

The role of glycoproteins in glomerular pathophysiology

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'Nothing in life is to be feared, it is only to be understood.'

Marie Curie

ABSTRACT

Chronic kidney disease (CKD) is increasing worldwide and has a prevalence of around 10%. With time patients are at risk of losing their renal function and will need dialysis or transplantation for survival. There are no specific treatments available and mechanisms behind the onset and progression of CKD are still not fully investigated. Two of the most common examples of CKD are diabetic kidney disease (DKD) and IgA nephropathy (IgAN). This thesis is focusing on the role of specific glycoproteins in these diseases and possible biomarkers for diagnostic purposes. The first paper demonstrates the importance of proteoglycans (PGs) in the endothelial cell surface layer for an intact glomerular filtration barrier. Loss of PGs from this layer led to increased proteinuria in rats, and analysis of human glomerular tissue and cells cultured in diabetic-like conditions revealed an altered PGs expression. Paper II focused on the role of PGs in the mesangial matrix in IgAN. One of the main reasons for onset of IgAN is considered to be galactose deficient IgA (gd-IgA) containing immune complexes deposited in the mesangium of the kidney. Analysis of human glomerular tissue in combination with mesangial cells treated with gd-IgA revealed increase in PG expression and an altered glycosylation profile of the PGs in IgAN. The last paper concerns the possibility of using gd-IgA as a biomarker for IgAN for early detection and follow up of the disease. Patient urine and serum from the time of the diagnostic biopsy as well as follow up samples were analyzed. Patients with IgAN had higher levels of gd-IgA compared to healthy individuals and patients with other renal diseases. gd-IgA levels in urine did reflect severity of disease but had no prognostic value and at this stage we cannot conclude that gd-IgA is a valuable biomarker for IgAN.

In conclusion, PGs are important for a normal function of the glomerular filtration barrier and loss of PGs leads to proteinuria. On the contrary increased levels of PGs in the mesangial matrix is part of the progression of IgAN. These findings highlight the importance of PGs in glomerular function and disease. In addition, we investigated the possibility of using gd-IgA as a biomarker for IgAN, but with inconclusive results calling for further investigation.

Keywords: Proteoglycans, extracellular matrix, IgA nephropathy, diabetic kidney disease

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SAMMANFATTNING PÅ SVENSKA

Glykokonjugat definieras som kolhydrater, kovalent bundna till variabla molekyler av andra kemiska klasser: proteiner, lipider och andra föreningar. Proteiner med kolhydrater bundna till sig namnges som glykoproteiner. Proteoglykaner (PG) är komplexa glykoproteiner, som består av ett protein med en eller flera negativt laddade glykosaminoglykaner (GAG) bundna till sig som sidokedjor. På grund av variationen i sidokedjor och kombinationer med olika proteiner är proteoglykaner molekyler med många olika funktioner i människokroppen. Deras viktigaste roller är bl.a. att stödja vävnadsstrukturer och fungera som reservoarer för signalmolekyler för att vid behov tillhandahålla korrekt intercellulär kommunikation. PGs är värdefulla komponenter i den glomerulära filtrationsbarriären. Således kan sjukdomsrelaterade förändringar i laddning eller struktur hos proteoglykanerna leda till progression av njursjukdom och som konsekvens av detta proteinuri, vilket är ett kännetecken för glomerulär sjukdom. IgA-nefropati (IgAN) och diabetisk njursjukdom (DKD) är de vanligaste globalt spridda kroniska njursjukdomarna. Båda orsakas och förvärras av en komplex förändring av underliggande molekyllära mekanismer. Denna avhandling fokuserar på förändringar av glykoproteiners profil och molekyllära uppbyggnad vid njursjukdomar. Studierna utfördes med cellkulturer från humana njurceller, samt med djur och patientprover. Vi har visat att förlusten av PG-komponenter på ytan av endotelceller påverkar njurfunktionen och inducerar en förtunning av det glomerulära basalmembranet med samtidig proteinuri. Minskat PG-innehåll i endotelet kan betraktas som ett sjukdomstecken och kan möjligen delvis förklara förändringen av den glomerulära funktion vid DKD. För det andra har det antagits att under-galaktosylerade (gd) IgA-innehållande cirkulerande immunkomplex är orsaken till mesangiell extracellulär expansion på grund av att dessa komplex utlöser en överproduktion av PG av mesangiala celler. För att studera detta utförde vi experiment på mesangiala celler (MC), behandlade med gd-IgA eller IgA, framrenade från serum från IgAN-patienter eller friska givare. Vi använde oss av ett nytt glykoproteomiskt analysätt, som möjliggjorde masspektrometrisk analys på prover utan att förlora GAG infästningen till själva proteinet. Vi identifierade flera PG, både i behandlade och obehandlade grupper (dekorin, kollagen alfa-1 XVIII och syndecan-4). CD44 och bikunin hittades endast i behandlade prover. Vi upptäckte också en övergång från heparan sulfat (HS) till chondroitin sulfat (CS) innehållande PG i behandlade MC jämfört med obehandlade. En patientgrupp med IgAN studerades också för att undersöka gennuttrycket av PG i glomeruli. Vi fann 19 PG och av dessa var 10 uppreglerade jämfört med friska kontroller. Baserat på dessa resultat kunde vi dra slutsatsen att PG-sammansättningen av MCs

extracellulära matrix förändras i förhållande till sjukdomsutveckling och PG biosyntes/nedbrytning kan vara ett bra terapeutiskt mål för att förhindra sjukdomsprogression. Modifierat IgA, gd-IgA, har tidigare undersökts som en möjlig biomarkör för IgAN, dock inte prospektivt och med varierande resultat. gd-IgA bildar cirkulerande immunkomplex, och när de deponeras i glomeruli kan de orsaka inflammation och sjukdomsprogression med nedsatt njurfunktion som följd. Vi har undersökt koncentrationen av gd-IgA och IgA i förhållande till sjukdomens svårighetsgrad och progression hos IgAN-patienter och patienter med andra typer av kroniska njursjukdomar. Inga signifikanta förändringar kunde uppmätas för IgA eller gd-IgA nivåer hos IgAN patienter, men gd-IgA koncentrationen i serum och urin blev lägre med tiden hos IgAN-patienter, vilket kan bli föremål för framtida forskning. I detta skede finner vi dock inte att gd-IgA har potential att bli en ny biomarkör för IgAN mer än att det korrelerar till sjukdomens svårighetsgrad.

LIST OF PAPERS

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

I. Proteoglycans contribute to the functional integrity of the glomerular endothelial cell surface layer and are regulated in diabetic kidney disease

Khramova A, Boi R, Friden V, Björnson Granqvist A, Nilsson U, Tenstad O, Oveland E, Haraldsson B, Ebefors K and Nyström J.

Scientific Reports (2021) 11:8487

II. Adaptive remodeling of mesangial extracellular matrix proteoglycan composition during IgA nephropathy

Khramova A, Noborn F, Buvall L, Larson G, Ebefors K and Nyström J.

Manuscript

III. Galactose-deficient IgA levels in blood and urine in patients with IgA nephropathy

Khramova A, Eliasdottir S, Saeed A, Guron G, Ebefors K and Nyström J.

Manuscript

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ABBREVIATIONS

ADH	Antidiuretic hormone
CD44	CD44 antigen
CD89	Immunoglobulin alpha Fc receptor
cDNA	Complementary deoxyribonucleic acid
CKD	Chronic kidney disease
CS	Chondroitin sulfate
CSPG	Chondroitin sulfate proteoglycan
DKD	Diabetic kidney disease
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DS	Dermatan sulfate
ECC	Endothelial cell coat
ECM	Extracellular matrix
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme linked immunosorbent assay
ESL	Endothelial cell surface layer
FBS	Fetal bovine serum
FGF23	Fibroblast growth factor-23
GAG	Glycosaminoglycan
GalNAC	N-acetylgalactosamine

GBM	Glomerular basement membrane
gd-IgA	Galactose-deficient immunoglobulin A
GFR	Glomerular filtration rate
GP	Glycoprotein
HA	Hyaluronic acid
HG	High glucose
HO	High osmolarity solution
HS	High salt solution (Paper I) or Heparan sulfate (Paper II)
HSA	Human serum albumin
HSPG	Heparan sulfate proteoglycan
IgA	Immunoglobulin A
IgAN	Immunoglobulin A nephropathy
IgG	Immunoglobulin G
IL-18	Interleukin-18
KS	Keratan sulfate
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
MCP1	Monocyte chemoattractant protein 1
MCs	Mesangial cells
MS	Mass spectrometry
NS	Normal salt solution
PA	Palmitic acid

PCR	Polymerase chain reaction
PG	Proteoglycan
RNA	Ribonucleic Acid
RT-PCR	Real time polymerase chain reaction
SGLT-2	Sodium/glucose cotransporter 2
TEM	Transmission electron microscopy
VCAM-1	Vascular cell adhesion protein 1

1 INTRODUCTION

Kidney pathologies are widely spread. They usually develop slowly, without showing any clinical symptoms, and generally have a huge impact on the quality of life of the patients. Chronic kidney diseases (CKD) are often hard to diagnose and without curative treatment, the only option is to try to delay the progression. Patients with chronic kidney disease may eventually reach end stage disease with a need for dialysis or kidney transplantation. As of today, there is still no specific, curative treatment for CKD.

Glycoproteins such as proteoglycans (PGs) have previously been shown to play an important role in glomerular function. They are negatively charged molecules taking part in the filtration process, both directly and indirectly. PGs are found in several areas of the glomerulus, including the mesangial matrix. Alterations in molecular composition and structure of PGs could affect both onset and progression of renal disease.

IgA is another glycoprotein, involved in glomerular disease. Patients with IgAN have under-galactosylated IgA (gd-IgA) in their circulation, involved in the onset of the disease. It has been suggested as a biomarker for IgAN, although the results have been inconclusive.

1.1 THE KIDNEY

Kidneys are essential organs responsible for many functions in our bodies: maintaining homeostasis, regulating the acid-base composition and volume of body fluids, excretion of harmful agents and synthesis of hormones (renin, erythropoietin, and 1, 25-dihydroxycholecalciferol).

The filtration of the blood takes place in the nephron, the functional unit of the kidneys. Each kidney contains approximately one million nephrons. This number is declining with age and in renal disease progression. The nephron consists of the glomerulus surrounded by Bowman's capsule and the renal tubules. The first step of urine formation takes place in the glomeruli where blood is being filtered over the capillary wall and the primary urine is formed, whereas the second step of urine formation take place in the renal tubules which are responsible for reabsorption and secretion (Eckardt et al., 2013). The final urine is formed in the collecting ducts of the tubular system. After this final step of urinary modification including concentration of the final urine under the influence of ADH, the urine is lead out of the kidney towards the ureters to the bladder (Figure 1).

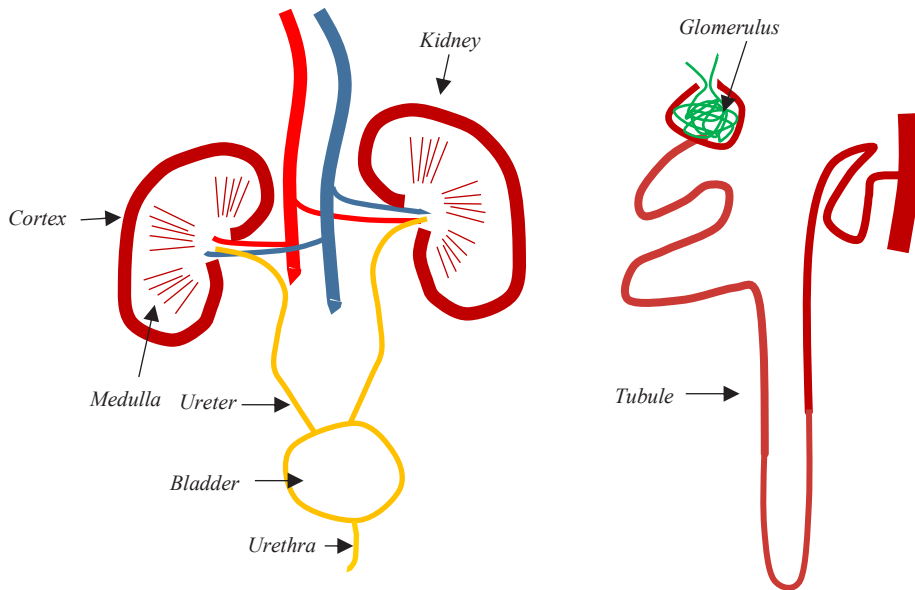


Figure 1. Structure of the renal system and a nephron.

1.2 THE ANATOMY AND MOLECULAR COMPOSITION OF THE FILTRATION BARRIER

In adult humans there are approximately 180 liters of primary urine produced each day, formed by filtration of the blood over the filtration barrier located in the glomerulus (Figures 2A and 2B). The barrier consists of 3 layers: the fenestrated endothelial cells with the endothelial cell surface layer, the glomerular basement membrane and the podocytes.

The permselectivity of the barrier is of utmost importance for maintaining large plasma proteins in the blood, while allowing small solutes and water to pass freely through the barrier. It is generally believed that the permselectivity depends on charge and size of molecules passing through the barrier. Negatively charged parts of the barrier (endothelial cells with glycocalyx and the basement membrane) restrain the passage of large negatively charged molecules, for example, acidic proteins and albumin (Y. M. Chen & Miner, 2012; Miner, 2012). The next part of the barrier is made out of specialized epithelial cells, called podocytes, and acts as a size-dependent selection barrier. This complex organization of the filtration unit assure a correct filtration process (Daehn & Duffield, 2021). The third cell type, presented in glomeruli,

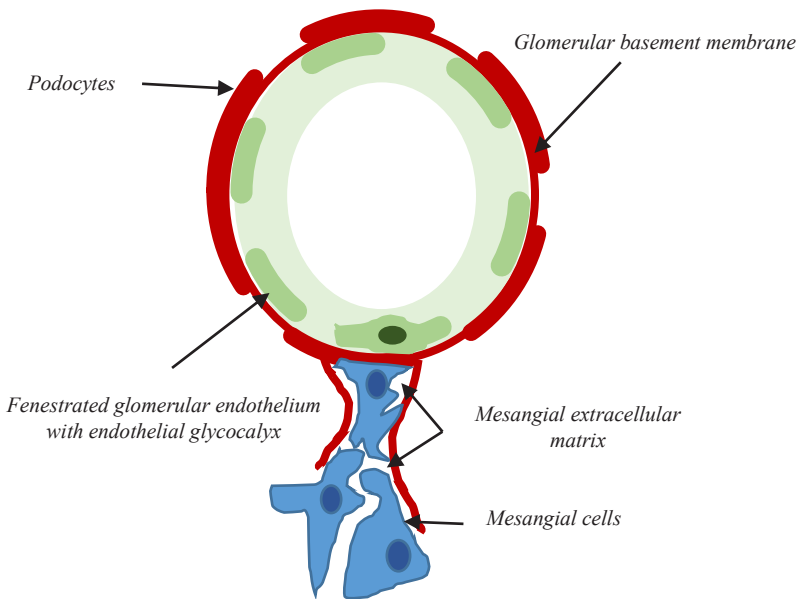


Figure 2A. Schematic illustration of a cross section of a glomerular capillary and the mesangial cells, the image is not drawn to scale. All cells are involved in the filtration process: endothelial cells with endothelial glycocalyx (green), mesangial cells (blue) and podocytes (red).

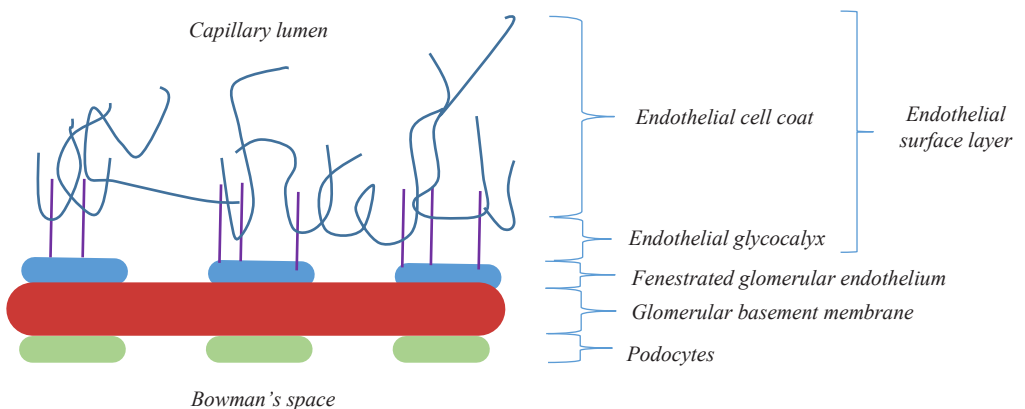


Figure 2B. Schematic illustration of the filtration barrier. Endothelial surface layer composed of glycocalyx and endothelial cell coat. The 'backbone' of ESL is the glycocalyx with cell membrane bound proteoglycans. The endothelial cell coat is a much thicker structure, represented by proteoglycans, glycosaminoglycans and proteins derived from the endothelium and/or plasma.

are mesangial cells (MCs), situated in between the capillary loops in the glomerulus.

MCs are surrounded by extracellular matrix (ECM) and form a central stalk in the glomerulus. They are in close contact with both endothelial cells and podocytes. MCs produce growth factors and cytokines and are thought to be of great importance in the cross-talk between cells in the glomerulus (Schlondorff & Banas, 2009). Changes in molecular structure in any layer of the filtration barrier, or even the MCs, might lead to disruption in urine formation and protein loss (Haraldsson, Nystrom, & Deen, 2008).

1.3 PROTEOGLYCANS

PGs are proteins that consist of a core protein with one or more glycosaminoglycan (GAG) chains covalently attached to it (Lindahl, Couchman, Kimata, & Esko, 2015). The GAG chains are negatively charged due to presence of acidic sugars and sulfate groups (Khoury, Baliban, & Floudas, 2011).

PGs can be divided based on cellular and subcellular localization to: extracellular (secreted, ECM PGs), pericellular, cell surface PGs and intracellular PGs. Another classification involves the type of GAG chain attached: chondroitin/dermatan sulfate (CS/DS), heparin/heparan sulfate (HS), hyaluronic acid (HA) and keratan sulfate (KS) (Iozzo & Schaefer, 2015; Kjellen & Lindahl, 1991; Stanley, 2011). Depending on type and location, PGs are involved in organ development, maintaining the tissue architecture and tissue repair. Many biologically potent molecules can bind GAG chains as a key part of their function in the ECM, at the cell surface and in some intercellular locations (Figure 3). Thus some PGs regulate enzymatic activity, serve as cell surface receptors and control gradients and availability of growth factors, chemokines, cytokines etc. (Reily, Stewart, Renfrow, & Novak, 2019). Since PGs have many functions in the human body, the role of PGs in disease progression is receiving increased attention (Couchman & Pataki, 2012). Even minor changes in their charge and structure can affect cell function (Noborn et al., 2016), causing the formation of unique motifs that allows binding sites for anomalous molecules instead of the physiological ones. These changes could further generate signaling misinterpretation between different cell types. As a result, such dysregulation may lead to development/progression of disease. Thus, PGs can be part of disease onset and could potentially be used as markers for early renal disease alterations, but at present the clinical use remains to be established.

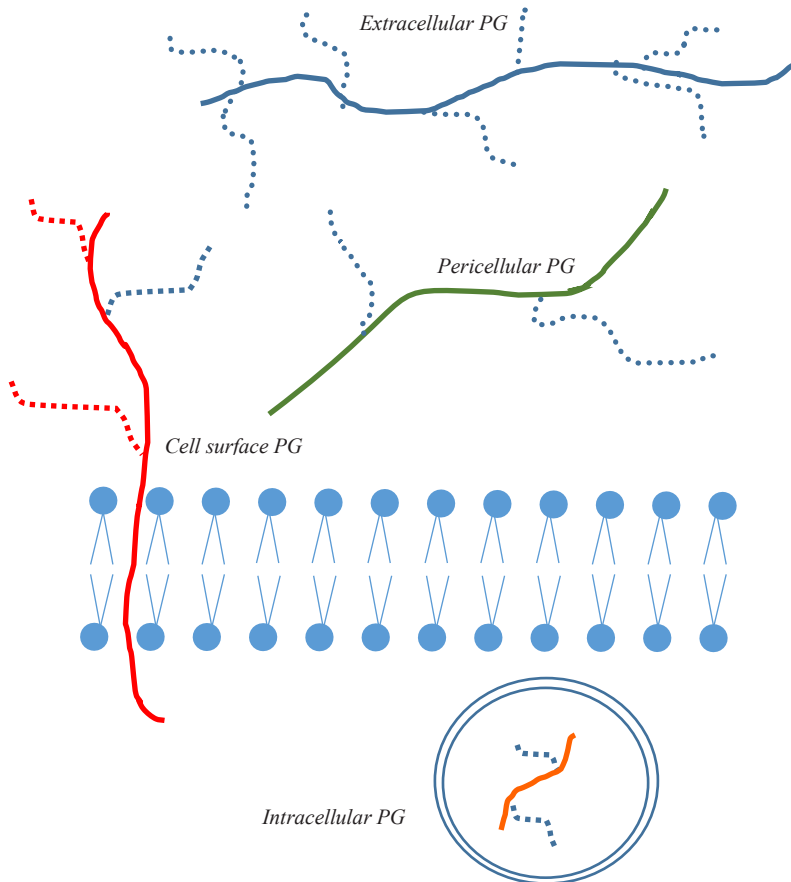


Figure 3. Proteoglycans can be found outside the cells (secreted), attached to the cell membrane or intracellular.

1.3.1 PROTEOGLYCANS IN THE ENDOTHELIAL CELL SURFACE LAYER

Most eukaryotic cells are surrounded by an ECM. The main functions of the ECM include regulation of signaling, acting as a molecular protective shield and support tissue architecture. The composition and thickness of the ECM varies between cell types and physiological vs pathological conditions. Proteoglycans, glycoproteins and glycolipids are some of the main components of the ECM together with collagens and other structural proteins (Dogne, Flamion, & Caron, 2018).

In the glomerulus, there are several ECMs since all cells are covered with an ECM of different composition. The endothelial cell surface layer (ESL) is one

of these ECMs, and probably the one of highest importance when it comes to permselective properties. This layer consists of two components: the glycocalyx, containing membrane-bound components, and a more loosely attached endothelial cell coat (ECC). The ECC is a relatively thick layer, which covers the fenestrations of endothelial cells. Together, they form a stagnant mucosal layer – an additional barrier to prevent leakage of high molecular weight proteins such as albumin. The loss of ability to retain macromolecules is a critical step during kidney disease progression and changes in molecular composition of this layer may lead to proteinuria (W. R. Zhang & Parikh, 2019).

The role of PGs, one of the most abundant components of ECC and the main supporters of the anionic mesh composition, has been shown in several studies (Friden et al., 2011; Singh et al., 2007), where properties of the ESL were changed after enzymatic treatment. Those experiments involved treatment with the GAG depolymerizing enzymes e.g. hyaluronidases, heparinases and chondroitinase, and revealed no effect on cell morphology but increased albumin passage across monolayers. Thus, PGs have been shown to support the restricting passage ability of endothelial ECC (Singh et al., 2007) and to prevent circulating plasma proteins to pass the filtration barrier, having an impact on development of several systemic processes (inflammation, hyperglycemia, albuminuria) (Dogne et al., 2018).

1.3.2 CHALLENGES OF STUDING THE ENDOTHELIAL GLYCOCALYX

The study of endothelial ESL and its structure-related function has been challenging due to complex molecular architecture and presence of loosely attached components, which could be destroyed during fixation, dehydration and sectioning procedures. Thus, classical tissue handling or animal perfusion might lead to partial loss of glycocalyx and the ECC. This means, that a completely different approach is required to investigate the ESL. There are a few non-traditional techniques proven to be effective in imaging and characterizing the endothelial glycocalyx. They include measuring circulating markers of glycocalyx, exclusion of macromolecules, intra lipid injection, tracer dilution technique and red blood cell-endothelial cell gap and sidestream darkfield imaging (Dane et al., 2015). However, these techniques are all challenging and indirect.

1.3.3 ROLE OF PROTEOGLYCANS IN FORMATION AND FUNCTION OF THE MESANGIAL MATRIX

The main function of the mesangial ECM is to form a unique milieu that allows communication between cells, vasculature and interstitium and to support the structural integrity of glomerulus. MCs normally keep the balance between matrix production and degradation and this delicate equilibrium is maintained by several mediators, hormones and enzymes (Rupperecht, Schocklmann, & Sterzel, 1996).

Disturbances of this balance can lead to increased mesangial ECM, a key finding in IgA nephropathy (IgAN) and in diabetic kidney disease (DKD) (Schlondorff & Banas, 2009; Singh et al., 2007). In IgAN, one of the most spread forms of glomerulonephritis worldwide, mesangial cell proliferation and ECM expansion in glomerulus is induced by gd-IgA-containing immune complexes. This leads to increased expression of several PGs, the main structural elements of ECM in the mesangium. These PGs could be considered both as effectors and biomarkers of disease progression.

In DKD, a serious complication of diabetes mellitus, one of the main hallmarks is the mesangial expansion. The increased amount of ECM could be explained by increased production of matrix proteins, such as collagens and PGs, with regular and modified structure. The turnover of PGs in DKD is affected by several growth factors, signaling pathways and hyperglycemia (Anders, Huber, Isermann, & Schiffer, 2018; Kolset, Reinholt, & Jenssen, 2012). In time, changes of ECM lead to glomerulosclerosis.

1.4 CHRONIC KIDNEY DISEASE

Chronic kidney disease (CKD) is a collection of diagnoses which are characterized by clinical changes in kidney function and structure. It is irreversible and most of the diseases have slow progression (Ammirati, 2020), where the decline of kidney function may not be linear (Zhong, Yang, & Fogo, 2017). CKD develops in 8-16% of the population and is often unrecognized in early stage due to its silent development, since patients remain asymptomatic for a long period of time (T. K. Chen, Knicely, & Grams, 2019). For an adult patient, CKD is defined when glomerular filtration rate (GFR) is lower than 60 ml/min/1.73 m² for at least 3 months and when the presence of albuminuria is at the minimum of 30 mg per 24 hours (Ammirati, 2020; T. K. Chen et al., 2019). Almost all of CKDs need to be diagnosed by a renal biopsy. Other relatively new molecular markers, such as kidney injury molecule-1, neutrophil gelatinase-associated protein, apolipoprotein A-IV, and soluble

urokinase receptor might be used to support the diagnosis but they are not fully validated yet (Zhong et al., 2017).

The most common causes of CKD are diabetes/hypertension, chronic glomerulonephritis (Ammirati, 2020), autoimmune diseases, infections and prolonged acute renal disease (T. K. Chen et al., 2019). Without treatment, CKD might develop to end stage kidney disease and cardiovascular complication, with increased mortality rate. As curative treatments are lacking, the general treatment aims to slow down the progression of disease and includes optimizing renin-angiotensin-aldosterone system blockade and immunosuppression (Ammirati, 2020; Lv et al., 2017; Rauen et al., 2015). Other medications might be targeting endothelin, transforming growth factor- β and oxidative stress (Zhong et al., 2017). Recent developments include locally acting slow-release corticosteroids, complement inhibitors and SGLT-2 blockers that have shown positive effects on kidney function in large trials (Neal et al., 2017; Perkovic et al., 2019).

1.4.1 DIABETIC KIDNEY DISEASE

Diabetes mellitus (DM) is a metabolic disorder with high risk of morbidity and disability (Shao, Zhang, Li, Meng, & Chen, 2021), affecting approximately 9% of adult population worldwide. The characteristic feature of DM is a chronic hyperglycemic condition, provoked by insulin depletion and loss of action (Flannick, Johansson, & Njolstad, 2016; Gembillo et al., 2021; Ilonen, Lempainen, & Veijola, 2019; McGrath & Edi, 2019). Additional hallmarks involve increased blood pressure together with increase in cardiovascular diseases and reduced GFR, thickness of basement membrane and expansion of the mesangium (Chew & Lennon, 2018; Mahtal, Lenoir, & Tharaux, 2021).

One of the most serious consequences of DM is renal failure already within 7-10 years after diagnosis (Gembillo et al., 2021; Mahtal et al., 2021). Both immune and renal cells are involved in the development of inflammation and complications of the circulation (Shao et al., 2021). The renal cells, stressed by the abnormal diabetic conditions, lose their function and by communicating with other cell types, enhance the glomerular damage and the development of proteinuria (Mahtal et al., 2021), resulting in sclerosis, loss of nephrons and consequently loss of renal function (Thomas et al., 2015; Vallon & Thomson, 2020).

1.4.2 IMMUNOGLOBULIN A (IGA) NEPHROPATHY

IgAN is the most common form of primary glomerulonephritis, with at least 2.5 new cases per 100,000 adults in a year with the highest prevalence in Asia (Kiryluk et al., 2012; Lafayette & Kelepouris, 2018; Schena, 1990).

IgAN is often a silent disorder accompanied by an inflammatory process (Canetta, Kiryluk, & Appel, 2014), generally associated with poor prognosis as 40% of cases results in end stage kidney disease within 20 years of biopsy-proven diagnosis (Irabu et al., 2020; H. Suzuki et al., 2016). The treatment strategy may vary depending on disease progression and between patient groups (Selvaskandan, Shi, Twaij, Cheung, & Barratt, 2020). Depending on individual features, patients might show hematuria and/or proteinuria or not. There is no specific therapy for IgAN (H. Suzuki, 2019), thus treatments are mostly targeting symptoms (Lafayette & Kelepouris, 2018).

gd-IgA plays a key role in a development of IgAN (Sun, Zhang, Zhang, & Liu, 2016). Based on the multi-hit hypothesis, gd-IgA production is followed by secretion of IgA and IgG autoantibodies towards the gd-IgA. Together they form circulating immune complexes, which can deposit in the kidney glomerulus, more specifically in the mesangium, and trigger inflammatory processes (Knoppova et al., 2016; Tomana et al., 1999). IgAN patients have higher levels of gd-IgA compare to healthy individuals, but it is clear that gd-IgA alone can't trigger the disease. Other factors involve genetic predisposition, inflammatory events, immune dysregulation and possibly more beyond this for IgAN to develop (Irabu et al., 2020; H. Suzuki et al., 2016).

1.4.3 MESANGIAL MATRIX EXPANSION IN IGAN AND DKD

Changes in MC biology are present in all forms of glomerular damage (Schlondorff & Banas, 2009). The accumulation of mesangial matrix with time is a hallmark of progressive renal disease both of immunological and non-immunological etiology (Floege et al., 1991). Being characterized by IgA deposition in mesangium, mesangial cell proliferation and matrix expansion, IgAN is the most common pure form of mesangioproliferative glomerulonephritis (Mestecky, Novak, Moldoveanu, & Raska, 2016; Monteiro et al., 1985; Nguyen et al., 2019; Schlondorff & Banas, 2009) (Figure 4). Mesangial matrix expansion is also a key finding in DKD.

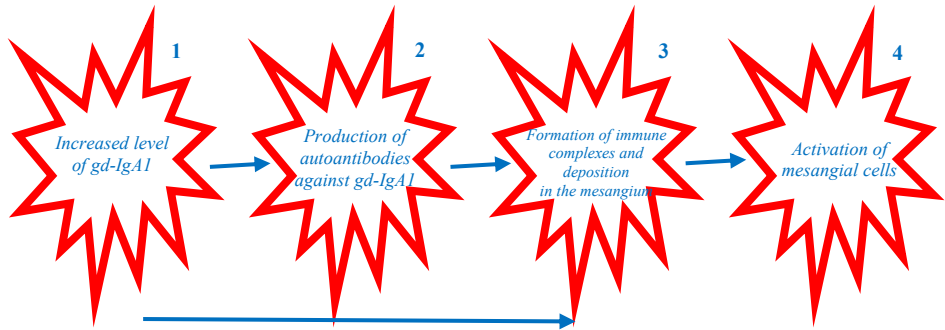


Figure 4. The four hit hypothesis of IgAN pathogenesis.

The production of IgA by plasma cells takes place in the respiratory and gastrointestinal tracts, bone marrow and lymph nodes. It could be potentially initiated by infection, stress or exposure to toxins (Lafayette & Kelepouris, 2018). The formation of the undergalactosylated IgA (gd-IgA) has been suggested to be genetically determined and related to decreased activity of core 1 β 1,3-galactosyltransferase (C1GalT1) and elevated activity of α -2,6-sialyltransferase 2 (ST6GalNAc-II) (Ohyama et al., 2020; H. Suzuki, 2019).

Contrasting to regular IgA having clusters of *O*-glycans in their hinge region (Placzek et al., 2018), gd-IgA lacks some of these modifications (Hastings et al., 2013; Hiki et al., 2001; Knoppova et al., 2016; Yu et al., 2021) (Figure 5). This specific feature favors the formation of circulating immune complexes with gd-IgA and IgG and/or IgA autoantibodies, prone to deposit in the mesangium and activate mesangial cells (Mestecky et al., 2013; Nguyen et al., 2019; Placzek et al., 2018). The deposition of immune complexes leads to local inflammation, extracellular matrix expansion, release of cytokines and growth factors, followed by podocyte and tubular injury. The described events result in a slowly progressing loss of kidney function (Irabu et al., 2020; Lafayette & Kelepouris, 2018; Mestecky et al., 2016; H. Suzuki et al., 2011).

A key episode in disease-related mesangium proliferation is the change in molecular composition of the ECM itself. These significant alterations could be applied to characterize changes of mesangial matrix composition for both IgAN and DKD. The healthy mesangial matrix consists of collagens (mainly IV and V), laminin, fibronectin and several different PGs. When the pathology-related remodeling develops, an increase in regular, 'healthy' PGs and an appearance of abnormal collagens occur. At this stage, associated mechanisms such as hemodynamic factors, lipid accumulation and glomerular hypertrophy also start to play a role in sclerosis formation (Floege et al., 1991).

Transforming growth factor- β (TGF- β) is a key player in this process, both in health and disease. The molecule is secreted by monocytes-macrophages, platelets or MC themselves in its inactive form and stored in mesangial ECM, with the main purpose to orchestrate the stability of ECM (H. S. Lee & Song, 2009; Rossert, Terraz-Durasnel, & Brideau, 2000).

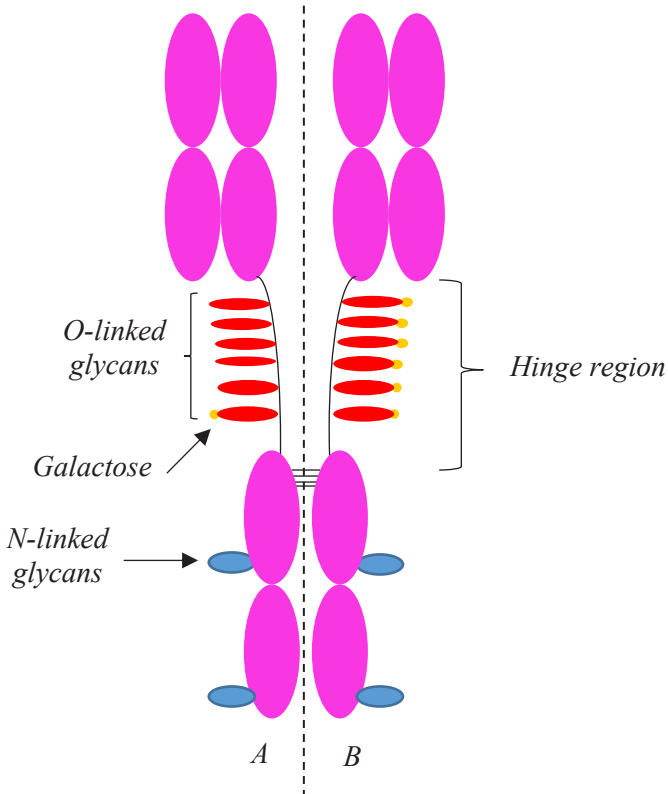


Figure 5. Schematic illustration of galactose deficient IgA (*gd-IgA1*) (A) and IgA (B) molecules (both in monomeric form). The IgA molecule has 3-6 O-linked glycans (red) at the hinge region. *gd-IgA* lacks galactose (yellow) in this region.

1.4.4 TREATMENT

Presently, a microscopic examination, including immunohistochemistry, of a kidney biopsy is the golden standard to diagnose and estimate the degree of damage in IgAN. Yet, a biopsy is invasive and might be questionable to perform multiple times due to possible complications and dependence of pathological findings at one single time point (Irabu et al., 2020; H. Suzuki,

2019). Thus, a noninvasive diagnostic marker is on high demand, both to detect IgAN and to estimate the effectiveness of treatment.

Current treatment strategies for IgAN patients are based on several biomarkers, collected as a part of the clinical routine. They include non-specific tests such as proteinuria, blood pressure, eGFR, creatinine clearance (Lai et al., 2016; Selvaskandan et al., 2020). Unfortunately, it is difficult to diagnose IgAN based only on results of those measures, but a decision on the necessity of a biopsy could be made accordingly. Histological assays reveal specific features of IgAN such as IgA and IgG depositions, mesangial and endocapillary hypercellularity, segmental glomerulosclerosis and tubular atrophy/interstitial fibrosis (Hastings et al., 2013).

IgAN therapy is usually a combination of a number of medications (Selvaskandan et al., 2020). Treatment strategies for IgAN are focused on reducing the inflammation by using immunosuppressive medicines (corticosteroids, azathioprine, and cyclophosphamide) or tonsillectomy (only used as treatment in some parts of the world). To improve kidney function and reduce proteinuria, renin-angiotensin system (RAS) inhibitors are widely used. Other more general recommendations in some regions include changes in life style, low salt diet and fish oil supplements (Gutierrez et al., 2020; Lafayette & Kelepouris, 2018; Rauen et al., 2015; Rodrigues, Haas, & Reich, 2017; H. Suzuki et al., 2011).

DKD is one of the major causes of morbidity and mortality in patients with diabetes mellitus, thus the prevention of DKD in diabetic patients is one of the main treatment goals. The main focus is to control the glycemic status already from the early stages of the disease (Nadkarni, Yacoub, & Coca, 2015; Subramanian & Hirsch, 2018). The next focus is to control the blood pressure (Patney, Chaudhary, & Whaley-Connell, 2018; Thomas & Atkins, 2006). Initial antihypertensive medication could include thiazide-type diuretics, a calcium channel blocker, an angiotensin-converting enzyme inhibitor, or an angiotensin receptor blocker (Alicic, Rooney, & Tuttle, 2017; Zagkotsis, Markou, Paschou, Papanikolaou, & Sabanis, 2018).

Novel treatment strategies involve acting on mechanisms, related to kidney damage, such as glomerular hyperfiltration, inflammation, and fibrosis. Promising results at different stages of testing have been obtained for a number of substances. The most promising include SGLT2 inhibitors (Neal et al., 2017; Perkovic et al., 2019; Wanner et al., 2016; Webster, Nagler, Morton, & Masson, 2017), baricitinib, a selective Janus kinase 1 and Janus kinase 2 inhibitor (Tuttle et al., 2018); ruboxistaurin, a protein kinase C- β inhibitor

(Tuttle et al., 2005); atrasentan, a selective endothelin A receptor antagonist (Heerspink et al., 2019); pentoxifylline, an anti-inflammatory and antifibrotic agent (Donate-Correa et al., 2019); finerenone, a highly selective nonsteroidal mineralocorticoid receptor antagonist (Bakris et al., 2020) and paricalcitol, a vitamin D analog (Lin, Chang, Yang, Wu, & Chu, 2018).

1.4.5 BIOMARKERS

In general biomarkers can be divided into 4 groups: diagnostic (non-invasive early detection of pathology), prognostic (gives information regarding likely course of disease), predictive (prediction of patient's response to selected therapy) and therapeutic (target for therapy, usually molecular) (Carlomagno et al., 2017).

The, in most cases, silent progression of IgAN doesn't allow for detection of the disease at initial stages or to distinguish it from other kidney pathologies. For now, there is no simple laboratory test to confirm IgAN and thus a renal biopsy is required for diagnosis.

The serum levels of IgA, of gd-IgA, or of autoantibodies to gd-IgA or are in most favor to become diagnostic markers for IgAN due to their initial participation in disease development (Bagchi et al., 2019; Hastings et al., 2013). These and other potential biomarkers are described below:

Serum total IgA. The level of total IgA was found to be 33-50% higher for IgAN patients than for controls but could not be considered as specific or sensitive enough for acting as a specific IgAN biomarker (Lai et al., 2016; Selvaskandan et al., 2020). The difference could e.g. be due to an increase in IgA production or to a decrease in IgA catabolism.

Serum gd-IgA. Serum gd-IgA has been suggested as a biomarker for IgAN, since IgAN patients have gd-IgA containing immune complexes, circulating in the blood. But only having gd-IgA in the circulation does not necessarily lead to development of IgAN. Based on the multi-hit hypothesis, gd-IgA production needs to be followed by secretion of IgA and IgG autoantibodies towards the gd-IgA with further formation and deposition of gd-IgA containing immune complexes in the kidneys (Moldoveanu et al., 2007; Novak et al., 2015; Rodrigues et al., 2017; H. Suzuki et al., 2011). The role of gd-IgA as a biomarker has been investigated by others and by us in this thesis.

Serum anti-glycan antibodies. Glycan-related IgG and/or IgA autoantibodies directed towards gd-IgA are involved in formation of immune complexes during IgAN and are able to form deposits in kidneys, which leads to the

possibility of reduced kidney function (Hastings et al., 2013; Selvaskandan et al., 2020). Unfortunately, circulating immune complexes have not as of today been proven to have any diagnostic value, despite high levels found in IgAN patient sera (Lai et al., 2016).

Proteins involved in complement activation. Complement activation fragments C3 in plasma and complement levels were detected in serum due to activation via alternative and/or lectin pathways in IgAN patients (Hastings et al., 2013; Maillard et al., 2015; Novak et al., 2015).

Soluble CD89 and CD89-IgA complexes. One of the functions of CD89 (myeloid Fc α R1-receptor) is being a receptor for the Fc component of human IgA. After interacting with IgA, CD89-IgA complex is shed from the surface of the cell and can be a cause of mononuclear cell influx into glomerulus. Some observations suggest transglutaminase 2 to be involved in CD89-IgA complex deposition in kidneys with future mesangial cell activation (Hastings et al., 2013; Selvaskandan et al., 2020) but its role as a biomarker is still not fully investigated.

Other biomarkers. There are also other possible biomarkers of IgAN. Levels of IL-18 (associated with renal injury), VCAM-1 (endothelial ligand, involved in inflammatory process due to moderate adhesion of leucocytes to endothelial cells), FGF23 (circulating hormone, involved in phosphate homeostasis) were found increased in IgAN patients (Hastings et al., 2013; Mestecky et al., 2013). Although those findings are promising, these targets are still not specific enough to be considered as biomarkers of IgAN.

Urinary biomarkers. Urine is more stable and less complex compared to plasma or serum, and thus has a great potential for clinical use (Dajak, Ignjatovic, Stojimirovic, Gajic, & Majkic-Singh, 2011; Gatto, Maruzzo, Magro, Basso, & Nielsen, 2016; E. Y. Lee et al., 2003). Complete molecular complexes or molecular fragments could be excreted in the urine and represent specific biomarkers of IgAN (Rudnicki et al., 2020). Urinary cytokines and chemokines, such as EGF (epidermal growth factor), MCP1 (monocyte chemoattractant protein 1), urinary complement factor H, podocalyxin could be associated with IgAN related kidney injury (Hastings et al., 2013; Lai et al., 2016) but this needs further investigation.

2 AIMS

The aim of this thesis was to investigate the role of glycoproteins and potential disease mechanisms, as well as markers of disease in glomerular kidney pathology.

Specific aims are stated below:

- To define the role of PGs as part of the endothelial cell coat in the glomerular filtration barrier (Paper I)
- To find and determine the change in PG content and composition of mesangial extracellular matrix in development and progression of IgA nephropathy (Paper II)
- To estimate the value of gd-IgA as a non-invasive biomarker of IgAN (Paper III)

3 METHODOLOGICAL CONSIDERATIONS

This section describes the general methodological approaches, used in this thesis. For more detailed information, please refer to respective papers.

3.1 ETHICS

For experiments, conducted on rats (Paper I), the ethical approval was given by the Regional Laboratory Animal Ethics Committee of Gothenburg (#236-2010). All experiments were performed in accordance with the ARRIVE guidelines and relevant regulations. Experiments, done with human material (Papers I, II, III) were conducted under the Declaration of Helsinki. All patients gave informed consent. The permission to collect biopsies, as well as blood and urine samples can be found in the ethical approvals of Gothenburg Regional Ethical Board (#552-02, #653-05, #413-09, #432-09).

3.2 ANIMAL MODELS

Female Sprague-Dawley rats were used as a research models in Paper I. Compared to male rats, female rats have a slower growth rate and a lower amount of visceral fat. Our group has previously used female rats for similar set-ups. Rats were anesthetized by isofluran inhalation, which allows for adjustments during the experimental procedure.

3.2.1 RENAL MORPHOLOGY AND SAMPLE COLLECTION FROM ANIMAL MODELS

In vivo experiments were designed with the hypothesis that high ionic strength solution can potentially remove non-covalently bonded molecules of ESL, in agreement with the main principles of ion exchange chromatography.

According to the method used, described by Friden et al. (Friden et al., 2011), glomerular PG components of ESL could be eluted using a perfusate with high concentration of NaCl. The renal vascular system was pulsatively perfused during 3 min with 12 ml of different solutions: 1 M NaCl (high osmolarity), 0.15 M NaCl (normal osmolarity) or 1 M mannitol (osmotic control) solutions. Solutions were based on Tyrode's solution – a solution with the same isotonic power as interstitial fluid and widely used in physiological experiments. The composition was as follows: normal salt (NS, 0.15 M NaCl) (in mM): 148.1 Na and 133.5 Cl, high salt (HS, 1 M NaCl) (in mM): 1032.65 Na and 1018.05 Cl and the high osmolality (HO) (in mM): 148.1 Na, 133.5 Cl, and 692.0

mannitol. All solutions were set to pH 7.4 and contained 25.04 HCO₃, 0.49 H₂PO₄, 0.83 Mg, 4.29 K, 2.50 Ca and 5.60 glucose. They were protected from light, bubbled with 5% CO₂ in O₂ and used at 37 °C.

After perfusion, kidneys were rinsed with 1 ml Tyrode with 0.15 M NaCl. Eluates were collected and analyzed with Liquid Chromatography Mass Spectrometry using LTQ-Orbitrap.

The ⁵¹Cr-EDTA content in urine samples was analyzed in a γ -counter and used for calculations of GFR.

To measure the thickness of the ESL after perfusion, transmission electron microscopy was used (Andersson, Nilsson, Hjalmarsson, Haraldsson, & Nystrom, 2007). Acquired micrographs of glomerular capillaries (102 capillaries from NS, 143 from HS and 133 from HO rats) were blindly measured for the following parameters: GBM thickness, podocyte foot process width and width of filtration slits.

Infusion of intralipid droplets was used as a method to estimate the thickness of ESL. The fat droplets distribute evenly in the perfusate. At a given time, micrographs taken by TEM to display the number of lipid droplets per capillary. Their proximity to the capillary wall was measured. If the ESL is intact, the exclusion zone between the lipid droplet and the vessel wall is wider compared to capillaries where the ESL may be damaged and reduced. This exclusion zone may be estimated and a mean distance calculated for the different treatment groups.

3.3 PATIENT COHORT AND SAMPLES

Patient biopsies were used in Paper I and Paper III. The biopsies were collected in collaboration with the Sahlgrenska University Hospital (Gothenburg, Sweden) and the Karolinska Hospital (Stockholm, Sweden), starting from 2003 and onwards. Patients admitted to the Department of Nephrology who were assigned for a diagnostic biopsy, and agreed to participate in the study to donate the material not needed for diagnostic purposes, were included. Biopsies were stored in RNAlater at 4 °C for 24 h prior to transferring to -80 °C for long term storage. Before analysis, the biopsies were micro dissected by hand to enable analysis of the glomerular compartments. Serum and urine samples were stored at -80 °C until use. Patients who had been diagnosed with DKD (Paper I) or IgAN (Paper III) were selected for further experimental work consecutively based on diagnosis. Patients where samples were missing or with

other diagnoses, besides DKD and IgAN, were not included. Samples from healthy kidney donors (Paper I) were collected and stored similarly.

For Paper III, patients with biopsy proven IgAN were selected consecutively, and the only criteria for not including the patients were missing data/biopsy or other diagnoses beside IgAN. The classification of IgAN biopsies was done according to MEST-C scores (Barbour et al., 2016; Bellur et al., 2020; Hassler, 2020; Trimarchi et al., 2017; Yeter et al., 2020) and performed by a clinical pathologist at Sahlgrenska University Hospital. Clinical parameters such as creatinine and albumin values were analyzed at the Clinical Chemistry Laboratory at Sahlgrenska University Hospital and have been prospectively followed up during the study. Other data (age, sex, blood pressure, blood pressure medications and immunosuppressive medications) were recorded similarly.

3.3.1 PURIFICATION OF IGA

Serum purified IgA was used in Paper II to stimulate mesangial cells. The serum was donated by IgAN patients and healthy donors under ethical permit #432-09. IgA was purified according to a technique described by Kim et al. (M. J. Kim et al., 2012). Briefly, the method involves binding of IgA to jacalin agarose, known for specifically binding to IgA (Gregory, Rundegren, & Arnold, 1987; Roque-Barreira & Campos-Neto, 1985). The concentration of purified IgA was analyzed by nephelometry at the Clinical Chemistry Laboratory, Sahlgrenska University Hospital. The serum concentration of purified IgA was between 0.7 to 1.6 g/L.

3.4 IN VITRO CELL MODELS

Cell culture models are widely used since they allow an in-depth analysis in the cells of choice and the possibility of well controlled experimental conditions. Using cell cultures, it is possible to estimate the individual contribution of a specific cell type to a process of interest. Despite obvious advantages of cell culture models, there are disadvantages. Cell culture models are traditionally restricted to one cell type and don't allow the study of a whole, complete processes, which often involves other cell types and growth matrices as well. Another disadvantage is that cell cultures give a limited information when it comes to the action of mechanistic forces (blood flow, surface tension etc.).

In Papers I and II primary human glomerular cell cultures were used to estimate the action of stimuli on cells and how that affects the possible development of pathology.

3.4.1 ENDOTHELIAL CELLS

In Paper I we determined how high glucose and palmitate levels may change the function of endothelial cells *in vitro*. EC are fenestrated cells in glomeruli, situated closest to the blood flow. These cells are covered by a thick protective layer, consisting of PGs and GPs. The layer could be divided into a loosely attached luminal surface layer and a glycocalyx. The malfunction of the endothelium is one of the first signs of DKD, where alterations in the glycocalyx components may lead to dysfunction of the filtration barrier and proteinuria.

Primary human glomerular endothelial cells (Cell Systems, Kirkland, WA) were cultured in Complete Normal Glucose Medium as described by the manufacturer up to passage 11. Palmitic acid was prepared in NaCl 150 mM pH 7.4 solution, and conjugated with human serum albumin (HSA) in a 6:1 molar ratio palmitate/HSA for 1 h at 37 °C. Cells were starved in medium containing 0.5% FBS and no added culture boost for 24 h prior to stimulation with 30 mM high glucose/HSA, palmitate/HSA (Sigma-Aldrich, Saint Louis, MO) 100 µM or a combination of the both for 24 h. Normal glucose concentration media (5 mM) was used as control.

3.4.2 MESANGIAL CELLS

As a model in Paper II, primary human mesangial cells from Cell Systems (USA) were used. The main function of mesangial cells is to support the structure of glomeruli. Mechanically, mesangial cells provide the central stalk for glomeruli which allow to maintain the filtration function by upholding the vessel architecture. Since they are being positioned in the middle of filtration unit, mesangial cells are important for communication between the cell types of the glomerulus. This feature reflects in the ability of MC to react on any non-physiological condition, and the most predictable reaction is cell proliferation and ECM production. Both are known to be hallmarks of IgAN. In Paper II, MC were stimulated with gd-IgA or IgA, purified from blood of a IgAN patient or a healthy donor, at the concentration of 100 µg/ml, to estimate the effect of those molecules on ECM production and structure. Cells were cultured in DMEM:F12 according to the manufacturer's instructions up to passage 11. The duration of treatment was 24 h or 48 h. Cells were starved for 24 h prior to the experiment.

3.5 GENE EXPRESSION ASSAYS

Gene expression analysis provide information on specific gene products and their potential contribution to the process of interest if regulated compared to their normal physiological state. Having more information regarding the roles of specific genes can give a powerful tool to understand the molecular background of pathology. The general procedure includes RNA purification, reverse transcription (where complementary cDNA is transcribed on relatively unstable RNA), and gene expression assay of choice. The gene expression assays were included in Paper I (RNA seq) and Paper II (gene microarray, TaqMan).

3.5.1 GENE MICROARRAY OF IGAN

The previously published data from a gene expression microarray study by our group was re-analyzed in Paper II to estimate the level of PGs expression. In this study, 19 IgAN patients (age 43 ± 15 years old, women-to-men ratio of 1:2) were included and 22 healthy donors (age 48 ± 13 years old, women-to-men ratio of 1:1.27). IgAN patients biopsies used in those experiments were scored according to the Oxford MES scoring system.

In short, biopsies of kidney cortex were dissected to separate the glomerular part from the tubulointerstitial part and RNA was extracted using the RNeasy Micro Kit (Qiagen, Netherlands). Samples were run on microarrays by Liu and co-authors (Liu et al., 2017) and normalized according to routine analysis. The sample quality was checked using a normalized unscaled SEM plot, a relative log expression plot, an RNA degradation plot, and a principal component analysis plot. To correct the batch effect, the empirical Bayes algorithm (Irizarry et al., 2003) was used and background signal threshold filtering technique (Makarenkov et al., 2007) to filter microarray probes. Data were clustered with the hierarchical Ward averaging method (Ward, 1963) and principal coordinate analysis (Gower, 1966) and statistically analyzed using SAM (Tusher, Tibshirani, & Chu, 2001) as implemented in MultiExperiment Viewer (MeV; USA). Significantly regulated genes were chosen with SAM q value < 0.01 and unlogged fold change < 0.67 as downregulation and > 1.5 as upregulation (equivalent logged fold change ± 0.58). The fold change threshold was set to capture a robust differentiation in expression and an experimentally reliable validation using quantitative RT-PCR.

3.5.2 RNA SEQUENCING

RNA sequencing or RNAseq was used in Paper I to evaluate the gene expression of PGs and PG related genes. This technique allows to analyze the

whole transcriptome. The procedure includes transcription of RNA or RNA fragments into cDNA, tagging and sequencing. The last steps include aligning of transcriptome, sample normalization and statistics (Stark, Grzelak, & Hadfield, 2019). High resolution of the method allows to identify new splicing variants with or without a reference genome, but the method is highly dependent on sample handling due to the risk of RNA degradation (Byron, Van Keuren-Jensen, Engelthaler, Carpten, & Craig, 2016; Hong et al., 2020; Kukurba & Montgomery, 2015).

The RNA sequencing or RNA seq data from our previous study by Levin et al. (Levin et al., 2020) was used in Paper I to evaluate the expression of PGs and PG related genes.

Nineteen patients with DKD (median (range) age: 61 (30–85), DKD stages 1–4) and 20 healthy kidney donors (median (range) age: 56 (30–70) years) were included in this study. Tissues were micro-dissected into a glomerular and a tubulointerstitial fraction with following RNA extraction. Next-generation sequencing libraries were made using the Nextera^{XT} transposon-based library generation kit (Illumina Inc.). Twelve cycles were used to amplify DNA libraries followed by purification with Ampure^{XP} beads. Preprocessing of the sequencing data was done using BCBio v1.0.1 (<https://github.com/chapmanb/bcbio-nextgen>). The DESeq2 R package was used to variance-stabilizing transformation (VST) normalization of the data (Love, Huber, & Anders, 2014). Differential expression analysis (DEA) was done with DESeq2 accounting for the research center factor. P-values were adjusted for multiple testing using Benjamini–Hochberg method, and genes with adjusted P <0.01 (transcriptome-wide analyses) and <0.05 (targeted analyses) were considered differentially expressed (Levin et al., 2020).

3.5.3 TAQMAN QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

TaqMan PCR is highly specific RT-PCR tool to perform relative quantification of gene expression. This assay requires a pair of primers and a non-extendable fluorescent labelled probe, which binds within the selected region between primers. The probe is labelled with fluorescence dye on 5' terminus and quencher on 3'. During the amplification, the probe hybridizes to the target sequence. As soon as upstream primer begin to extend, the *Taq* polymerase mediates 5'→3' hydrolysis of the probe, inducing the quenching effect. The value of fluorescent signal is proportional to PCR product (Nagy et al., 2017).

TaqMan assays were used in this thesis to estimate the change in gene expression of PGs and PG related genes. In Paper I, the assay was performed on RNA extracted from endothelial cells, treated with high glucose and/or palmitate. Cells were treated for 24 h with palmitate, high glucose or both and starved for 24 h prior to experiments. The change in gene expression level in Paper II was estimated on mesangial cells, treated with IgA purified from blood of healthy donor or IgAN patient at the end concentration of 100 µg/ml. Human mesangial cells were treated for 24 h or 48 h with starvation of 24 h prior to experiment.

In both papers, RNA was extracted and purified from cells with RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. cDNA was generated using High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific). Quantitative PCR was completed using Taqman probes (Thermo Fisher Scientific) with GAPDH used as housekeeping gene using the QuantStudio 7 Flex System (Thermo Fisher Scientific). All assays were performed in biological triplicates and technical quadruplicates.

3.6 PROTEIN EXPRESSION ANALYSIS

Proteins are one of the four important elements of living systems. The structure and related functions of proteins may be modified on different levels, where 3 of most important are: transcriptional, translational and post-translational. The complexity of proteins requires different approaches and types of assays performed and there are numerous ways to assay proteins. In this thesis we have used the methods described below.

3.6.1 WESTERN BLOT

Western blot is one of the most commonly used techniques in molecular biology to characterize and semi-quantify proteins of interest. During the procedure the protein sample, native or denaturated, is introduced into usually agarose or polyacrylamide gel with a certain pore size, which allows proteins to migrate according to their charge and size. The next step includes transfer of proteins onto a charged membrane and detection of the protein of interest using specific antibodies, which may be monoclonal or polyclonal (Ghosh, Gilda, & Gomes, 2014; Mishra, Tiwari, & Gomes, 2017; Pillai-Kastoori et al., 2020).

Western blot was used in Paper I to estimate the changes in protein expression of lumican in endothelial cells, after treatment with palmitic acid and/or high glucose. All experiments were performed in triplicates. For protein analysis

cells were harvested with lysis buffer (Triton X-100 1% Tris-HCl 50 mM, NaCl 150 mM, pH 7.5) with phosphatase and protease inhibitors (Sigma Aldrich). The protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher Scientific). Western blot was run using Mini-Protean TGX Stain free Gel 4–15%. Lumican was detected using an anti-lumican antibody (R&D Systems). Images were acquired with a ChemiDoc Touch Imager (Bio-Rad). Relative quantification of lumican was done using the Bio-Rad V3 Western Workflow by normalizing the lumican band intensities to the total lane volume. The band corresponding to lumican was analyzed for protein abundance.

3.6.2 MASS SPECTROMETRY

Mass spectrometry (MS) is a valuable tool to identify proteins or mixtures of peptides with high specificity. The sample specific labelling reduces the variation between samples, but sample preparation itself and analysis requires specific procedures and precision. The raw data visualizes as a spectrum, where the plot indicates the relative intensities of each ion presented as its mass-to-charge (m/z) ratio or a property related to m/z in Daltons (Da) per unit charge (Glish & Vachet, 2003). All MS analyses were run at the Proteomic Core Facility at the University of Gothenburg.

In Paper I the analysis of renal eluates was performed as a pooled sample of eluates after perfusion (7 for high salt perfusate, 5 for high osmolarity and control). Peptides and proteins were analyzed with Mascot protein discoverer, matching the data against *Rattus norvegicus* and *Mus musculus* Swiss-Prot protein databases.

In Paper II the quantitative MS data regarding PG expression was reanalyzed from a previous study by our group (Liu et al., 2017). In this study, MCs were treated with gd-IgA purified from serum of IgAN patients. The results were expressed as difference of PG expression in treated cells compared to untreated.

Samples for MS were prepared using the filter-aided sample preparation method. Proteins were digested with trypsin, reduced with dithiothreitol (Sigma-Aldrich) and diluted with 400 μ l 8 M urea and 0,1 M TEAB. After that, samples were applied on Nanosep 30k Omega Filters (Pall Life Sciences, Portsmouth, United Kingdom) (Wisniewski, Zougman, Nagaraj, & Mann, 2009). Trypsin (Sequencing Grade Modified Trypsin; Promega) was added in the sample at 1:100 dilution with 1% sodium deoxycholate and 20 mM TEAB. According to the next step of the protocol, pH was adjusted to approximately

8. After that, samples were incubated at 37°C overnight. Another portion of trypsin was added and incubated, then peptides were collected by centrifugation, labelled with a unique isobaric mass tagging reagent, and combined according to the manufacturer's instructions (Thermo Fisher Scientific). Samples were acidified to a pH of approximately 2 with formic acid to precipitate sodium deoxycholate; afterwards, it was made basic with 1 M NH₃ solution and fractionated. Peptides were analyzed on an Orbitrap Fusion Tribrid Mass Spectrometer interfaced to an Easy-nLC1000 (Thermo Fisher Scientific). The raw data obtained from LC-MS/MS were merged and identified using Proteome Discoverer, version 1.4 (Thermo Fisher Scientific). Database searches were done by Mascot search engine (Matrix Science Ltd, London, United Kingdom) using SwissProt *Homo sapiens* protein database (Swiss Institute of Bioinformatics).

Expression ratios of the samples were calculated based on average quantity of all the control samples. Expression ratios of samples, treated with IgA, were compared with control expression ratios using *t* test with Benjamin–Hochberg multivariable adjustment (Benjamini & Hochberg, 1995). A technical variance of 10% is observed using unique isobaric mass tagging reagent labeling in the Orbitrap Fusion Tribrid Mass Spectrometer. Thus, a 15% proportion change of the protein content is used for cutoff for the fold changes: fold change <0.85 indicated downregulation and >1.15 indicated upregulation (equivalent logged fold change ± 0.2). Proteins were considered differentially significantly expressed at $P < 0.05$ (Liu et al., 2017).

3.7 GLYCOPROTEOMICS

Most proteins in the human body undergo post-translational modifications. One of the most common and most complex type of modification is glycosylation. Glycomics characterizes all glycans in a cell or tissue. Glycoproteomics aims to identify the position and determine the complete range of glycosylated proteins and glycans in a cell or tissue of interest. A glycoproteomic approach for characterizing PGs was used in Paper II.

Glycoproteins have many functions, including supporting the structure of cells and tissue, cell signaling and taking a part in the immune response. Since they are having important functions and complex organization, glycoproteins require a complex approach to study and different strategies have been developed (Singh, 2021). Traditional methods consist of two principal steps, where the first is to separate all glycans from their core proteins and the second step is to analyze the glycans separately. But by the use of these methods, the information regarding attachment site of the glycan will not be obtained

(Varki, 2017). Another approach, used in Paper II, allows to preserve the glycosidic link between protein and glycan and access a more complete picture of the glycoprotein, in our case proteoglycans (Noborn et al., 2016; Noborn et al., 2015; Toledo et al., 2020).

Glycoproteomics analysis could be done using bottom-up and top-down approach. In the bottom-up approach, used in Paper II, glycopeptides derived from glycoproteins are retrieved and analyzed by LC-MS/MS, while in top-down the whole undigested glycoproteins are analyzed by LC-MS/MS.

In Paper II, the cell lysate and culture media from MCs were collected for analysis. Cell lysates were harvested using RIPA or urea buffer. We performed the sample preparation at the Departments of Physiology and Clinical Chemistry, Gothenburg University. Samples were trypsinized with a subsequent enrichment of GAG peptides using ion exchange chromatography. Peptides and glycopeptides were analyzed by reversed phase LC-MS/MS, followed by identification using Mascot search, where the search was set to trypsin and semi-trypsin, and manual verification of primary and secondary ions.

3.8 IMMUNOASSAYS

Immunoassays are a group of methods based on antibody-antigen specificity. They can be used to detect the presence or quantity of certain specific molecular sequence. Immunoassays are frequently used in clinics, drug and food testing. Immunoassays of different types were used in all 3 papers, included into this thesis.

3.8.1 IMMUNOFLUORESCENCE

Immunofluorescence is a convenient microscopic method to detect molecules of interest in cells or tissues. During the assay, the specific primary antibody recognizes and binds to the protein of interest. The secondary antibody, labelled with fluorophore, interacts with primary antibody, which allows the detection in cell or tissue. Using the combination of different secondary antibodies, it is possible to detect a few proteins in the same sample. Not only the localization, but quantification is also possible using immunofluorescence. In this case, the intensity of fluorescence could be measured, but pictures taken must have the same acquisition parameters and pictures should preferably be analyzed blindly.

Immunofluorescence was used in Paper I to check the co-localization of PGs, found in renal eluates of rats, and if they are expressed and localized in human

kidneys of DKD patients. The endothelial cell marker *Ulex europaeus* agglutinin I (Bjornson, Moses, Ingemansson, Haraldsson, & Sorensson, 2005; Mundel, Gilbert, & Kriz, 1991; Mundel et al., 1997) was used in tissue sections in combination with antibodies against the PGs of interest. In Paper II the amount of CS and HS in mesangial cells was quantified using immunofluorescence. The specificity of antibodies was confirmed by sample treatment with heparinase II and III or chondroitinase ABC.

3.8.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-linked immunosorbent assay (ELISA) is an assay which is based on highly specific antigen-antibody recognition. The antigen in this type of assay is immobilized on a surface directly or via a capture antibody. The antigen or antibody-antigen complex is revealed after recognition by an antibody conjugated to a detection molecule. ELISA is highly specific but yet a relatively simple and fast method to detect molecules in a sample and it is widely used both in research and in clinical practice. The disadvantages of ELISA are possible cross reactivity and availability of antibodies (Charan & Gautam, 1984; Houben, Callebaut, & Pensaert, 1995; Menezes, Rossener, da Silva, Rodrigues, & Manguiera, 2020).

ELISAs for mouse albumin were used in Paper I to determine spot-urine albumin and to estimate the concentrations of hyaluronan in rat kidney eluates.

In Paper III, the serum and urine samples from IgAN patients and healthy donors were tested using commercially available ELISA kit for total IgA and a novel ELISA kit for gd-IgA detection. Earlier, the level of gd-IgA was quantified with ELISA, containing lectin from *Helix aspersa* or *Vicia villosa*, specific to *O*-linked terminal N-acetylgalactosamine (GalNAc), where lower terminal galactosylation and sialylation showed higher lectin binding (Irabu et al., 2020; Nguyen et al., 2019). While using this assay, it had to be kept in mind that stability and comparability between results of different runs may be slightly different due to difference of activity and stability of lectins, purified from natural sources. The new ELISA kit is based on a monoclonal KM55 antibody directed against the gd-IgA molecule, and it recognizes a specific epitope in the hinge region of the modified IgA molecule. This assay is more stable, less variable and takes around two hours to perform (Irabu et al., 2020; H. Suzuki et al., 2018; Y. Suzuki, Suzuki, Yasutake, & Tomino, 2015; Yasutake et al., 2015).

Also, in Paper III another ELISA was used to detect creatinine in spot urine samples. For this assay, urine samples were diluted 1:25 and the assay was then

performed according to manufacturer's instruction. Creatinine is a metabolite of creatine phosphate, which could be utilized by cells for ATP production. Creatinine is released from muscle tissues and is excreted in the urine continuously since it is freely filtered by the kidneys. If creatinine is being build up in the blood outside the normal range, it is an indicator of kidney disease.

3.9 ALCIAN BLUE STAINING

The difference in total GAG content in treated and untreated cells in Paper II was estimated on cells stained with Alcian blue. Alcian blue is a dye which binds to sulfated groups of GAGs of CS, HS and dermatan sulfate (DS) (Meyerholz, Rodgers, Castilow, & Varga, 2009; Scott & Dorling, 1965).

3.10 STATISTICAL ANALYSIS

The statistical analyses in papers included in this thesis were done in Graphpad prism 8 statistical software. In Papers I, for normally distributed data one-way ANOVA with multiple comparisons with Sidak's test was used. For non-normally distributed data, Kruskal-Wallis test with multiple comparisons using Dunn's test was performed. In Paper II, for comparison between more than two groups one-way ANOVA with multiple comparisons with Sidak's test was done, for 2 groups comparison unpaired t-test for normally distributed data and Mann-Whitney for non-normally distributed data. In Paper III linear regression with Spearman's correlation coefficient was used as a statistical significance test. To calculate the difference between samples, taken at two different time points, Wilcoxon's matched pairs signed rank test was used.

In Papers I and II error bars represent standard errors of the means (SEMs) unless stated otherwise. $P < 0.05$ was considered significant for all papers presented in this thesis.

4 RESULTS AND DISCUSSION

This thesis is based on 3 papers, where we investigated glycoproteins and their role in chronic kidney disease and how these diseases may be diagnosed. Proteoglycans are involved in the maintenance of the filtration process in the glomeruli and are being altered in pathological processes occurring in glomerular disease. Considering this, Paper I was focused on proteoglycans present in the endothelial cell surface layer and how the loss of proteoglycans may cause kidney damage and lead to the development of albuminuria. In Paper II we investigated changes of molecular composition of mesangial matrix in relation to the accumulation of gd-IgA containing immune complexes. In Paper III the possibility of using gd-IgA as a clinical biomarker of IgA nephropathy was evaluated.

4.1 Paper I: Proteoglycans contribute to the functional integrity of the glomerular endothelial cell surface layer and are regulated in diabetic kidney disease

The integrity of the glomerular barrier is the key to the maintenance of a normal filtration process in the kidneys. Damage at any level of the filtration barrier can lead to proteinuria. The endothelial surface layer (ESL) consists of a highly negatively charged two-layered gel like substance: the glycocalyx with membrane-bound PGs and the endothelial cell coat (ECC), which includes secreted PGs, GAGs and soluble proteins from the surrounding environment. PGs themselves are molecules consisting of a protein core with covalently attached negatively charged GAG chains. Negative charge, carried by PGs, is one of the main contributors to the permselectivity of the glomerular filtration barrier (Haraldsson et al., 2008). The presence of a high amount of charged molecules determine the function of the ESL as a dynamic system with not only structural contribution, but also as a valuable storage point of hormones and growth factors (Iozzo & Schaefer, 2015; Karamanos et al., 2018). The change in charge selectivity and thickness of filtration barrier is one of the key steps in development of proteinuria in patients with diabetic kidney disease (Jeansson, Granqvist, Nystrom, & Haraldsson, 2006).

In this paper, we performed experiments, which allowed us to better understand how the composition of ESL affects protein loss during pathophysiological conditions. To do so, we used an approach, partly described

in a previously published paper (Friden et al., 2011), to elute the ESL from perfused kidneys of rats with similar high salt solutions as before, but now with longer perfusion times in order to release larger PGs.

High salt solution (HS) was used to elute highly negatively charged components of the ESL and physiological salt solution (NS) as a control. Since HS solution has a high osmotic power, which could be a reason to elute some of ESL components, high osmotic solution (HO) of mannitol at the same concentration as NaCl in HS solution was introduced as one of the controls. Previous results (Friden et al., 2011) show that a 10 to 15 sec time of flush perfusion was not enough to elute large molecules from ESL. The flush perfusion approach increased albumin clearance, but the only PG found in eluate was relatively small lumican. Longer elution time, as used in this paper, lead to irreversible increased fractional clearance of albumin, stronger GFR reduction. Seventeen PGs were now identified in the eluate. These observations allowed us to conclude that PGs and GAGs are valuable parts of function and integrity of ESL and losing the integrity of ESL leads to an increase in proteinuria.

After the perfusion, the GFR (HS perfusion) was changed significantly. Starting with an average flow of 1.2 ml/min/g wet kidney, GFR remained unchanged for NS perfused rats, was reduced by approximately 50% for HO perfused rats and was reduced approximately by 83% for HS perfused animals. The latter group became anuric 40 min after perfusion. Fractional clearance of albumin was increased in HS rats. Using transmission electron microscopy and the intralipid infusion technique, we could determine a significant reduction of the ESL in HS perfused rats (compared to NS and HO perfused rats) and conclude that there was no major damage to the other parts of the filtration barrier.

To identify the PG content in the eluates, we performed mass spectrometric analysis. In total, we identified 658 proteins including 17 PGs in the combined mouse and rat databases and 25 proteins, related to ECM organisation. The approach of analysing both databases allowed us to identify a wider range of proteins without false positive results, taking into account the homology between the two species.

The concentration of hyaluronan, a PG lacking the core protein, was determined by ELISA. Hyaluronan is a huge non-sulfated anionic GAG and an essential component of ECM (Toole, 2004). The reduced production of hyaluronan in a mouse model lead to a reduction of the glycocalyx thickness (van den Berg et al., 2019), oppositely to experiments, where the hyaluronidase

(enzyme, which cleaves hyaluronan), was protecting against proteinuria. Our results revealed the same amount of hyaluronan in both HS and HO eluates, but not in NS eluate. This means that increased fractional clearance of albumin in HS perfused animals found in our study is, in contrast to other studies (Dogne et al., 2016; van den Berg et al., 2019), most likely not due to a loss of hyaluronan.

The loss of PG components and a thinner ESL with simultaneous development of proteinuria were shown previously for DKD patients as a reason behind proteinuria. To find out the role of PGs, identified in eluates, in development of this disease, we reanalysed the next generation sequencing data from DKD patient samples, previously published by our group (Levin et al., 2020). In general, we discovered the change in expression of a number PGs, as well as enzymes, involved in GAG chain biosynthesis, and PG degradation. These findings indicate changes in the ESL charge and its contribution to proteinuria. The data show that AGRN and DCN were significantly downregulated, while LUM, GPC4, COL15A1, COL18A1 and CD44 were upregulated in patient biopsies. Additionally, six PGs were significantly regulated in DKD patients biopsies compare to control. Enzymes, involved in PG side chain synthesis, were significantly downregulated while PG degradation enzymes were, vice versa, upregulated. In general, these findings indicate decreases in PG and GAG compounds in DKD patient glomeruli, not only by reduced amounts of core proteins, but also by changes in GAG composition.

To determine if PGs found in murine kidney eluates and DKD patient kidneys are expressed at the surface of endothelial cells, immunofluorescence experiments were performed on kidney sections from pre-transplant biopsies. Selected PGs were analyzed by specific antibodies in combination with the endothelial cell marker *Ulex europaeus* agglutinin I (Bjornson et al., 2005; Mundel et al., 1991; Mundel et al., 1997) on human kidney tissue sections. Partial co-localization was detected for decorin and collagen alpha-1 (XV) chain, while complete co-localization was obtained for lumican, glypican-4, agrin, collagen alpha-1 (XVIII) chain and CD44.

Analysis of the DKD cohort revealed alterations in PG mRNA expression profiles during the disease, mainly with an increase in expression of PGs. We found that PG expression in endothelial cells is changed by the diabetic milieu compared to untreated cells. Cells were treated for 24 h with high glucose (HG) or palmitate conjugated to human serum albumin (PA), or both. An increase of COL18A1 and CD44 was detected in cells treated with HG and combination of HG and PA. AGRN was increased in cells treated with only HG. No significant changes were detected for COL15A1 or GPC4. The gene

expression assay didn't reveal significant changes in expression of DCN or LUM, but lumican was highly expressed on protein level in samples, treated with HG and PA. Lumican has been identified in the healthy glomerular endothelium previously. Decorin, on the other hand has been found mainly in sclerotic areas in glomeruli (Friden et al., 2011; Schaefer et al., 2001). Lumican is known to be involved in inflammation (Iozzo & Schaefer, 2015), ECM assembly (Chakravarti et al., 1998) and has been suggested to be a plasma molecular marker for DKD (Schaefer et al., 2001).

To conclude, the ESL has a valuable functional role in preventing protein loss especially in terms of charge selectivity in the filtration process. Any changes in ESL composition could lead to development of pathology with albuminuria and disrupted cell signalling. We hypothesise that preventing or restoring the alterations in the ESL provoked by for instance a diabetic milieu and enhancing PG recovery could reverse pathological changes in kidney filtration function.

4.2 Paper II: Adaptive remodeling of mesangial extracellular matrix proteoglycan composition during IgA nephropathy

IgAN is characterized by inflammation in the glomerular area due to deposition of immune complexes (Nakazawa et al., 2019; Novak, Barratt, Julian, & Renfrow, 2018). These complexes contain modified galactose deficient IgA (gd-IgA), a hallmark of IgAN. The deposition is considered to be a trigger for overproduction of chemokines, cytokines and growth factors, leading to increased proliferation of MC and matrix expansion (Ebefors et al., 2016; Kashgarian & Sterzel, 1992; Katz et al., 1991; Libetta, Rampino, Palumbo, Esposito, & Dal Canton, 1997; Ruef, Kashgarian, & Coleman, 1992). PGs are one of the main components of the MC ECM (Ebefors et al., 2011). The aim of this paper is to estimate the PG content itself and modifications in PG structure as a part of the development of IgAN.

To investigate the expression of PGs and PG related genes we analyzed the glomerular gene expression in IgAN patients and healthy donors, using gene array data from a previously published paper from our group (Liu et al., 2017). Thirty-one PGs were found according to our criteria, and of those 6 were significantly regulated in IgAN patients: SDC1 was downregulated, while MXRA5, CD44, TNC, FMOD and PRELP were upregulated. Also, some enzymes involved in PG biosynthesis were identified, but no significant changes were detected at the gene expression level. While this data can't reflect

the enzymatic activity, it does give an overview of the gene expression profile of the whole glomerulus.

A proteomic data set from the same paper, previously published by our group (Liu et al., 2017), was used to estimate the proteomic expression of PGs by MS. In this set up, mesangial cells were stimulated with gd-IgA, purified from serum of IgAN patients. Control cells remained unstimulated. This data describes core proteins of PGs. In total, 19 PGs were found, 10 of them were significantly regulated: chondroitin sulfate proteoglycan 4, glypican-1, agrin, versican, lumican, AMBP protein, tenascin, collagen alpha-1 (VII) chain and laminin subunit alpha-4. The only downregulated PG was aggrecan. Lumican and versican were the only PGs identified in treated cells, but not in patient samples. Both of the data sets reveal an increase in PG biosynthesis in IgAN, both in glomeruli and in a cell culture setting.

The data set experiments only investigate the core protein expression. To investigate the covalently attached GAG chains which have a wide structural diversity two approaches were used: glycoproteomics and immunofluorescence staining. These experiments were aimed at finding changes in the composition of PGs as well as the localization and levels of GAGs in the mesangial cells.

First, we used a new glycoproteomic approach which allow investigation of the site-specific structural information regarding HS- and CS-linkage regions (Noborn et al., 2016, Noborn et al., 2021). In general, glycoproteomics is not giving quantitative results and requires further studies to estimate the amount of PGs in treated cells. To find out the best yielding preparation for glycoproteomics, two extraction buffers were tested: RIPA buffer and urea buffer. Cells were either treated with gd-IgA from patients with IgAN, IgA from healthy subjects or left untreated. RIPA buffer was more efficient when it comes to the numbers of PGs detected, and allows a minimum of non-specific interactions and is compatible to the BSA protein assay. Urea buffer was chosen due to its ability to inhibit proteases, but urea and thiourea can hydrolyze to cyanate and isocyanate, which can modify amino groups of proteins (Ngoka, 2008). The two extraction procedures gave similar results, but generally RIPA buffer worked better for cell lysates and urea buffer for cell media.

With this approach, the PGs identified in both the treatment groups and control group were decorin, collagen alpha-1 XVIII chain and syndecan-4. CD44 and bikunin were found only in treated samples. Perlecan was found only in unstimulated cell lysate. Conversely, perlecan was found in stimulated cell media. Aggrecan, amyloid-beta A4 protein isoform H, laminin subunit 4,

decorin, matrix remodeling associated protein 5 and nidogen-2 were identified in unstimulated cell media. Interestingly, tenascin C was identified in gd-IgA treated cells and was found significantly upregulated in both IgAN patient glomeruli and gd-IgA treated cells. Increased expression of tenascin C was earlier described in pathologically expanded glomerular ECM (Truong, Pindur, Foster, Majesky, & Suki, 1996) and during IgAN development (Masaki et al., 1997). In combination with our data, tenascin C might be suggested for further investigation as to its involvement in of MC ECM overproduction in IgAN.

Bikunin is one of the proteins of cleaved AMBP protein, together with trypstatin and alpha-1-microglobullin and has been shown to be expressed in kidneys (Itoh et al., 1996), but not in glomeruli itself. Bikunin was detected only in stimulated cells, but AMBP gene wasn't found to be significantly upregulated in the IgAN cohort. On the contrary, AMBP expression was significantly upregulated in gd-IgA stimulated cells.

Another interesting finding of this set up was that nidogen-2 was identified as chondroitin sulfate PG (CSPG) and collagen alpha-1 VII as heparan sulfate PG (HSPG).

The second step of our analysis was the investigation of the expression of HS and CS content in treated MCs using immunofluorescence staining and analysis of intensity. Alcian blue staining, a general staining method for GAGs, revealed no changes in total GAG amount between treated and untreated samples. Specific HS or CS staining showed a switch between expression of those two GAGs in treated and untreated cells. CSPGs were significantly upregulated compared to HSPGs in treated MC, while the HSPG content was higher in untreated cells in comparison to CSPGs. This data is aligning with our findings in the IgAN cohorts, as well as in the gd-IgA treated cells where CSPGs were significantly upregulated compared to HSPGs. It had been shown previously that MC, stimulated with gd-IgA, increase their cytokine production (Ebefors et al., 2016), which triggers the increase in CSPG content (Davies, Thomas, Shewring, & Mason, 1992).

To understand the underlying process between this CSPG/HSPG switch in treated MC, we investigated the expression of enzymes involved in the biosynthesis of CSPG and HSPG at the gene level. The enzymes in focus were XYLT1 (starting the synthesis of linker region for both CS and HS), EXTL1 (initiating the first step of HS polymerization) and CHSY1 (polymerization of CS). No significant changes at the gene expression level of two enzymes were detected when comparing treated to untreated cells. The switch in GAG type

may be difficult to explain by just expression analysis but may need measuring the activity of these two enzymes. Further investigation is needed to better understand the underlying mechanisms behind the switch and how it affects the pathophysiological processes in IgAN.

In conclusion, in this paper we focused on how PG composition in MC ECM changes during IgAN, how these changes affect the ECM expansion and possible underlying processes. PGs are not only the structural component of ECM, but also a storage for signaling molecules and receptors. Changes in structure of ECM and related PGs could affect intercellular communication and trigger the development of pathology (Katta et al., 2015; Truong et al., 1996). The increased PG expression and switch between HSPG and CSPG in gd-IgA stimulated MCs could be an important step in the mechanisms behind IgAN development.

4.3 Paper III: Galactose-deficient IgA levels in blood and urine in patients with IgA nephropathy

IgAN is often a silent disorder, which is not easy to detect at early stages. Presently, there are no non-invasive clinical tests available to diagnose IgAN, although general clinical tests such as albumin/creatinine ratio and eGFR could indicate glomerular disease and, potentially, IgAN development. A renal biopsy is still necessary for a proper diagnose. Taking into account the medical risks for patients and high costs for an invasive diagnostic methods such as a renal biopsy, a non-invasive biomarker of IgAN is highly demanded. In Paper III we hypothesized that the level of gd-IgA could possibly be used as a non-invasive marker of IgAN. Similar studies had been performed for Asian and Caucasian populations, suggesting either serum or urinary gd-IgA as a possible marker of IgAN (H. Suzuki et al., 2016; K. Zhang et al., 2019; Zhao et al., 2012).

Recently it has become possible to measure the level of gd-IgA in a stable and reliable fashion due to a newly developed ELISA kit, based on monoclonal KM55 antibody against gd-IgA (P. Chen et al., 2019; Yasutake et al., 2015; K. Zhang et al., 2019). This antibody recognizes a specific epitope at the hinge region of gd-IgA and provides the opportunity of more stable test results. The technique used before was based on the principles of ELISA, using the same approach as the newly developed kit. The recognition was possible due to lectin from *Helix aspersa* or *Vicia villosa*, specific to terminal O-linked N-acetylgalactosamine (GalNAc), where lower terminal galactosylation and sialylation show higher lectin binding (Irabu et al., 2020; Nguyen et al., 2019).

Those lectins were purified from a natural source, meaning that activity and stability of assay was depending on the batch.

In our study, we checked levels of gd-IgA and IgA in serum and urine from a Swedish cohort of IgAN patients with biopsy proven IgAN. Samples were collected in collaboration with Sahlgrenska University Hospital over a time period of approximately 9 years. Patients were followed up during this time in terms of clinical parameters.

Oppositely to earlier studies (J. S. Kim et al., 2020; K. Zhang et al., 2019), the result of our experiments detected a trend but didn't show any significant correlation between IgA and gd-IgA in serum. To avoid misinterpretation of results due to sampling time and volume of urine samples, the concentrations of IgA and gd-IgA were normalized to urine creatinine levels measured prior to the experiments.

Investigation of the relationship between gd-IgA levels in plasma and urine to clinical parameters revealed a correlation with albuminuria, i.e. patients with lower albuminuria show lower levels of both IgA and gd-IgA in the urine samples. Albuminuria indicates a general degree of kidney malfunction (Levey, Gansevoort, et al., 2020; Levey et al., 2009; Levey, Titan, Powe, Coresh, & Inker, 2020) and the presence of increased levels of IgA or gd-IgA may be due to the loss of barrier function as well. Only the amount of gd-IgA in urine correlated significantly with eGFR. The higher level of gd-IgA in urine correlated with lower eGFR, where lower eGFR indicates reduction in kidney function. The ratio between gd-IgA and IgA in urine was significantly higher when compared to serum, suggesting the possible enrichment of gd-IgA in urine. These results suggest that gd-IgA in urine may have a clinical prognostic value.

In addition, we performed follow up measurements of gd-IgA in serum and urine samples taken again approximately 9 years after the first biopsy. To our knowledge, the change in gd-IgA levels with time has not been the subject of research before. We saw a general decrease in gd-IgA concentrations both in serum and urine compared to the levels at the time of biopsy while the disease is still progressing.

In conclusion, in the cohort we analyzed, neither IgA nor gd-IgA could be considered as a diagnostic marker per se. The IgAN diagnosis will still require a renal biopsy for diagnostic and prognostic purposes. The leakage of gd-IgA and IgA to the urine may be explained by the damage of the glomerular barrier

during the progression of disease. The reduction of gd-IgA in the urine in the follow up samples may be an interesting subject for future investigation.

5 CONCLUDING REMARKS

This thesis work is a combination of three studies. The general aim was to discover and estimate changes in glycoproteins, with focus on PG content and composition and its relation to both normal and pathological conditions in the kidney such as DKD (Paper I) and IgAN (Papers II and III).

The approach used in Papers I and II is the general-to-specific, where physiological aspects of healthy and diseased kidney states have been analyzed from the point of changes at the molecular level. In Paper I, the hypothesis was set on the statement that PGs of ESL, together with related GAGs, are an essential part of the kidney filtration barrier and changes in those compounds may affect the kidney function. The loss of PG components by elimination with a highly concentrated salt solution led to ESL modifications. We have been able to identify high numbers of PGs in eluates, even some previously not known to be part of the ESL. Removing those PGs from ESL led to proteinuria in animal models. Changes in PG expression were detected for PGs in glomeruli from DKD patients, as well as in human endothelial cell culture under diabetic like condition. The reduction of endothelial cell PG content is a clear sign of pathology and a possible explanation for DKD glomerular malfunction.

From Paper II, we can conclude that PGs play an essential role in ECM expansion during IgAN. Gd-IgA-containing immune complexes are known to be one of the triggers of IgAN development, but the mechanisms underlying this process are not clearly understood. To study the pathological processes taking place in the ECM in the presence of gd-IgA, mesangial cells were treated with gd-IgA, purified from IgAN patients or IgA from healthy donors. The novel glycoproteomic approach used in this study allowed us to detect PGs by MS without losing the GAG attachment site. Based on data sets from MC and IgAN patients, we have been able to conclude that MC ECM PG composition is subjected to changes during IgAN and may be considered as a possible therapeutic target to prevent ECM expansion in IgAN.

The research presented in Paper III was focused on finding a possible biomarker of IgAN, which would allow detection of disease or disease progression without the need to perform a renal biopsy. The assumption of this research was based on the fact that gd-IgA containing immune complexes, deposited in glomeruli of IgAN patients, might be released into urine and serve as a biomarker for disease. In this study, we used a recently developed ELISA kit for gd-IgA quantification. IgA and gd-IgA levels of urine and serum

samples from approximately 40 IgAN patients, 20 patients with CKD and 20 healthy controls were analyzed. So far, 19 patients agreed to provide follow up samples approximately 9 years after the initial diagnosis and sampling. Our results showed that neither IgA nor gd-IgA levels in IgAN patient sera had any diagnostic value and there was no correlation to disease severity at the time of diagnosis for any of the two biomarkers. The gd-IgA levels were however declining with time and were significantly lower in the follow up samples, providing a promising result for future research.

In conclusion, the processes underlying development of kidney pathology are variable and complex. PGs are an important group of molecules involved in maintaining the integrity of the filtration system in kidneys, providing not only structural support and charge selectivity, but also serving as a dynamic deposition pool for signaling molecules. In other words, PGs are important for normal renal function in a multitude of processes. The understanding of how the composition and amount of PGs change in glomerular disease is one of the ways to develop a targeted therapy for patients, suffering from kidney disease, in the future. This impelling need for new kidney disease biomarkers led us to investigate the role of gd-IgA in IgAN in this aspect. Even though we cannot conclude that gd-IgA is a valuable biomarker, new and interesting aspects of the course of IgAN progression and development was found and will constitute a starting point for further research.

6 FUTURE PERSPECTIVES

The results from the studies, presented in this thesis, reflect the complex organization and importance of a functional filtration barrier in the glomeruli. Many molecular entities are important for preserving the integrity of the barrier. PGs are contributing to normal kidney function, both as receptors and for storage of signaling molecules. The variety of PG functions is likely to be due to their highly variable structure, including both core protein and charged GAG side chains.

Since PGs are complex molecules, there is a need for advanced and sophisticated methodological approaches to be able to investigate their role in biological processes, both in physiological and pathophysiological settings. Another crucial point is the accurate and precise collection of patient's data and sampling protocol. Carefully and methodologically approaching these analytical and sampling challenges will facilitate the discovery of PG related malfunctions in patients with kidney diseases. The general goal is to unveil new possible therapeutic targets in future.

In Paper I, we estimated the role of the ESL and effects on the charge selectivity in development of albuminuria. An intact and functional ESL has a relevant part in the prevention of protein loss from blood. Any change in the ESL PGs content can lead to the harmful development of albuminuria. Possibly, these alterations could be reversed by boosting PG production or slowing down PG degradation processes. This could be interesting therapeutically. However, before this will become possible, more details regarding the individual PGs and their respective role in the permselectivity needs to be determined. There may be common ways to restore PGs functionality, but this demands further investigation.

Changes in PG content and composition during IgAN were in focus of Paper II. The alterations of both the PGs pool and PGs structures were detected in this study. PGs are not only an important part of the ESL, but they are also expressed by MCs and are an important part of the ECM production by the MCs. Aligning with our hypothesis regarding pathology related adjustment of MC ECM, our results indirectly prove the dependence of abnormal expansion of ECM in response to presence of gd-IgA in the MC area. Targeting molecules involved in PG modification and shift of GAG chain composition could be considered as a way to interfere with the ECM expansion. Potentially, this could be used as a tool to modulate the proliferation and expansion of the ECM, a hallmark of IgAN development.

In Paper III gd-IgA and IgA concentrations were estimated in serum and urine of IgAN patients. IgAN is a silent disease and it still requires a biopsy to confirm the diagnosis, thus a non-invasive biomarker, both to diagnose and follow disease progression is highly needed. Our results indicate that in our cohort of IgAN patients, neither gd-IgA nor IgA could be considered as predictive biomarkers for disease progression. The levels of gd-IgA in the urine do correlate with disease severity, but not beyond what proteinuria already may reveal. We did detect a significant reduction of gd-IgA content in patients after approximately 9 years follow up and the reason behind this finding demands future investigations. We speculate that the amount of gd-IgA may be of highest relevance in the onset of IgAN because of its deposition in the mesangium. Without doubts, the role of gd-IgA in the onset and later phases of the disease calls for further research.

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