (135), was decreased in all samples with silenced CKAP4. Activation of PLCγ ultimately leads to increased influx of Ca\textsuperscript{2+} into the cells and increased cell proliferation through mechanisms still being elucidated (136). In our PGDF-treated mesangial cells, the phosphorylation of PLCγ on tyrosine 783 was less pronounced in cell with silenced CKAP4 compared to virus control cells, while total levels of the protein seemed unchanged. A similar pattern was observed for mitogen-activated protein kinases ERK1/2 and JNK, which are also activated in response to growth factors such as PDGF and mediate proliferative signaling. Activation of AKT was seemingly not affected by silencing of CKAP4, indicating that the PI3K/AKT pathway might be less affected by decreased expression of the PDGFRs. The results taken together suggest that CKAP4 silencing downregulates expression of the PDGFRs and decreases activation of proliferative signaling through the PDGF pathway.

The effects on PDGFR expression when silencing CKAP4 were also seen in mass spectrometry analysis of mesangial cell lysates. Cells with either silenced or overexpressed CKAP4 were compared to their respective virus control in a semi-quantitative analysis of total protein expression. The results confirmed the previous findings regarding downregulation of both PDGFRα
and PDGFRβ in mesangial cells with silenced CKAP4 expression, while overexpressing CKAP4 did not seem to affect either receptor. Expression of other growth factor receptors such as EGFR and the TGFβRs were less affected by silencing of CKAP4 than the PDGFRs, and unchanged by overexpression of the protein. Notably, the data obtained from overexpressing cells did not cluster separately from virus control cells during hierarchical clustering and principal component analysis, indicating that there was no significant difference in protein expression between the groups. This finding also suggests that the mesangial cells are dependent on CKAP4 for normal function, as silencing of the protein was seemingly more impactful to the cells than overexpressing it. Another observation supporting this hypothesis was that the mesangial cells with silenced CKAP4 changed morphology in culture, and some were detaching from the culture dish, possibly due to cell death or dysregulation of proteins necessary for attachment. When assessing the reducing capacity of the cells as an indicator of viability by Alamar blue assay, there were no indications of dramatic changes in viability due to virus transduction or silencing of CKAP4. Although further experiments assessing e.g. expression of proteins associated with apoptosis could be done to further investigate possible differences in viability between cells with silenced CKAP4 and their controls.

Considering the evidence for a connection between CKAP4 and the PDGFRs and previously known functions of CKAP4, for example linking the microtubules to the ER (73), translocating to the nucleus to affect transcription (83), and functioning as a receptor at the cell surface (81, 84, 137), we theorized that disruption of either of these mechanisms due to diminished CKAP4 expression could be responsible for the effects on PDGFR expression and downstream signaling, either by a direct or an indirect effect. However, since CKAP4 seemingly differs in expression and function between cell types, the just mentioned functions might not all be represented in the mesangial cells. As we had found expression of CKAP4 among cell surface proteins, and the PDGFRs localize to the cell surface in unstimulated cells, we hypothesized that they might interact. This hypothesis was tested by co-immunoprecipitation, and preliminary results suggested an interaction between CKAP4 and PDGFRβ. Using an antibody to block CKAP4 at the cell surface and subsequently treating the cells with PDGF-BB did however not seem to affect activation of the PDGF-signaling pathway more than a marginal effect on phosphorylation of tyrosine 1009. The experiment however requires repetition for more reliable data.

Finally, in a collaboration with Astra Zeneca in Mölndal they kindly shared glomerular sections and RNA sequencing data from BTBR ob/ob mice; a
model for severe diabetes and progressive diabetic nephropathy. Immunofluorescence analysis of CKAP4 expression in the glomeruli from mice 8 to 14 weeks old showed a progressive increase in CKAP4 expression, mainly localized to the mesangium and the podocytes, and which was exaggerated in mice fed a high protein diet. The same progressive increase was observed for CKAP4 in data from RNA sequencing. Although the reason for this finding is not clear, one theory could be that the cells overexpress CKAP4 as a protective mechanism. Further experiments are needed to determine the validity of that hypothesis, and through what mechanism it might function.

In conclusion, the findings of this study show that CKAP4 is an abundantly expressed protein in human mesangial cells and in podocytes, and that the protein is likely necessary for signaling through the PDGF-pathway and normal expression of the PDGFRs. The reduced activation of proteins downstream of the PDGFRs involved in proliferative signaling after CKAP4 silencing and addition of PDGF-BB suggests a role for CKAP4 in regulation of cell proliferation through the PDGF-signaling pathway. One clue to this connection is the possible interaction between CKAP4 and PDGFRβ. The upregulation of CKAP4 in glomeruli of diabetic mice is in agreement with previous findings of CKAP4 overexpression in other disease states, and might be due to a protective mechanism. Further research is however needed to clarify the cause of this upregulation. The role of CKAP4 in mesangial proliferative signaling makes it a relevant to study in the context of diseases with dysregulated mesangial cell proliferation, such as IgAN, LN and DN.

4.4 Paper IV: Cytoskeleton-associated protein 4 (CKAP4) is essential for podocyte integrity

One of the findings in Paper III that was not further explored in that study was the abundant expression of CKAP4 in podocytes, seen in immunofluorescence analysis of human glomerular sections. Podocytes express neither of the PDGFRs under physiological or pathological conditions (64). The high expression of CKAP4 and exclusion of a function involving PDGFRs in the podocytes encouraged us to look for other potential CKAP4 functions in this cell type.

Since the immunofluorescence and the electron microscopy analyses of glomeruli in Paper III suggested that CKAP4 was expressed in the cell body and major processes of podocytes but not in the foot processes, a complementary analysis was done, staining human glomerular tissue samples
using antibodies against CKAP4 and synaptopodin. The latter is a marker of the podocyte foot processes, as it links other proteins to the actin cytoskeleton near the slit diaphragm and the GBM (138). Co-staining showed little to no co-localization between the two proteins, supporting our previous findings. There was however clear co-localization between CKAP4 and the ER-marker PDI3A in cultured immortalized human podocytes, and simultaneous staining of CKAP4 and tubulin suggested the presence of many anchorage points for tubulin in the ER in proximity of CKAP4. This would be in agreement with the known function of CKAP4 linking the rough ER to microtubules (73).

In a collaboration with Professor Mario Schiffer and Dr. Janina Müller-Deile from Friedrich-Alexander-Universität Erlangen-Nürnberg in Germany, we planned and performed experiments on zebrafish, with the aim to learn more about CKAP4 function in vivo. The zebrafish homolog to CKAP4, zgc:84975 (139), was silenced using an ATG-blocking morpholino injected into zebrafish embryos at the one- to four-cell stage. Analysis of proteinuria was done on a transgenic fish strain with systemic expression of EGFP coupled to a vitamin-D binding protein, where loss of fluorescence in the retinal vessel plexus indicates protein leakage. Embryos given CKAP4 morpholinos in three different concentrations had lower average eye fluorescence than those given a non-silencing control morpholino at 96 hours post fertilization, indicating proteinuria. However, only the highest concentration of CKAP4 morpholino induced proteinuria at 120 hours post fertilization. Transmission electron microscopy analysis of the glomerulus of zebrafish embryos with silenced CKAP4 confirmed damage to the podocytes and showed foot process effacement. The two highest concentrations of CKAP4 morpholinos were also associated with more embryos showing edema, and with increased severity of edema compared to control. The results from the zebrafish experiments however need to be evaluated for specificity of the CKAP4 silencing, for instance by showing that the phenotype can be rescued by cRNA coding for either zebrafish or human CKAP4.

The results from the zebrafish experiments indicated an important function for CKAP4 in podocytes, and prompted us to move on by silencing CKAP4 in human immortalized podocytes in vitro. The same method of lentiviral mediated shRNA transduction was used as in Paper III, and the protein was also overexpressed tagged with EGFP as previously described. When assessing the efficacy of silencing or overexpressing CKAP4, the results implied a slow turnover of protein in the cultured podocytes. The relative abundance of CKAP4 in the cells treated with shRNA was rarely lower than 50%, while the gene expression was usually diminished to about 10%. A long
half-life of CKAP4 had previously been observed in HEK293, HeLa and C2C12 cells, ranging between 42 and 156 hours (79).

With the aim to clarify differences in total protein expression between podocytes with either silenced or overexpressed CKAP4 and their respective virus controls, a mass spectrometry (MS) analysis was performed. To enable relative comparisons between groups, each sample was labeled with a tandem mass tag (TMT) before analysis. After normalization and quality check of the protein expression data from MS, the relative protein abundances were analyzed by the Ingenuity Pathway Analysis (IPA) tool for identification of significant differentially regulated pathways. A cutoff for fold changes was applied considering the technical variance of 10% when using the TMT system, where $0.8 < \text{fold change} > 1.5$ was considered down- or up-regulation, respectively. The samples from cells overexpressing CKAP4 did not pass the quality check, where they failed to cluster separately from controls. The cells with silenced CKAP4 did however separate well, suggesting biologically relevant differences compared to their controls. In the pathway analysis, there were 8 out of the 20 top regulated pathways concerning the cytoskeleton or cell attachment, suggesting that CKAP4 is relevant for normal function of the cytoskeleton in podocytes.

One of the main events associated with proteinuria is podocyte foot process effacement, where retraction, widening and shortening of the foot processes leads to loss of their interdigitating pattern, and ultimately to increased permeability of the filtration barrier to proteins such as albumin (140). Since both effacement and proteinuria were detected in the zebrafish after silencing of the CKAP4 homolog, we analyzed the effect of CKAP4 silencing on the actin cytoskeleton structure of the immortalized human podocytes by immunofluorescence. The cells were stained with fluorescence labeled phalloidin, a peptide isolated from the death cap mushroom with high affinity for F-actin (141). Subsequent microscopy analysis showed dramatic changes in both overall morphology and actin distribution in the cells. Firstly, many podocytes had retracted and shrunk after treatment with shRNA against CKAP4. Secondly, the actin stress fibers were reorganized with loss of transverse stress fibers and an increase in cortical actin. Thirdly, cell density was affected and many cells seemed to have detached from the surface of the culture dishes. Overexpressing CKAP4-EGFP did however not appear to affect cell morphology or actin organization. From these findings, we hypothesized that CKAP4 is essential for maintenance of the actin cytoskeleton structure in podocytes, while the mechanistic link between CKAP4 and actin was still to be determined.
To assess the effects of a stressor mimicking chronic disease on the function of podocytes with silenced or overexpressed CKAP4, we treated these cells with puromycin aminonucleoside (PAN). PAN induces oxidative stress in cultured podocytes and reportedly causes an apoptotic response and DNA damage as well as lead to loss of actin stress fibers and rearrangement towards cortical actin (142-144). Cells with silenced or overexpressed CKAP4 and subsequent treatment with 50 μM PAN for 24 or 48 h were stained using phalloidin, with results suggesting that PAN and CKAP4 silencing are both deleterious to the podocytes, but possibly through different mechanisms. Overexpressing CKAP4 did not rescue the phenotype caused by 24 h PAN treatment, although a slight trend of restored stress fiber count was seen after 48 h treatment with PAN. Additionally, cells with diminished CKAP4 expression or only treated with PAN seemed to differ morphologically by observation, which could also suggest different mechanisms of disruption of the actin microfilament structure.

A closer look into the effects of CKAP4 silencing or overexpression with or without additional treatment with PAN was done for proteins associated with the cytoskeleton and podocyte injury. Preliminary results from Western blots suggested little effect of either treatment on expression of the actin bundling protein α-actinin-4 or the microtubule building block α/β tubulin. Clearer differences were seen when analyzing expression of the gap-junction protein connexin 43 (Cx43), which increased in all groups in response PAN, except for in podocytes where CKAP4 was silenced. Also without PAN treatment there was a clear difference in expression of Cx43 between virus control cells and cells with silenced CKAP4. An additional analysis of Cx43 gene expression gave similar results, although the Cx43 expression significantly increased after 48 hours of PAN treatment, albeit not to the same levels as the PAN treated virus control.

The protein integrin β1, which is one of the integrin subunits attaching podocytes to the GBM (145), was also visibly diminished in the Western blot results for podocytes with silenced CKAP4, when compared to expression in the virus control cells. There were no corresponding differences in integrin β1 expression observed in the cells where CKAP4 was overexpressed. Previous studies with conditional knockouts of integrin subunit α3 and integrin subunit β1 in mice indicate that they are the most important integrins for maintenance of glomerular structure (146). Integrins are also known to interact with both extracellular matrix proteins and the intracellular cytoskeleton, promoting actin assembly (147). Since the podocytes with silenced CKAP4 seemed to detach from the surface of the culture dish, the
possible link between CKAP4, actin and integrin could be interesting to pursue in further studies.

Another possible reason for the observed detachment of the podocytes could be cell death, which prompted an assessment of cell viability by Alamar Blue. The analysis indicated that transduction with the pLKO.1 vector used for CKAP4 silencing might have a slight effect on viability, while no such indications were observed for the VVPW-EGFP vector. PAN treatment only appeared to affect cell viability of the overexpression cells marginally after virus transduction, which might have been due to the use of a relatively low dose of PAN in the experiment (50 μM). There was however a decrease in viability observed after 24 hours of PAN treatment for cells with silenced CKAP4 expression compared to untreated cells, which was also detectable after 48 hour treatment with PAN. After 48 hours of PAN treatment there was additionally a difference in viability between treated and untreated virus control cells.

As many results in this study are preliminary, some might change after repetition of experiments. Considering the results included in the present report however, there are some limitations possible to discuss. One is the challenge of low sequence homology between human and zebrafish CKAP4. Although they have been reported to be homologous in one publication, our analysis has shown 19% RNA sequence homology and 23% protein sequence homology, which is not optimal. It also increases the need for a verification of specificity of the silencing morpholino, which could not be done within the time frame of the zebrafish experiments due to challenges in constructing the rescue RNA. Another one is translatability of the results from the podocyte cell culture to in vivo conditions. The immortalized human podocytes differ from physiological podocytes e.g. in morphology and in formation of connection between cells. Podocytes in vivo also have a different distribution of their cytoskeleton compared to the cultured podocytes. A way to manage this discrepancy could be to include experiments with conditional knockout of CKAP4 in mice when going forward with the study.

In conclusion, this study shows preliminary results indicating that CKAP4 has an important function in podocytes maintaining their cytoskeletal integrity. The protein is abundantly expressed in the ER of human immortalized podocytes, and has a previously known function of linking the ER to microtubules. Silencing of the homolog for CKAP4 in zebrafish resulted in proteinuria and podocyte injury, which was supported by results seen in silencing experiments using human podocytes in vitro. There the
silencing of CKAP4 led to deleterious effects on actin microfilament structure, and altered expression of connexin 43 and integrin β1, which was sustained also after PAN injury. Overexpressing CKAP4 did however not seem to change the phenotype of the cells, or to rescue them from the damaging effects of PAN. From these results, we hypothesize that CKAP4, potentially through its interaction with the microtubules, is necessary to maintain the actin cytoskeletal structure in podocytes as well as its attachment to the GBM through integrins.
5 CONCLUDING REMARKS

Generating new knowledge about the pathophysiology of chronic kidney diseases is a collaborative effort involving researchers from many disciplines. To delineate the often complex onset and progression of these diseases requires expertise on the physiology of the kidney, from its function in the body and interaction with other organ systems, to its own molecular biological machinery. In this thesis, the focus has been on the latter, with the aim to contribute to the current knowledge of glomerular function and the molecular causes of glomerulonephritis.

Paper I

The aim in Paper I was to test if mesangial cells obtained from patients with IgAN had a pathogenic phenotype, and would then react differently to stimuli associated with disease than healthy control cells.

We found that IgAN patient mesangial cells increased their proliferation more than healthy control cells in response to both IgA1 purified from IgAN patient serum and to the cells’ main mitogenic factor PDGF. The patient cells also released more of some growth factors and cytokines (PDGF, IL-6) and equal amounts or less of others (CCL5, IL-8, MCP-1) than healthy control cells. These results encouraged us to suggest an additional hit to the multi-hit hypothesis on IgAN development, which would consider susceptibility of the resident mesangial cells to pathogenic signaling associated with disease.

Paper II

The aim of Paper II was to identify differentially regulated genes, proteins and signaling pathways in IgAN, and to assess the applicability of cell-type specific standard genes and their expression in distinguishing between healthy and diseased states, as well as in predicting progression of IgAN.

We found that mesangial cell standard genes identified in our data set were more often differentially regulated than the podocyte standard genes in IgAN patient glomeruli, and that the mesangial cell standard gene expression could be used to separate healthy from diseased samples by principal coordinate analysis. Mesangial but not podocyte standard gene expression also correlated with serum creatinine concentration and eGFR from the IgAN patients after z-score transformation, indicating effects of these genes on clinical parameters important for disease progression. Differentially regulated
pathways shared between the glomerular transcriptome in IgAN and the proteome from mesangial cells exposed to IgA1 from IgAN patients’ serum highlighted the role of inflammation in disease development. Both the pathways identified in that analysis and the mesangial cell-specific standard genes could be of use in further research into the molecular mechanisms of IgAN.

**Paper III**

The aim of Paper III was to investigate the function of CKAP4 in mesangial cells, where special focus was put on the observed link between CKAP4 and proliferative signaling through the PDGF pathway.

We found that CKAP4 is an abundantly expressed protein in both mesangial cells and podocytes in the glomerulus, while less in the endothelial cells. shRNA mediated silencing of CKAP4 in cultured mesangial cells downregulated both gene and protein expression of PDGFRα and -β, and decreased activation of proteins involved in proliferative signaling downstream of the receptors. Overexpressing CKAP4 did not appear to significantly affect the cells in culture, although progressively increased expression of the protein was observed in glomeruli from mice with diabetic nephropathy, which was exaggerated if the mice were also fed a high protein diet. From our findings we conclude that CKAP4 seems to be an important protein for proliferative signaling in mesangial cells, and further research would help us understand its potential role in diseases such as IgAN and DN.

**Paper IV**

The aim of Paper IV was to explore the function of CKAP4 in podocytes and its relation to actin cytoskeletal stability.

We found that silencing of the CKAP4 homolog in zebrafish embryos led to proteinuria and effacement of the podocytes in the fish glomerulus. Silencing of the protein in immortalized human podocytes was associated with detrimental changes in the actin cytoskeleton structure of the cells, leading to shrinking and isolation from other cells in culture. Overexpression of CKAP4 did not seem to change the cells’ phenotype, similar to the results in the mesangial cells. Inducing cell injury with PAN after silencing or upregulation of CKAP4 led to changes in protein abundance of cytoskeleton related proteins connexin 43 and integrin β1. These preliminary results provide an incentive for further research into the role of CKAP4 in podocyte cytoskeletal stability.
6 FUTURE PERSPECTIVES

This thesis includes both hypothesis-driven and exploratory studies of the molecular physiology and pathophysiology of glomerular cells. The transcriptomic and proteomic analysis in Paper II as an example of the latter resulted in identification of signaling pathways differentially regulated between IgAN patients and healthy kidney donors, which would be interesting to compare to results from similar analyses in other cohorts. It would also be of interest to extend the analysis of the data from microarrays to include endothelial cell standard genes as it was missing in previous study. The next step would be to assess cell specific standard gene expression in other glomerular diseases, such as DN, FSGS or MCD. This would enable us to hopefully find disease signatures and gain a deeper understanding about the molecular background to these diseases.

The hypothesis-driven study in Paper I suggested that mesangial cells from IgAN patients were more susceptible to disease-associated stimuli than healthy cells. The next step from that study would be to evaluate what might cause that susceptibility, which could be analyzed through similar methods as were used in Paper II. A transcriptomic and proteomic analysis of the IgAN patient material from Paper I could provide an interesting bridge between the two studies, and it would be intriguing to include the IgAN patient cells as an additional group in the mass spectrometry analysis of IgAN treated and untreated cells that was performed in Paper II.

The studies of CKAP4 function in Paper III and Paper IV are still works in progress. Despite the lack of PDGF receptors and proliferation in podocytes, CKAP4 might have a similar function in these cells and in mesangial cells. Since CKAP4 was found on the surface of mesangial cells, it could in the future be an interesting target for drug development, which it already has been suggested for in cancer cells (84). To evaluate if CKAP4 has overlapping functions in mesangial cells and podocytes, it would therefore be most relevant to start by assessing its eventual expression and function in the podocyte cell membrane. In both studies it would also be of interest to investigate the function of CKAP4 through conditional knockout of the protein in the specific glomerular cell type studied, which would probably be done using the Cre-loxP system in mice. Today this is only possible for podocytes and not mesangial cells, as it requires expression of cell-type specific proteins. It would for example be intriguing to know if a conditional knockout of CKAP4 in mice would induce proteinuria as it did in the zebrafish.
In the long-term perspective, the results from the transcriptomic and proteomic analyses in Paper II highlight the previously known role of inflammation in the early stages of IgAN. Results from several previous studies in the field support the conclusion that IgAN is an autoimmune disease, and this is considered in the four-hit hypothesis of pathogenesis. The ongoing research into the enzymatic processes responsible for glycosylation of IgA1 in B-cells is of great interest in this regard, and was well summarized within a review from 2015 by Dr. Jan Novak et al. (41). The research being done to identify genetic loci associated with risk or protection from IgAN in genome-wide association studies (GWAS) is also very important, and these studies have already identified 18 susceptibility loci, out of which some are common in autoimmune diseases, e.g. involved in antigen presentation, mucosal defense or the alternative complement pathway (148). A different study on familial IgAN identified four genes associated with this type of IgAN, among them the gene coding for Myc target 1 (MYCT1) (149), a protein recently reported to interact with CKAP4 (76). The continuing research into cellular mechanisms of IgAN has been challenged by the difficulty of finding a representative model for disease, as was summarized by Dr. Hitoshi Suzuki et al. (96), in part because only hominoid primates produce the IgA1 subtype. If such a model was available it could facilitate studies of the important glomerular crosstalk taking place during disease onset and progression. In the area of clinical studies, the ongoing multicenter study CureGN have already yielded interesting findings about patients with IgAN and IgAV (45), and future publications from this study will likely be useful also for pre-clinical research.

Solving the complex problem presented by different forms of glomerular disease I think requires both focus on the glomerular cells affected by disease, as well as their environment in the kidney and around the individual the glomerular cells belong to. The research included in this thesis can hopefully open up for new questions about the first of these three; about the altered phenotype of mesangial cells in IgAN, about signaling pathways relating to IgAN development and the use of cell specific standard genes as predictive tools for onset and progression of disease, as well as about the function of CKAP4 under physiological conditions and in glomerular disease.
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