Glomerular and tubular proteome markers of progressive IgA nephropathy

Flavia Teodora Ioana Paunas

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

The work presented in the thesis was initiated in 2014, at Department of Medicine at Haugesund Hospital, and the Renal Research Group, Department of Clinical Medicine, University of Bergen.

Main supervisor was Professor Bjørn Egil Vikse and co-supervisor was PhD Kenneth Finne.

Laser capture microdissection was performed at the Molecular Imaging Center (MIC) at the University of Bergen.

Protein extraction and protein analyses were performed at the Proteomics Unit at the University of Bergen (PROBE).

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Abbreviations

AA	Amino acid
Ab	Antibody
ACEi	Angiotensin-converting enzyme inhibitors
Ag	Antigen
AKI	Acute kidney injury
AMN	Amnionless
AMR	Antibody mediated rejection
AP	Alternative pathway
ARB	Angiotensin II receptor blocker
ATL	Ascending thin limb
AUC	Area under the Curve
BP	Blood pressure
CI	Confidence interval
CFH	Complement Factor H
CFHR	Complement Factor H–related
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration Equation
CP	Classical pathway
DRI	Direct renin inhibitor
DT	Distal tubule
DTL	Descending thin limb
ECM	Extracellular matrix
eGFR	Estimated Glomerular Filtration Rate
ESL	Endothelial surface layer
ESRD	End Stage Renal Disease
EMT	Epithelial-mesenchymal transition
FB	Factor B
FC	Fold Change
FD	Factor D
FH	Factor H
FFPE	Formalin-fixed paraffin-embedded
GAL	Galactose
GalNAc	N-acetyl galactosamine
Gd-IgA1	Galactose-deficient IgA1
GN	Glomerulonephritis
GBM	Glomerular basement membrane
GWAS	Genome Wide Association Study
HR	Hazard Ratio
IHC	Immunohistochemistry
IgAN	Immunoglobulin A Nephropathy
Il	Interleukin
KDIGO	Kidney Disease Improving Global Outcomes
LC	Liquid chromatography
LH	Loop of Henle
LTBP-1	Latent Transforming Growth Factor Beta Binding Protein 1
MAC	Membrane attack complex
MASP-2	Mannan-binding lectin-associated serine protease-2
MCD	Minimal change disease
MDRD	Modification of Diet in Renal Disease
MHC	Major Histocompatibility Complex
MMF	Mycophenolate mofetil
MMPs	Matrix metalloproteinases
MS	Mass spectrometry
NKBR	National Kidney Biopsy Registry
NPR	Norwegian Population Registry
NRR	Norwegian Renal Registry
PEC	Parietal epithelial cells
PT	Proximal tubule
PTEC	Proximal tubular epithelial cells
RPC	Reversed-phase chromatography
RPGN	Rapidly progressive glomerulonephritis
ROC	Receiver Operating Characteristic
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SA	Sialic acid
SPSS	Statistical Package for the Social Sciences
SYK	Spleen tyrosine kinase
VALIGA	Validation of the Oxford Classification of IgAN in a European cohort
TAMPs	Tight junction associated Marvel proteins
TEC	Tubular epithelial cells
TGF-β	Transforming growth factor beta
TIF	Tubulointerstitial fibrosis
TJ	Tight junctions
TNF	Tumor necrosis factor
TRF-budesonide	Targeted-release formulation of budesonide
TCC	Terminal complement complex

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Abstract

Background: IgA nephropathy (IgAN) is the most common glomerulonephritis in the world, with a clinical course ranging from asymptomatic non-progressive to aggressive and progressive disease to kidney failure. The exact mechanism of progression is not fully understood and further research is needed. Proteomics could help to investigate the mechanism of progression in IgAN and define potential biomarkers.

The aims of this study were:

- To identify novel tissue biomarkers for progressive IgAN that may be used for prognostication.
- To improve understanding of underlying mechanisms in IgAN.
- To separately investigate glomerular and tubular biomarkers in IgAN.

Methods: Formalin-fixed paraffin-embedded kidney biopsy of patients with IgAN and control from the Norwegian Kidney Biopsy Registry were used. The IgAN group was divided in progressive or non-progressive based on progression to kidney failure over 10 years. Glomerular and tubulointerstitial tissues were microdissected, the proteome was analysed using mass spectrometry and the protein abundance was compared between groups.

Results: As compared to IgAN patients without progressive disease, glomeruli from patients with progressive IgAN had significantly higher abundance of components of the classical and the terminal complement pathways (Paper I), and the tubulointerstitial tissue had higher abundance of proteins related to inflammation (Paper III). As compared to controls, glomeruli from patients with IgAN showed significantly higher abundance of extracellular matrix structural proteins and extracellular matrix associated proteins (Paper II). Periostin was the protein with the highest fold change between groups both in glomeruli and tubuli.

Conclusions: Microdissection of glomeruli and tubuli allowed for compartment-specific analyses of prognostic markers and a better understanding of underlying mechanisms of progressive IgAN. The most promising marker seem to be periostin which associated with progressive disease both in glomeruli and tubuli.

List of included papers

Paper I

Paunas TIF, Finne K, Leh S, Marti HP, Mollnes TE, Berven F, Vikse BE: **Glomerular abundance of complement proteins characterized by proteomic analysis of laser-captured microdissected glomeruli associates with progressive disease in IgA nephropathy**. Clin Proteomics 2017;14:30

Paper II

Paunas FTI, Finne K, Leh S, Osman TA, Marti HP, Berven F, Vikse BE. **Characterization of glomerular extracellular matrix in IgA nephropathy by proteomic analysis of laser-captured microdissected glomeruli**. BMC Nephrol. 2019 Nov 14; 20(1):410.

Paper III

Paunas FTI, Finne K, Leh S, Marti HP, Berven F, Vikse BE: **Proteomic signature of tubulointerstitial tissue predicts prognosis in IgAN** (Accepted BMC Nephrol 2022 March)

Introduction

The kidneys

The functional unit of the kidney, the nephron, consists of the renal corpuscle with the glomerulus, the Bowman capsule and the tubular system.

The glomerulus

The glomerulus is a spherical cluster of capillaries and matrix housed within a connective tissue structure, the Bowman capsule [1]. Along their outer portion, the glomerular capillaries are supported by the podocytes, which reside in the urinary space and have interdigitating foot processes (Figure 1). Between the capillaries, the mesangial cells and the mesangial extracellular matrix (ECM) make up the mesangium [2].

The three types of cells that reside in the glomeruli are: podocytes, endothelial cells and mesangial cells. The extracellular matrix of the glomerulus is present in anatomically distinct areas with different functions depending on its molecular components: the glomerular basement membrane, the Bowman's capsule and the mesangial ECM [3].

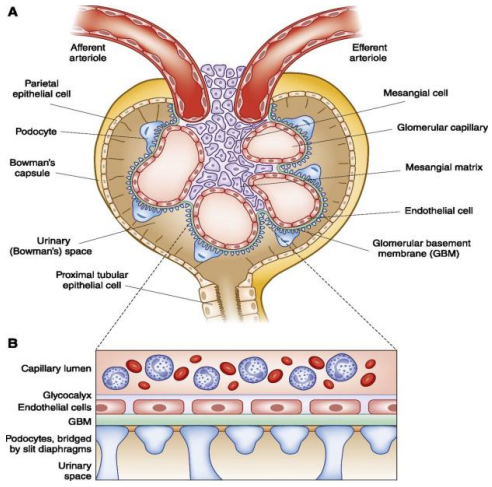

Figure 1. Basic structure of the glomerulus and the glomerular filtration barrier. Reprinted with permission from A. Richard Kitching and Holly L. Hutton 2016 [4]

Cellular components of the glomerulus

Podocytes

Podocytes are highly specialized cells, that have a voluminous cell body, from which long primary processes arise. From each primary process, numerous foot processes project toward the capillaries [5]. Podocyte foot processes are involved in a complex series of interdigitations with foot processes from other cells, forming intercellular junctions that make slit diaphragm filtration barriers [6]. Podocytes synthesize the proteins of the slit diaphragm and many extracellular matrix components of the glomerular basement membrane (GBM) [7].

Nephrin is the major component of the slit diaphragm extending out into the centre of the slit and interacting with nephrin from adjacent podocyte foot processes [7]. Other proteins of the slit diaphragm complex include podocin, podocalyxin, NEPH 1/2/3 and glomerular epithelial protein 1 (GLEPP-1) [7]. Podocytes adhere to the underlying GBM using transmembrane adhesion receptors [8]. Adhesion molecules α3β1 integrin and α-dystroglycan are linked to laminin. Integrin is coupled to the actin cytoskeleton through a complex of talin, vinculin and paxillin proteins, whereas the adhesion molecule α-dystroglycan links to actin through utrophin [7]. When podocytes become injured, they undergo a process of effacement in which they lose their structure and become diffused, spreading out and leading to a reduction in the filtration barrier function [6].

Mesangial cells

Mesangial cells are situated between glomerular capillary loops, embedded in the mesangial matrix, forming a supporting framework that maintains the structural integrity of the glomerular tuft [9]. Mesangial cells have contractile properties generated by anchoring filaments to GBM opposite podocyte foot processes, thereby assisting in the maintenance of capillary organization [10]. The GBM is not present at the endothelial cell–mesangial cell interface, i.e., the mesangial angle [9] where mesangial cells make direct contact with endothelial cells through fenestrations [11] (Figure 2). In the normal human glomerulus, there should not be more than two mesangial cells per mesangial area [1]. Mesangial cells can secrete mesangial matrix

components, such as type IV and type V collagen and fibronectin [2], and have receptors for vasoactive agents, such as angiotensin II and endothelin [9]. Mesangial cells generate and are embedded in their own extracellular matrix. In addition, components of mesangial matrix can influence mesangial cell growth and proliferation [10]. Mesangial cells make contact with both endothelial cells and podocytes (Figure 2) [12].

Cross-communication between mesangial cells and endothelial cells is achieved through platelet-derived growth factor β (PDGFRβ), integrin αvβ8 and TGF-β signalling pathways [13]. Communication between podocytes and mesangial cells is also believed to exist, although so far direct *in vivo* evidence is limited. Mesangial cells have many functions: a) structural support for the glomerular capillary loops, b) they generate and control the turnover of the mesangial matrix, c) they contribute to the regulation of glomerular capillary flow and ultrafiltration surface and hence glomerular filtration and d) they contribute to the pathophysiology of a number of glomerular diseases [10] such as IgAN [14].

Mesangial cell proliferation is a prominent feature of glomerular diseases, including IgA nephropathy, membranoproliferative glomerulonephritis, lupus nephritis and diabetic nephropathy [15]. When treated with galactose-deficient IgA, mesangial cells from patients with IgAN express and release more PDGF, resulting in an increased proliferation rate of cells [16]. Also, mesangial cells cultured from patients with IgAN express and release more IL-6 than controls and have a higher expression of matrix genes [16].

Endothelial cells

Endothelial cells line the inner surface of the glomerular capillary loops and have round fenestrated regions measuring 70-100 nm in diameter [1]. Endothelial cells are connected to each other by gap junctions and shallow occluding junctions and they form gap junctions with mesangial cells [1]. The endothelial cells also produce the endothelial glycocalyx that forms and important part of the glomerular filtration barrier [17].

Parietal epithelial cells

Parietal epithelial cells (PEC) line the outer aspect of the glomerulus, playing a structural role in maintaining Bowman's capsule. Additionally, they are also speculated to have the ability to differentiate into podocytes to replace damaged or old podocytes [18]. The parietal layer is not directly involved with filtration from the capillaries [18]. Recently, it has been shown that PEC can be either activated, undergoing proliferation, manifesting as crescent formation, or can deposit extracellular matrix proteins, which contributes to the development and progression of glomerulosclerosis [19].

The Glomerular Matrix

Glomerular Basement Membrane

The GBM is the extracellular matrix component of the glomerular filtration barrier which lies between the glomerular endothelial cells that line the glomerular capillaries and the podocytes [20]. The GBM is thicker than most other basement membranes and is composed of four major macromolecules: laminin, type IV collagen, nidogen and heparan sulphate proteoglycan (the major one being agrin) [21]. Because of its negative charge, it has been believed that GBM is important in keeping away albumin through charge selectivity. However, more recent studies [22,23] suggested that the endothelial glycocalyx may be more important in the charge selective properties of the glomerular filter than the GBM [24].

The mesangial matrix

The mesangial extracellular matrix (ECM) differs substantially from GBM, in that its composition allows larger molecules to pass to the mesangium [3]. The mesangial matrix consists of proteins such as fibrillin-1, emilin, microfibril-associated proteins (MAPs) 1 and 2, and latent transforming growth factor-binding protein-1 (LTBP-1) [1]. The mesangial matrix contains also type IV and type V collagen, laminin and fibronectin [1]. Small amounts of the proteoglycans decorin and biglycan are found in the mesangial matrix [10]. The composition and amount of mesangial matrix are tightly controlled in health but can be markedly altered during disease such as IgAN [10], for example decorin and biglycan were shown to be up-regulated in IgAN [25].

Matrix metalloproteinases (MMPs) seem to play an important role in the homeostasis of the mesangial matrix by degrading ECM [26] and MMPs have been shown to be involved in both acute and chronic renal pathophysiologies [27].

The filtration barrier

The glomerular endothelium is highly fenestrated with pores estimated to be 60-80 nm in diameter. The endothelium is covered by the glycocalyx (polysaccharide-protein gel-like structure), also known as the endothelial surface layer (ESL) [28]. The ESL consists of membrane-bound molecules, such as proteoglycans and glycoproteins, and soluble molecules, both endothelium-derived and from plasma, including hyaluronic acid and other soluble proteoglycans [29]. The glomerular endothelium functions as a filtration barrier for large molecules, such as albumin.

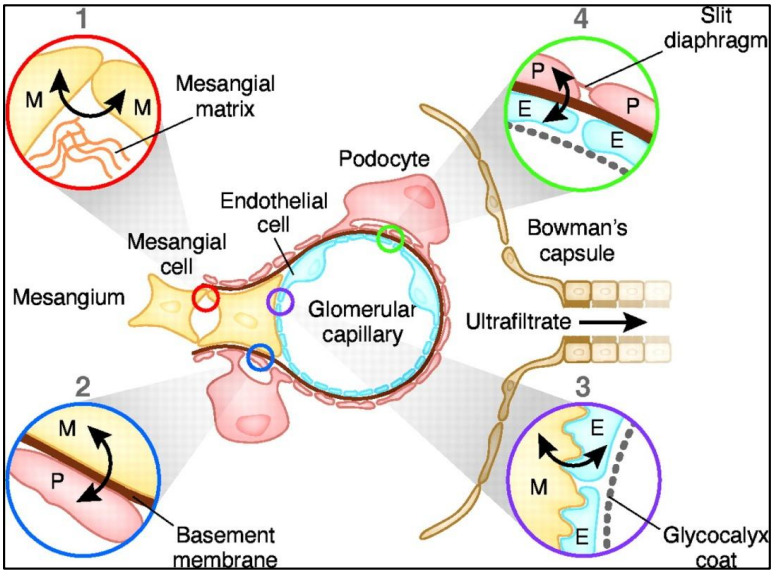

Figure 2. Cell–cell signalling between intrinsic glomerular cells M=mesangial cell, P=podocyte, E=endothelial cell. Reprinted with permission from Detlef Schlöndorff, and Bernhard Banas JASN 2009;20:1179-1187 [10]

The tubular system and tubulointerstitium

The segments of the tubules that arise from the glomerulus are the proximal tubule (PT), the loop of Henle, and the distal tubule which empties into the collecting duct.

The proximal tubule

The proximal tubule (PT) differs histologically from other tubular segments, since it has cuboidal/columnar epithelial cells with round, centrally-located nuclei and densely eosinophilic cytoplasm, due to numerous mitochondria [1]. The mitochondria are concentrated in the basolateral region of the cell, where the active ion pumps are located. The apical surface of the cells, facing the lumen of the tubule (the luminal surface), is covered with densely packed microvilli and forms the brush border.

The cell membrane between the microvilli invaginates to form a system of pits and tubules, which are coated with proteins, such as clathrin, and contain the gp330 Heymann nephritis antigen [1]. A large part of solutes and water are reabsorbed from the PT through the interstitium and then into peri-tubular capillaries. The transport of ions and water is achieved by sophisticatedly arranged channels, carriers and transporting ATPases, both in the apical and basolateral membranes of the epithelial cells [30]. At the basolateral pole, the tight junctions (TJ) are of the "leaky" type, allowing for high paracellular transport [31]. The proteins of this TJ have been identified: claudin-2, claudin-10, claudin-11, claudin-17 and the tight junction associated Marvel proteins (TAMPs), occludin and tricellulin [30].

At the apical pole, the Na^+-H^+ exchanger 3 (NHE3) is important in H^+ extrusion [32]. Proteins such as NHERF-1, ezrin, megalin and clathrin can regulate NHE3 activity [33]. Aquaporin 1 (AQP1), present in high abundance at the apical and basolateral membranes, plays a central role both in the reabsorption of H_2O and HCO_3^- [34]. Angiotensin II acts on the PT via angiotensin II receptors type 1, which are G protein-coupled receptors for both its stimulatory and inhibitory effects [34]. Three important membrane proteins are responsible for glucose reabsorption from the glomerular filtrate in the proximal tubule: sodium−glucose cotransporters SGLT1 and SGLT2, in the apical membrane, and GLUT2 [35] at the basolateral membrane.

The limb of Henle

The limb of Henle (LH) has a descending thin limb (DTL) and an ascending thin limb (ATL) with several types of epithelia, that reflect functional differences in solute and

water permeability [1]. Aquaporin-1 expression is a marker of DTL. In the ascending limb, the Na^+, K^+ and Cl^- are co-transported across the apical membrane by the Na-K-Cl cotransporter (NKCC2), that is sensitive to loop diuretics [36]. ROMK protein at the apical membrane has a critical role in Na^+-Cl^- absorption by the ATL [36]. At the basolateral membrane, the chloride channels CLC-K1 and CLC-K2 participate in Cl^- transport.

Distal tubule

The distal tubule (DT) plays crucial roles in NaCl reabsorption, K^+ secretion and Ca^{2+} and Mg^{2+} handling [37]. The cells of the DT have a thiazide-sensitive NaCl cotransporter (also called NCC; SLC12A3 or TSC) responsible for the reabsorption of Na^+ [37]. The cotransporter is one of the major targets of thiazide-type diuretics and loss-of-function mutations in the *SLC12A3* gene causes Gitelman syndrome, a salt wasting disorder that commonly presents with low-to-normal blood pressure (BP), elevated renin levels, hypokalemia, metabolic alkalosis, hypocalciuria and hypomagnesemia [38]. Aldosterone, angiotensin II, insulin and vasopressin stimulate salt reabsorption in the distal nephron by increasing NCC activity through the WNK-SPAK/OSR1 signaling pathway [37]. At the basolateral pole, the Na^+/K^+-ATPase participates in Na^+ transport, and K^+ channels in the K^+ efflux [38]. A chloride channel ClC-Kb with its accessory subunit Barttin, together with potassium chloride cotransporter 4 (KCC4; SLC12A7) are important in chloride transport [37].

The interstitial ECM

The ECM of the tubulo-interstitial compartment consists of basal membranes of peritubular capillaries and tubules, and of the interstitial space. The tubular basal membrane consists of collagen IV, agrin, perlecan and laminin [39].

The renal interstitial matrix is normally composed of collagen type I, III, V, VI, VII and XV, both sulphated and non-sulphated glycosaminoglycans, glycoproteins (e.g. fibronectin, versican, biglycan, decorin) and polysaccharides [39]. During fibrosis, the formation of scar tissue in the interstitial space is the result of excessive accumulation of ECM components [3]. For example, under physiological conditions, collagen type III is normally expressed at low levels in the tubulointerstitium, but during fibrosis the

expression levels increase [40]. Even though many cell types in the kidney are able to produce ECM, myofibroblasts in the interstitium are considered the main cellular mediators of interstitial fibrosis [3]. The origin of interstitial fibroblasts remains an area of controversy [41].

Recent studies have identified an extensive population of mesenchymal cells, called either pericytes (when attached to peritubular capillaries) or resident fibroblasts (when embedded in matrix) as the progenitors cells of myofibroblasts [42]. Other cells seem to be to a lesser extent the source of myofibroblasts, including endothelial cells (via endothelial-to-mesenchymal transition), tubular epithelial cells (via epithelial-to-mesenchymal transition) and fibrocytes [3]. Apart from ECM synthesis, fibroblasts can produce a wide array of pro-inflammatory cytokines and chemokines, as well as oxygen radicals [41]. During fibrosis, ECM is changed both in a quantitative, but also qualitative manner [43]. Biochemical modification of the fibrillar collagen, by covalent cross-linking by lysyl oxidases and transglutaminases, occurs as fibrosis progresses, making it resistant to proteolysis [42]. Very little is known about the composition of the basement membrane of the peritubular capillaries [39].

Chronic kidney disease (CKD) and progression of CKD

CKD is a heterogeneous group of disorders characterized by alterations in kidney structure and function, which manifests in various ways depending upon the underlying cause and the severity of disease [44]. In high-income and middle-income countries, diabetes and hypertension are the main causes of CKD, while in Asia, India, and sub-Saharan Africa, glomerulonephritis is a more common cause of CKD [45].

According to KDIGO 2012 Guidelines, CKD is defined as "abnormalities of kidney structure or function, present for more than 3 months, with implications for health" [46]. Diagnostic criteria for CKD imply either a GFR threshold <60 mL/min/1.73 m^2 or the presence of kidney damage, characterized by proteinuria, albuminuria, urinary sediment abnormalities, pathologic/imaging abnormalities, genetic disorders, or a history of renal transplantation [46].

The progression rate of CKD varies among individuals and the natural pattern of progression from CKD to kidney failure may often follow a more staccato and unpredictable course rather than a linear one [47]. That makes it difficult to identify those at high risk for progression. Low GFR at presentation, higher levels of proteinuria, and hypertension are known markers for a more rapid decline in GFR [48]. Other risk factors associated with CKD progression include: low birth weight or prematurity [49], male sex [50], obesity [51], dyslipidemia [52], diabetes [50] and disorders of bone and mineral metabolism [53]. Changes in the gut microbiota composition and structure (dysbiosis) are also believed to contribute to the progression of CKD [54].

The final common pathway for many chronic kidney diseases is **renal fibrosis**. Renal fibrosis represents the unsuccessful wound-healing of kidney tissue after chronic, sustained injury, and is characterized by a dysregulated remodelling of the extracellular matrix (ECM) [55]. Renal fibrosis can affect all compartments of the kidney being term "glomerulosclerosis" in the glomeruli, "tubulointerstitial fibrosis" in the tubulointerstitium and "arterio- and arteriolosclerosis" in the vasculature [55]. In initial stages, fibrosis formation can be found only in the primarily affected

compartment, but in advanced stages of renal diseases all kidney compartments are affected [56].

Glomerulosclerosis

Glomeruli are often first affected by pathological stimuli. Given that glomeruli, particularly podocytes, have a very limited regenerative potential, glomerulosclerosis is considered to be irreversible [56]. All cell types in the glomeruli seem to be involved in glomerulosclerosis. Glomerular microinflammation can be, for example, initiated by activation of endothelial cells in response to hypertension, with inflammatory cells such as macrophages, activating mesangial cells [45]. Activated mesangial cells produce chemokines and cytokines, which act on mesangial cells themselves and on other glomerular cells, which in turn will secrete mediators that act on mesangial cells, forming a paracrine loop [4]. Activated mesangial cells can also produce excessive ECM, leading to mesangial matrix expansion, an early sign of glomerulosclerosis [45]. Podocytes can also contribute to glomerulosclerosis by their detachment from the GBM which can lead to endothelial stress and injury, but also by activation of parietal epithelial cells and mesangial cells which further facilitate glomerulosclerosis [56].

The mechanisms by which glomerular damage leads to tubular damage and interstitial fibrosis are not fully understood. Several different insults have been shown to trigger tubulointerstitial pathology and fibrosis, such as proteinuria [57], leakage of glomerular filtrate into the periglomerular space [58], or tubulointerstitial hypoxia and peritubular capillary regression [59].

Proteinuria is especially interesting, as it is the currently used clinical parameter which has the best correlation with future clinical course; furthermore, reduction of proteinuria is a treatment target which is associated with a better prognosis [60]. Some patients do however progress to kidney failure with little proteinuria and it also seems evident that proteinuria is handled very differently by the renal tubuli in different patients. Experimental evidence has shown that proteinuria induces changes in gene

expression in tubular epithelial cells, ultimately leading to inflammation and progressive renal fibrosis [57,61,62].

Tubulointerstitial fibrosis

Factors contributing to tubulointerstitial fibrosis (TIF**)** include: 1) infiltration of inflammatory cells, 2) fibroblast activation and expansion from various sources, 3) production and deposition of a large amount of ECM components and 4) microvascular rarefaction and hypoxia and tubular trophy [63].

1) Inflammation has a crucial role both in the initiation of fibrogenesis after injury, and in the progression of fibrosis [63]. Inflammatory cells (T calls, macrophage), fibroblasts and tubular epithelial cells (TEC) contribute to these processes. The monocyte/macrophage is the most abundant immune cell in most models of chronic kidney injury, and they are believed to promote TIF progression through the production of pro-fibrotic cytokines that have paracrine effects on neighbouring fibroblasts/pericytes and epithelial cells [64]. Other immune cells contributing to inflammation are dendritic cells, inflammatory lymphocytes [63], T cells and natural killer (NK) cells [64]. Pro-fibrotic cytokines will activate the matrix-producing cells such as fibroblasts, tubular epithelial cells, vascular smooth muscle cells and a subset of macrophages [63].

2) Fibroblasts are considered the principal matrix-producing cells that generate a large amount of interstitial matrix components, including fibronectin and type I and type III collagen [63], but their origin remains an area of controversy [41]. 3) Upon activation by pro-fibrotic cytokines and regardless of their different origins, fibroblasts will proliferate and start producing a large amount of ECM components [63].

4) Renal hypoxia caused by microvascular rarefaction is also an important factor in fibrosis [63,65]. Hypoxia can lead to tubular epithelial-mesenchymal transition (EMT) or apoptosis, it can activate resident fibroblasts and impair peritubular capillaries, leading to a cycle of chronic hypoxia and progressive kidney failure [63]. Chronic hypoxia often coexists with increased oxidative stress and generation of reactive oxygen species (ROS) [63]. Alteration in mitochondrial metabolism of tubular

epithelial cells leading to the production of ROS and oxidative stress and apoptosis has been shown to promote fibrosis [66,67]. TEC can become arrested in the G2/M phase of the cell, which is associated with excessive production of pro-fibrotic growth factors and cytokines that promote inflammation and fibroblast activation and thereby fibrosis [68]. Arrested tubular cells cannot regenerate in order to replace the lost cells, leaving space for the proliferation of fibroblasts and deposition of ECM [69]. Interstitial inflammation, tubular epithelial cell apoptosis, oxidative stress and EMT can finally lead to tubular atrophy, a hallmark of CKD [70].

IgA nephropathy

IgA nephropathy (IgAN) is the most common glomerulonephritis in the world [71], and although in most cases the clinical course of IgAN is benign, some patients progress to kidney failure. A study using 901 patients with IgAN estimated that up to 26.8% have a 10-year risk of kidney failure or 50% reduction in eGFR [72]. Although some clinical and histological aspects have been linked to poorer outcome, the mechanism of progression is not fully understood. Progression in IgAN follows the pattern of other chronic kidney diseases and, thus, it is generally believed that IgAN can work as a model disease for several other glomerular diseases. The most important risk factors for progression of IgAN include established renal insufficiency at time of diagnosis, proteinuria and hypertension [73-75]. The risk factors for progression of IgAN can divide patients into high-risk and low-risk groups for progression, but there is a large group with moderate risk for progression, for whom we miss good prognostic markers [75]. More knowledge is therefore needed about the mechanisms for progression and risk markers for progression.

Epidemiology and clinical aspects

IgAN typically affects young adults and is more prevalent in men than women. Sice the diagnosis require a kidney biopsy, the true prevalence of IgAN is not really known [71]. The clinical presentation varies from asymptomatic microscopic hematuria to sustained proteinuria and rapid deterioration of renal function. Children and young adults usually present with macroscopic hematuria 1-2 days after upper respiratory or gastrointestinal illness, while adults can usually present with proteinuria, microscopic hematuria, or hypertension [11]. Nephrotic syndrome is uncommon.

Diagnosis

Histological aspects

Definitive diagnosis of IgAN is established by kidney biopsy. The diagnostic hallmark is the predominance of IgA deposits, either alone or with IgG, IgM, or both, in the glomerular mesangium, as demonstrated by immunofluorescence [71]. Members of the

complement system such as complement C3, properdin, C4 or C4d, mannose-binding lectin, and terminal complement complex (C5b–C9) are also frequently detected [71].

The histological patterns of IgAN on light microscopy can vary significantly. Focal or diffuse mesangial proliferative glomerulonephritis are the most common histological lesions, but the histology can vary from no detectable histologic lesion to diffuse proliferative and crescentic glomerulonephritis [11]. Crescents are also common and correlate with the risk of progression [76]. Tubulointerstitial lesions don't differ from those seen in other forms of progressive glomerulonephritis [77] and vary from normal findings to diffuse tubular atrophy and interstitial fibrosis.

The Oxford classification of IgAN provides a histo-pathological grading system for IgAN based on four variables: mesangial hypercellularity (M); (endocapillary hypercellularity (E); segmental glomerulosclerosis (S) and tubular atrophy/interstitial fibrosis (T) which are forming the MEST score [78]. In 2016, the MEST score was updated and the C for crescents was added [79] (Table 1). Biopsies with a minimum of 8 glomeruli are required, in order to classify according to the MEST score. The MEST-C score serves also as a prognostic tool as discussed further in the thesis.

Table 1. Oxford classification of IgAN. Modified with permission from Macmillan Publishers Limited, Nature Reviews Nephrology, Ian S D Roberts [80]

Histological variable	Definition	Score
Mesangial hypercellularity (M)	More than four mesangial cells in any mesangial area of a glomerulus	M0 mesangial cellularity <50% of glomeruli M1 mesangial cellularity in ≥50%
Endocapillary proliferation (E)	Hypercellularity due to an increased number of cells within glomerular capillary lumina	E0 absence of hypercellularity; E1 hypercellularity in any glomeruli
Segmental glomerulosclerosis (S)	Adhesion or sclerosis (obliteration of capillary lumina by matrix) in part but not the whole glomerular tuft	S0 absence of segmental glomerulosclerosis, S1 presence of segmental glomerulosclerosis in any glomerulus
Tubular atrophy/interstitial fibrosis (T)	Estimated percentage of cortical area showing tubular atrophy or interstitial fibrosis, whichever is greater	T0 0-25%; T1 25-50%; T2 >50%
Crescents (C)		C0 (no crescents), C1 (crescents in a least 1 but <25% of glomeruli), or C2 (crescents in at least 25% of glomeruli)

Indication for kidney biopsy

Renal biopsy is the gold standard for diagnosis of IgAN, but there are currently no internationally accepted guidelines for the indications of performing a biopsy. There is not an international consensus about the clinical indications of the procedure and the decision is usually based on single-center policies and personal opinion [81]. Kidney biopsy will usually be reserved for patients with signs of more severe or progressive disease, such as persistent urinary protein excretion of at least 1g/day or elevated serum creatinine concentration. Norway has a quite liberal biopsy policy due to low complication rate [82].

Differential diagnosis

IgAN cannot be distinguished from Henoch–Schönlein purpura nephritis from biopsy. The clinical difference is the concurrent presence of palpable purpura, due to leukocytoclastic vasculitis with IgA in the walls of dermal capillaries [71]. The histological similarities have led to the assumption that the two entities represent

different ends of the clinical spectrum characterizing a single disease [83]. Alport syndrome and thin basement membrane nephropathy (TBMN) can also present with persistent isolated hematuria. While Alport syndrome is often accompanied by hearing impairments and ocular defects, for TBMN approximately one-half of first-degree relatives have hematuria. The difference between IgAN after an episode of upper respiratory tract infection (URTI) and acute poststreptococcal glomerulonephritis lies in the onset of hematuria. While in IgAN the hematuria is typical observed in 1 to 3 days after URTI, in acute poststreptococcal glomerulonephritis, macroscopic hematuria is typically observed 1 to 2 weeks after an episode of pharyngitis or tonsillitis.

Pathogenesis

The pathogenesis of IgAN has been described as a multi-hit mechanism (Figure 3). First hit is characterized by aberrant glycosylation of IgA1 (lacking galactose on some O-glycans). Second hit is characterized by synthesis of antibodies directed against galactose-deficient IgA1. Formation of immune complexes between galactose-deficient IgA1 occurs and anti-glycan/glycopeptide antibodies, constitutes the third hit. Accumulation of these complexes in the glomerular mesangium to initiate renal injury constitutes the forth hit (Figure 3) [84].

The glomerular IgA1-containing immune complexes cause local activation of the complement system [85] and release of proinflammatory and profibrotic mediators (IL-6, transforming growth factor β (TGFβ), tumor necrosis factor α (TNFα) and monocyte chemo attractant protein (MCP-1)), leading to mesangial injury. Through mesangio-podocyte crosstalk [86], the glomerular IgA1-containing immune complexes lead to podocyte injury characterized by cytoskeletal disorganization and loss of adhesiveness, foot-process effacement and podocyte loss [87]. TNF-alpha released from the mesangium after IgA deposition has also been shown to activate renal tubular cells through mesangial-tubular crosstalk [88].

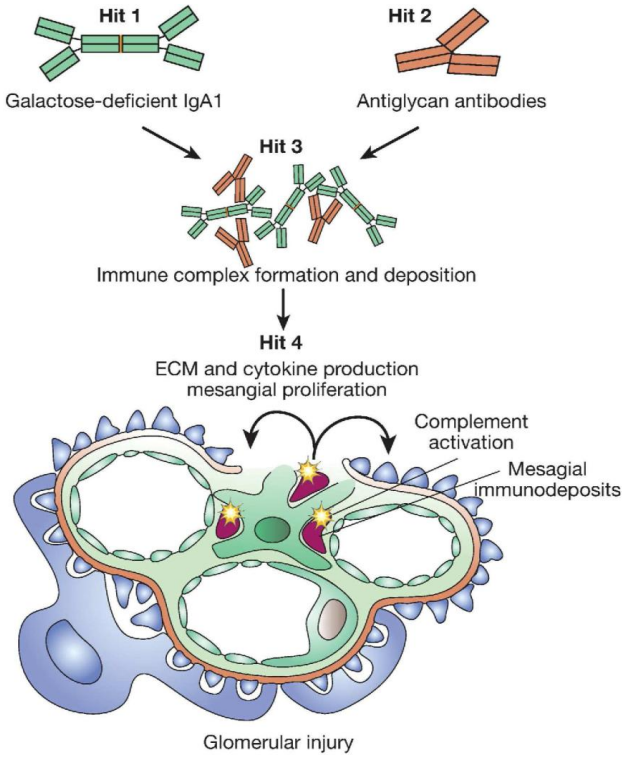

Figure 3. The multihit pathogenesis model of IgA nephropathy. Increased production of galactose-deficient IgA1 (Hit 1); formation of autoantibodies that recognize galactose-deficient IgA1 (Hit 2); formation of pathogenic immune complexes (Hit 3); deposition of pathogenic immune complexes in the mesangium, activation of mesangial cells, and induction of glomerular injury (Hit 4). With permission from Magistroni et al. 2015

Genetic factors

Several studies have reported familial clustering of IgAN, suggesting an autosomal dominant inheritance with various penetrance [89,90]. Genome-wide association studies (GWAS) led to the identification of 15 distinct genetic loci that correlated with the disease risk, including antigen processing and presentation (MHC region), the complement system (CFHR1/3 and ITGAM-ITGAX loci), regulation of mucosal IgA

production (TNFSF13 and LIF/OSM loci) and innate immunity against pathogens (DEFA, CARD9, ITGAM-ITGAX and VAV3 loci) [91-94].

Prognostic factors

Clinical prognostic factors

Several clinical prognostic factors have been identified for poor outcome, including increased serum creatinine concentration, impaired GFR, sustained hypertension, and proteinuria [95].

Renal function

Impaired renal function at the time of biopsy is a risk factor for kidney failure [96,97]. A Japanese study on 2269 patients showed that the risk of dialysis after 7 years from diagnosis was 90% in patients with creatinine above 220 µmol/l, compared to 2.5% in patients with creatinine level less than 110 µmol/l. In one study, eGFR at presentation was the strongest predictor of kidney failure [98].

Blood pressure

Hypertension at the time of biopsy is an independent risk factor for progressive disease. Uncontrolled hypertension during follow-up is associated with greater proteinuria and predicts a faster GFR decline [99,100]. According to the 2021 KDIGO guidelines, a target systolic blood pressure <120 mmHg is recommended in most adults patients with glomerulonephritis (GN) [101]. The blood pressure at diagnosis is included in the International IgAN Prediction Tool [102].

Proteinuria

According to the KDIGO guidelines published in 2012, proteinuria is the strongest prognostic factor in IgAN and has a dose-dependent effect that is independent of other risk factors [103]. Proteinuria is a recognized predictor of outcome in IgAN [104] both at the time of biopsy [96], but also on repeated measurements over time (proteinuria exposure over time/time-average proteinuria), the latter being a stronger predictor of the rate of renal function decline [105]. In addition, an analysis of 445 patients with IgAN from the Toronto glomerulonephritis Registry demonstrated that, at any given

timepoint within the first 10 years after biopsy, the current value of proteinuria is a better predictor of a 50% decline in eGFR or kidney failure than time-averaged proteinuria [106].

Histological prognostic factors

The Oxford classification of IgA nephropathy, or the MEST score, published in 2009, consisted of four histological features that could predict the clinical outcome [107]. Later, a fifth feature - crescentic lesion (C) - was added to the score [79]. Is been showed that M, S and T lesions each provide prognostic information by univariate analysis, although only T lesions were a consistent, independent predictor of renal outcomes, with more variable results for M and S [79]. E1 is predictive of outcome only in patients who have not received immunosuppression [108]. Other histological prognostic factors include deposits of IgG [109] and Cd4 [110,111]. The location of IgA are predictive of outcome. For example, glomerular capillary wall deposits may carry a worse prognosis than mesangial deposits alone [112].

The presence of tip lesions, podocyte hypertrophy, and adhesions correlate with higher proteinuria at the time of biopsy [113] and a more rapid decline in renal function.

Urinary and serum biomarkers

In the past years, several serum and urinary potential biomarkers have emerged. Serum galactose-deficient IgA1 (Gd-IgA1) [114] and CD89 complexes [115] have been associated with disease progression. Patients with IgAN have increased levels of serum C4a were compared to healthy controls [116], while decreased circulating C3 levels in IgAN patients were shown to be a risk factor for progression [117].

Urine EGF/MCP-1 ratio [118], KIM-1 [119], urinary mannose-binding lectin [120] have all been associated with poor renal outcome. According to one study, urine podocyte mRNAs correlated with disease activity [121]. In another study, urinary free κ light chains and laminin G-like 3 concentrations inversely correlated with the severity of clinical and histological [122]. Urinary concentration of transferrin receptor was found to be increased in patients with IgAN [123]. Currently there are no validated prognostic serum or urine biomarkers for IgAN other than eGFR and proteinuria [101].

The International IgAN Prediction Tool is an online-based tool that includes estimated GFR at time of biopsy, systolic and diastolic blood pressure, proteinuria at time of biopsy, age, race, MEST score and immunosuppression use at or prior to biopsy. The tool was designed to predict the risk of a 50% decline in eGFR or kidney failure after biopsy [102]. According to the 2021 KDIGO guidelines, the tool is a important resource to quantify risk of progression [101].

Treatment

As with most kidney diseases, there is no specific/curative treatment for IgAN and the objective of therapy is to delay the progression to kidney failure. No specific medical treatment is required for patients with isolated hematuria, but a long-term follow-up is advised for all patients, since it has been shown that even patients with assumed benign IgAN can have a progressive course [124]. Deciding which treatment is best suited for the patient can be a challenge and the discovery of biomarkers that will be able to better identify and stratify patients at risk would allow for better treatment with less possible side effects.

Supportive therapy

An optimal supportive regimen constitutes the cornerstone of therapy for IgAN patients and consists of management of proteinuria and hypertension.

Lifestyle advice, such as information on dietary sodium restriction, smoking cessation, weight control, and exercise are important. Other than dietary sodium restriction, no specific dietary intervention has been shown to alter outcomes in IgAN [101].

The KDIGO guidelines recommend initial therapy with either an ACEi or ARB if the patient has proteinuria >0.5 g/day, irrespective of whether they have hypertension [101].

Non-established/controversial supportive therapy in IgAN consisting of fish oil, antiplatelet and anticoagulant drugs and tonsilectomy [125] have no place in the treatment of IgAN, but tonsillectomy can be considered for Japanese patients with IgAN as recommended by KDIGO [101].

Renin-angiotensin system blockers

Blockade of the renin-angiotensin-aldosterone system (RAS) with ACEi and ARB are well-established components of supportive therapy in IgAN.

There is evidence of early activation of the renin-angiotensin system (RAS) in the kidneys of IgAN patients [126,127] and receptors for Angiotensin II are found on both mesangial cells (inducing mesangial matrix production and sclerosis) [128] and tubular cells [129]. In addition to its effect on blood pressure, RAS blockade has a well-established antiproteinuric effect, which is also seen in IgAN patients [130,131]. The KDIGO guidelines recommend long-term treatment with RAS-blockade when proteinuria is over 0.5 g/day, irrespective of whether they have hypertension or not [101]. Up to date, there is no evidence of superior therapeutic effect of ACEi compared to ARB or vice versa, concerning renal outcome in IgAN. The role of combining an ACEi with an ARB is uncertain and KDIGO recommends avoiding any combination of ACEi, ARB, and direct renin inhibitor (DRI) therapy in patients with CKD [132].

Immunosuppressive therapy

Steroid use in IgAN is still controversial. In the STOP-IgAN trial, immunosupressive treatment transiently reduced proteinuria over 3 years, but had no impact on eGFR and only resulted in significant, particularly infectious, adverse events [133]. Another trial (TESTING Low Dose Study -TESTING), which randomized patients to 6 months of steroids or placebo, was terminated early after an interim analysis revealed a high risk of infectious serious adverse events including lethal Pneumocystis jirovecii pneumonia [134]. There was a significant reduction in the risk of kidney failure in the steroid group and the beneficial impact was similar in patients with eGFR > or <50 ml/min per 1.73 m^2.

Other clinical trials also showed a benefit of immunosuppression at lower eGFR, but with an increased risk of adverse events [135,136]. Current guidelines recommend immunosuppressive drugs only in patients with IgAN who remain at high risk of progressive CKD (defined as proteinuria >0.75–1 g/day despite 90 days of optimized supportive care) [101]. These patients should be considered for a 6-month course of

glucocorticoid therapy, along with prophylaxis against *Pneumocystis pneumonia*, gastroprotection and bone protection [101].

Special situations/ Variant forms of IgAN

IgA deposition with minimal change disease (MCD), IgAN with acute kidney injury (AKI), and IgAN with rapidly progressive glomerulonephritis (RPGN) require specific and immediate treatment according to KDIGO 2021 [101].

IgA deposition with MCD

Patients with kidney biopsy indicative of mesangial IgA deposition accompanied by light and electron microscopy features otherwise consistent with MCD should be treated in accordance with the guidelines for MCD [101].

Rapidly progressive IgAN

Rapidly progressive IgAN (RPGN) is defined as a 50% decline in eGFR over 3 months, where other causes of RPGN have been excluded [101]. Patients with rapidly progressive IgAN should be offered treatment with cyclophosphamide and glucocorticoids in accordance with the guidelines for Antineutrophil Cytoplasmic Antibody–Associated Vasculitis [101].

Other immunosuppressive therapy

There is conflicting information for the use of **mycophenolate mofetil (MMF)** in the treatment of IgAN. While some studies showed no benefit [137,138], another Chinese study [139] showed complete proteinuria remission with MMF.

A combined treatment regimen of **cyclophosphamide** and corticosteroid on a small IgAN patient population with crescentic disease and rapidly progressive clinical course showed benefits on renal function, but serious side effects [140], while no benefit was seen in chinese patients with crescentic IgAN [141].

Other studies

Trials of **rituximab** and **tacrolimus** have had negative results [142,143].

Spleen tyrosine kinase (SYK), an important molecule involved in the production of proinflammatory cytokines, has been shown to be involved in the pathogenesis of IgAN [144]. A phase II randomized, double-blind, placebo-control trial on 76 patients was finished in 2019 showing a reduction in proteinuria after 24 weeks of treatment [145].

Targeted-release formulation of budesonide (TRF-budesonide). The unique formulation of TRF-budesonide allows the delivering of the drug directly to the distal ileum with the aim of directly targeting Peyer's patches [146].

The NEFIGAN Study, a randomized, double-blind, placebo-controlled Phase 2b trial, demonstrated a significant reduction in proteinuria after 9 months treatment with TRF-budesonide in patients already receiving maximal supportive care (RAS inhibition) [147].

The NefIgArd trial is an ongoing Phase 3, randomized, double-blind, placebo-controlled, multicentre study aiming at evaluating the risk–benefit profile of TRF-budesonide (at a dose of 16 mg/day) compared to placebo [148]. The primary endpoint, based on the first 199 patients treated for 9 months, showed a statistically significant 7% (3.87 mL/min/1.73m^2) treatment benefit on eGFR (p=0.0029), compared to placebo. There was a similar overall incidence of adverse events in the two treatment groups [148].

Complement Inhibitors

In recent years, complement inhibitors have shown promising results as therapeutic options for IgA nephropathy patients. I will describe the different complement inhibitors in this chapter on treatment options, but the complement system will be described in the next chapter and some readers might want to read this first.

Inhibition of the Alternative Pathway

Factor B Inhibitor: *Iptacopan* (LNP023) and IONIS-FB-LRx

Iptacopan is a selective factor B inhibitor of the alternative complement. A phase II primary endpoint results for investigational Iptacopan in IgAN

demonstrated effective and clinically meaningful reduction of proteinuria [149]. A phase III study APPLAUSE (NCT04578834) is currently underway to investigate whether LNP023 could delay disease progression and improve clinical outcomes [149].

IONIS-FB-LRx is an antisense inhibitor of factor B messenger ribonucleic acid (mRNA). A phase II, single-arm open-label clinical study (NCT04014335) is still evaluating the efficacy and safety of IONIS-FB-LRx in adults with primary IgAN.

Anti-C3: Compstatin and APL-2

Compstatin inhibits the activation of C3. APL-2 is a pegylated derivative of compstatin that binds C3 and prevents cleavage to C3a and C3b by C3 convertase [150]. A phase II study to evaluate the safety and biologic activity of APL-2 in patients with IgAN, lupus nephritis, primary membranous nephropathy, and C3 glomerulopathy is still ongoing (NCT03453619) [150].

Inhibition of the Lectin Pathway

MASP-2 Inhibitor: OMS721 (Narsoplimab)

Narsoplimab is a human monoclonal antibody targeting mannan-binding lectin-associated serine protease-2 (MASP-2) of the lectin pathway of the complement system. A stage 2 phase trial showed that Narsoplimab was well-tolerated and correlated with a substantial (61.4%) reduction in 24 h albuminuria excretion and a stable eGFR at 31–54 weeks of treatment, in eight patients with advanced IgAN [151]. A phase III, double-blind, randomized, placebo-controlled study is going to evaluate Narsoplimab in patients with IgAN with albuminuria > 1 g/day (NCT03608033), with results expected in 2023.

Inhibition of the Terminal Pathway

Anti-C5a Receptor Inhibitor: Avacopan

Avacopan is a small oral C5a receptor inhibitor that has been investigated in patients with IgAN on stable RAAS blockade (NCT02384317) in a Phase II trial. The drug led

to an improvement in urine protein to creatinine ratio or urine albumin to creatinine ratio of ~50% at week 12 [152].

Anti-C5 Monoclonal Antibody: Eculizumab and Ravulizumab

Eculizumab is a recombinant humanized monoclonal antibody (IgG2/4) that selectively inhibits C5, thus preventing C5a release and formation of the membrane attack complex (MAC and C5b-9) [150]. Eculizumab has been used only in isolated cases as "rescue therapy" in young patients with progressive IgAN who did not respond to immunosuppressive drugs and led to stabilization of renal function and proteinuria [153].

Ravulizumab, a long acting C5 antagonist Ravulizumab in IgAN is currently under evaluation in preclinical studies [154].

C5 Suppression by RNA Interference: ALN-CC5 (Cemdisiram)

Cemdisiram is a synthetic RNA interference (RNAi) which suppresses C5 production, thus reducing terminal complement pathway activation [150]. A phase II, double-blind, randomized, placebo-controlled study (NCT03841448) is ongoing and aims to evaluate the safety and efficacy of Cemdisiram in patients with IgAN and >1 g/day proteinuria.

The complement system

Complement activation occurs through three different pathways (Figure 4): alternate, classical and lectin [155-158]. The proteins of the complement system exist mostly as inactive zymogens that need to be converted to active enzymes that further activate new substrates. The activation of complement occurs both in tissue and in blood. The three activation pathways are triggered differently and the first steps are different, but they converge to a common terminal pathway. The complement system has more than sixty components and activation fragments, including nine central components of the cascade (C1 to C9) and their activation products, regulators and inhibitors, proteases and newly assembled enzymes or receptors for effectors molecules [159] (Figure 4).

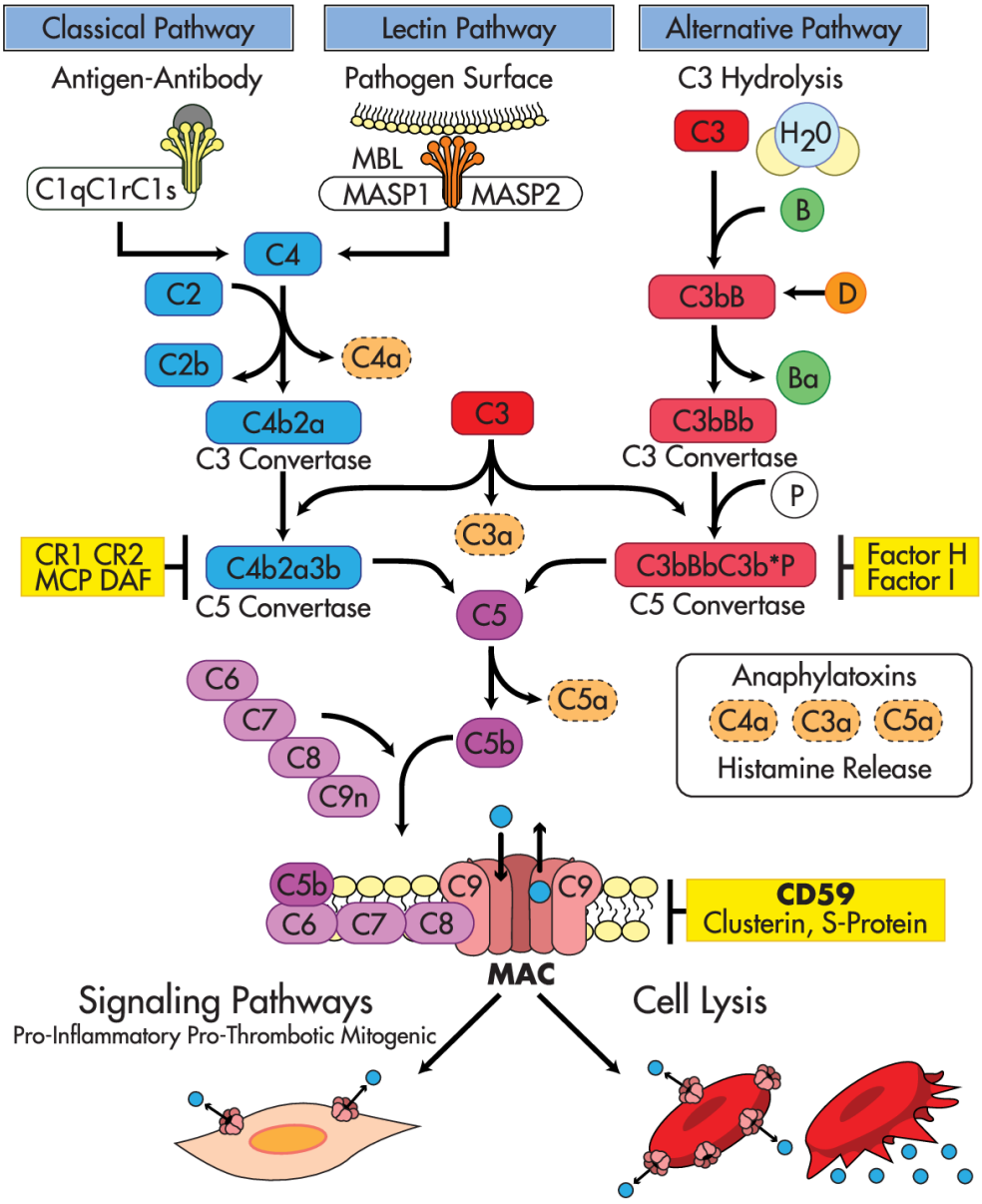

Figure 4. Complement activation through the three different pathways: alternate, classical and lectin. With permission from Ghosh P et al 2015 [160]

The Classical pathway (CP) activation of complement is triggered when C1 binds to the Fc region of an antibody from an antibody-antigen (Ab-Ag) complex. C1r and C1s are serine proteases that are activated when C1q binds to an antibody Fc region. C1r is autoactivated and cuts itself and C1s activating it. C1s than cleaves and activates C2 and C4. C4 is cleaved into a small fragment, C4a which diffuses away, and a large fragment, C4b. C4b that is bound to the cell, can act as a ligand for C2 which will be then split by C1s into C2a (which remains bound to C4b to form a C3 convertase (C4bC2a)) and C2b. The C3 convertase cleaves C3 to release C3a and C3b. From here, there is a common pathway for all three complement pathways: C3b together with C4bC2a (C3 convertase) form C4bC2aC3b also known as C5 convertases, which further cleave C5 to form C5a and C5b. C6 and C7 bind to C5b and C8 and multiple molecules of C9 to form the membrane attack complex (C5b-9, MAC) also called the terminal complement complex (TCC). The MAC complex forms a pore by inserting itself into cell membranes, resulting in cell lysis. Factor I can also cleave C4b which is bound to the cell, generating an inactive form of C4b, called inactive C4b (iC4b). Further cleavage of iC4b by factor I results in two fragments, soluble C4c, and surface-bound C4d [161]. C4d has no known biological function, but, due to its stable covalent bonds to the site of complement activation, it has been used as a marker for AMR (antibody mediated rejection). It has been shown that one C1q molecule is able to cleave 28 molecules of C4b [162], which would be able to generate just as many molecules of C4d. This amplification together with the fact that C4d bonds covalently to the site of complement activation, makes it an attractive marker for complement activation.

The Lectin pathway (LP) of complement is initiated by the binding of pattern recognition molecules from subfamilies of collectins and ficolins to carbohydrates on the surface of microorganisms or altered self-cells. Six recognition molecules have thus far been reported to be able to activate the lectin pathway: Mannose Binding Lectin (MBL), Collectin-10 (CL-10), Collectin-11 (Collectin kidney 1, CL-11), Ficolin-1, Ficolin-2 and Ficolin-3 [163]. Both MBL and Ficolins circulate in the serum as complexes with MBL-associated proteins (MASPs) [164]. Upon binding of the

complex to a specific ligand, MASP2 gets autoactivated, which cleaves C4 into C4a and C4b. C4b attaches to the surface of the pathogens or cell, inducing C2 to bind, which will further be cleaved by MASP2 to form C2b and C2a. C4b together with C2a has enzymatic activity and forms the C3 convertase for the lectin pathway, C4bC2a.

The Alternative pathway (AP) is an antibody-independent route for complement activation. Even in the absence of a pathogen, the alternative pathway maintains a constant activation (fluid phase activation) by a process known as tick-over. Tick over occurs through the spontaneous cleavage of the thioester bond in C3 to form C3(H_2O) [165]. The hydrolyzed C3 allows the binding of plasma protein Factor B. The C3(H_2O)-bound factor B (FB) is then cleaved by Factor D (FD), allowing formation of a fluid phase C3 convertase complex C3(H_2O)Bb. The Ba fragment is released into the fluid phase. During normal physiological conditions, this C3 convertase constantly generates small amounts of C3b, which is ready to bind to the pathogen, in case of infection. On host cells, bound C3b molecules are rapidly inactivated by complement regulators [165]. On pathogens that lack specific regulators of complement, C3b interacts with FB and FD to form a surface-bound C3 convertase (C3Bb) as part of the AP, which cleaves more C3 into C3a and C3b [165], in self-amplification and generation of the C5 convertase C3bBbC3b. The convertase can be stabilized by the binding of the only positive complement regulator - properdin (Factor P) [166]. Although the classical and the lectin pathway have critical role in pathogen recognition and initiation of the complement cascade, it has been suggested that AP insures more than 80% of the terminal complement activity during pathogen recognition [167].

Complement system and IgAN

The role of the complement system in IgAN was long suspected, given that complement components factors C3, properdin and factor H have been commonly detected in renal biopsy specimens [168,169]. Early studies showed that both the lectin pathway [170] and the alternative pathway [171] are involved in the pathophysiology of IgAN. Although deposits of C1q have been found in patients with IgAN [172], activation of the classical pathway was suggested to occur later in the damaged kidney and not being specific for IgAN [85].

Lectin pathway in IgAN

It is believed that that LP activates complement in IgAN, and that the classical pathway is unlikely to contribute to pathogenesis [173], since studies have not identified C1q in biopsies of IgAN patients, but rather mesangial C4 (and particularly C4d).

It has been shown that, in IgAN, mesangial deposits of MBL are co-localised with deposits of C4d [174]. In addition, glomerular deposition of MBL and L-ficolin was associated with more pronounced histological damage, as well as with significantly more proteinuria in IgAN [175]. A previous *in vitro* study showed that IgA purified from pooled normal human serum binds MBL-MASP complexes and concluded that, in the absence of classical pathway activity, it can activate complement LP [176].. Positivity for C4d was shown to be associated with a worse prognosis in IgAN [111]. Recently, an investigation of the deposits of glomerular complement factors by immunofluorescence has shown that 63% of IgAN patients had the deposition of at least one initiator of lectin pathway accompanied with C4d deposition, indicating the activation of the lectin pathway [177].

Alternative pathway in IgAN

C3 deposits are present in biopsies from IgAN patients [117,173,174]. It has classically been believed that the AP is the main complement cascade activator in IgAN and is principally responsible for C3 deposition [173]. The assumption is also based on the fact that since C1q was not found in IgAN biopsies, the presence of C3 implies AP activity [173]. Components of the AP such as properdin [174] and factor H

[178,179] were found in the renal biopsies of IgAN patients. Recent GWAS of IgAN have identified protective associations within the CFH locus on chromosome 1q32, where deletion of the FHR1 and FHR3 genes (*delFHR3-R1*) associated with protection from IgAN [180]. Studies have also shown that plasma levels of FHR1 were significantly higher in IgAN patients than controls, with a negative correlation between FHR1 levels and eGFR [181,182].

Mesangial deposits of the terminal pathway of complement (C5b-9) have been found in IgAN patients [174,183].

In conclusion, the complement and its different components seem to play an essential role in the pathology and prognostic of patients with IgAN.

Proteomics

Proteomics is the study of the proteome and it consists of structural, functional and quantitative protein studies. The terms “proteomics” and “proteome” were first used by Marc Wilkins and colleagues in the early 1990s and mirror the terms “genomics” and “genome,” which describe the entire collection of genes in an organism [184]. Rapid technical advances in the field of proteomic, allow to date, large-scale, high-throughput analyses for the detection, identification, and functional investigation of proteome [185]. In the last years, liquid chromatography (LC) coupled to mass spectrometry (LC-MS) has become the standard proteomic method, allowing the quantification of thousands of proteins.

Proteins and amino acids

The human genome has 19000–21000 protein encoding genes [186], each coding for one or more proteins, with a total number of human proteins been estimated to be close to one million [185]. Proteins are made of amino acids (AA). An AA consists of a central carbon atom linked to an amino group, a carboxylic acid group, a hydrogen atom, and a distinctive side chain that is different for each AA. The amino and carboxyl groups of amino acids can react in a head-to-tail fashion, eliminating a water molecule, forming peptides. A protein is often defined as a peptide chain of more than 50 AAs. Each type of protein differs in its sequence and number of amino acids.

Protein analysis

Extracting proteins from biological samples

Full length proteins are too large for effective chromatography and mass spectrometry (MS). In order to analyse tissue by MS, the mixtures of proteins must be separated into less complex mixtures of peptides. The tissue sample is disrupted (by pulverization, sonication etc) to yield a “soup” that contains cells, subcellular components, and other biological debris [187]. Proteins are then extracted from this soup by a number of procedures with the help of detergents, reductants, denaturing agents or enzymes [187]. Once the proteins are extracted from the sample tissue, they need to be cleaved into peptides to be further analyzed by MS. This is done by protein "digestion".

Trypsin, a serine proteinase, is the most widely used protease for protein digestion. Peptide fragments of about 6–20 amino acids are ideal for MS analysis and database comparisons [187]. After the proteins are cleaved, the resulting peptide mixtures must be cleaned, desalted and concentrated, before separated prior to analysis. The most used technique for peptide separation is reverse-phase chromatography (RPC).

Liquid chromatography–mass spectrometry

Liquid chromatography is a method used to separate peptides. A Liquid Chromatograph consists of 1) a column with a mobile phase - a liquid used to move the samples; 2) an injector which introduces the sample into the flow stream of the mobile phase 3) a pump which is used to force the mobile phase through the liquid chromatograph at a specific flow rate and 4) the column's stationary phase which separates the sample components of interest using various physical and chemical parameters.

Reversed phase liquid chromatography (RPLC) is a type of high-performance liquid chromatography (HPLC) that utilizes a nonpolar stationary phase (most frequently a hydrocarbon chain chemically bonded to porous silica particles) and a polar mobile phase constituted by water and at least a water-miscible organic solvent [188].

In proteomics, the RPLC column binds peptides, which are released in a gradient of increasing concentration of an organic solvent, e.g. acetonitrile (ACN). The peptides separated by RPLC are then ionized by electrospray and transferred into the mass spectrometer for direct analysis, so-called online coupling [189].

The mass spectrometer consists of an ion source, a mass analyzer and a detector. The mass analyzer measures the to-charge ratio (m/z) of the ionized analytes and the detector registers the number of ions at each m/z value.

In proteomics, MS measures the mass and charge of peptides. AAs that make up a peptide have distinct masses, therefore the measured peptide mass (calculated from the m/z multiplied by the charge) can be used to deduce the AA composition of the peptide, and ultimately the protein from which that peptide is derived by using a

database. The problem with complex mixtures of samples is that the possibility that more than one peptide from a peptide database fits the measured peptide mass is relatively large, therefore tandem mass spectrometry is a more appropriate method for analyzing complex mixtures. Tandem mass spectrometry (MS-MS) is a MS with an additional mass analyzing step, thus increasing its abilities to analyze complex samples. In MS-MS, peptides are further fragmented into smaller molecules and the fragmented molecules can be pieced back together to identify the exact amino acid sequence of a parent peptide [190]. The instrument's first records are referred to as MS1 or survey spectrum and the second mass spectral scan is referred to as MS2 or MS/MS. The peptide mass and fragment masses are used to identify the peptide sequence, and the intensity can be used for quantification [191]. The data from MS/MS can be represented as mass spectrum which is an intensity vs. m/z (mass-to-charge ratio) plot.

Quantitative proteomics

Because the MS cannot readily quantify the amount of proteins, several strategies can be used to assist MS for protein quantitation [192]. These strategies can be divided based by the method of quantitation: labeling versus label-free; and also by the quantitative goal: relative versus absolute quantitation [192]. Absolute quantitation determines changes in protein expression in terms of an exact amount or concentration (e.g. ng or nmoles/g of tissue, or ng or nmoles/ml of plasma), whereas relative quantitation determines the up- or down-regulation of a protein relative to the control sample, and the results are expressed as fold increases or decreases [193].

Label-based quantitation methods utilize stable isotope labels which are incorporated within the peptides. This labels have alternative different mass allowing detection based on specific change in mass **[194]**. In labelled quantitation samples of each condition are combined prior to the MS and analyzed at the same time. This offers little experimental bias, but the technique is costly and time consuming.

Label-free quantification uses commonly two types of strategies: 1) spectral counting—counting and comparing the number of fragment spectra and 2) peptide

chromatographic peak intensity measurements—measuring and comparing the chromatographic peaks of peptide precursor ions belonging to a specific protein [195]. In label free proteomics, each sample is analyzed by the mass spectrometer separately using the same protocol [196]. A disadvantage of the label free approaches is that is more prone to errors introduced by the measurement conditions (e.g. temperature, experimenter) and thus require strict sample processing [197]. The advantages are that is more cost effective and less time consuming.

Data analysis

Mass spectrometry produces various types of data, such as the mass spectrum, an intensity vs. m/z (mass-to-charge ratio) plot. Further analysis of these data is done with the help of specially designed programs, such as Progenesis and MaxQuant, which process this raw data from MS. The MaxQuant software consists of a set of algorithms that can identify and quantify proteins from large-scale mass-spectrometric data sets. Peptides are identified by matching the MS/MS spectra against a sequence database [198]. For each protein, several peptides are measured and each contribute to the overall identification [191]. To avoid false positive protein identification, the program uses a target-decoy-based false discovery rate (FDR) approach. The FDR is determined using statistical methods that account for multiple hypotheses testing. In addition, the database search includes not only the target sequences, but also their reverse counterparts and contaminants, which helps to determine a statistical cutoff for acceptable spectral matches [199]. For statistical analyses of the MaxQuant output, one can use the Perseus framework software developed by the same group. The raw file from MaxQuant can be directly loaded into Perseus.

Prior to any statistical analysis, data cleansing is usually performed which includes normalization, to ensure that different samples are comparable, and missing value handling, to enable the use of methods that require all data points to be present [200].

Other online platforms that can further help in data analysis are for example Uniprot, Reactome (https://reactome.org/) and String (https://string-db.org/).

UniProt (www.uniprot.org) is a freely accessible database of protein sequence and functional information, many entries being derived from genome sequencing projects.

Reactome (https://reactome.org/) is a manually curated open-source open-data resource of human pathways and reactions. The latest version (04.03.2022) describes 11200 human proteins organized in 2,553 pathways. Reactome allows for the list of proteins that one can obtain from Perseus analysis to be organized in biological pathways. Examples of biological pathways in Reactome include Immune system, Metabolism of proteins, Transport of small molecules, etc. The number of total entities in the pathway are displayed alongside with the number of entities from uploaded protein list, P-values, FDR and reactions. P value represents the probability that the overlap between the query and the pathway has occurred by chance. FDR represents the probability corrected for multiple comparations. According to the website, the Reactome curation process for a pathway is similar to the editing of a scientific review. An external domain expert provides his/her expertise, a curator formalizes it into the database structure, and an external domain expert reviews the representation [201]. A system of evidence tracking ensures that all assertions are backed up by the primary literature [201]. The significance of each pathway is measured by calculating the probability that the observed number of differentially expressed genes in a given pathway were simply observed by chance.

STRING is an online database of protein-protein interactions. The interactions include direct/physical and indirect/functional associations [202]. Interactions in STRING are imported from the following main sources: other interaction databases, by prediction algorithm from known genomic interactions, by automated text mining of scientific literature and by systematic transfers of interaction evidence from one organism to another [203-205]. For each protein-protein association stored in STRING, a confidence score (between 0 and 1) is provided indicating the estimated likelihood that a given interaction is biologically meaningful, specific and reproducible, given the supporting evidence [203].

Objective/purpose of the study

Rationale

The rationale for this thesis is that IgAN is a common glomerulonephritis with a variable and difficult to predict course with risk of progression to kidney failure. It is generally believed that IgAN can work as a model disease for several other glomerular diseases and that risk markers of progression seem to be similar to those seen in chronic kidney disease in general. The risk factors for progression of IgAN can divide patients into high-risk and low-risk groups for progression, but there is a large group with moderate risk for progression for whom we miss good prognostic markers [75].

More knowledge is therefore needed about the mechanisms and risk markers for progression. IgAN is diagnosed by kidney biopsy and we therefore had available kidney biopsies for IgAN patients, from the time of their diagnosis.

Overall aims for the project were to:

- Identify novel tissue biomarkers for progressive IgAN that may be used for prognostication.
- Improve understanding of underlying mechanisms in IgAN.
- Separately investigate glomerular and tubular biomarkers in IgAN.

Methods

The Norwegian Kidney Biopsy Registry

The Norwegian Kidney Biopsy Registry was established in 1988 and has, since then, stored extensive clinical and histopathological data on almost all kidney biopsies that have been performed in Norway. The Norwegian Renal Biopsy Registry merged in 2016 with the Norwegian Nephrology Registry in one register named Norwegian Renal Registry (Norsk Nyreregister). In 2015, Renal Biopsy Registry had collected clinical and pathological data of about 13,000 non-neoplastic kidney biopsies[206]. Formalin-fixed paraffin embedded (FFPE) tissue have been stored for almost all these biopsies and, in about 50% of cases, the biopsies are stored at Haukeland University Hospital. Very important for the present project was the possibility to use the 11-digit national identity number to link with the former Norwegian Nephrology Registry. This enabled us to identify subgroups that did or did not progress to kidney failure.

Formalin-fixed paraffin-embedded tissue

The golden standard for preserving and storing tissue uses formalin fixation, followed by embedding in paraffin wax, to produce formalin-fixed paraffin-embedded (FFPE) archived samples [207]. Tissues were first immersed for 24–48 h in formalin stock (containing water, formaldehyde and methanol) and de-hydrated using a dimethyl-benzene (i.e. xylene) and ethanol series and embedded in paraffin wax [207]. The paraffin blocks were cut in thin sections (3-10 μm) and stained (e.g. haematoxylin & eosin) to allow light microscopy-based assessment of tissue morphology. For our study, 5 μm slices were mounted on special adhesive glass for laser microdissection of glomeruli and tubulointerstitial tissue.

Patients

The same cohort of patients was used for all three studies. The most common indication for biopsy was hematuria and proteinuria. The control group consisted initially of 20 patients, but for the first two studies, 2 patients were excluded because

there was not enough glomerular tissue on the biopsies and 3 samples were lost during preparation. In the third study, we used only patients with IgA nephropathy.

The inclusion criteria for **the control group** were:

- normal or minimal morphological changes in the kidney biopsy:
- eGFR >60 ml/min/1.73m^2;
- urinary protein <0.5 g/day;
- no development of kidney failure during a follow-up period of at least 10 years.

The IgAN group was divided into two subgroups, based on whether or not they progressed to kidney failure during a 10 year follow up. The IgAN group consisted initially of 29 patients (12 progressive and 17 non-progressive), but in the first two studies, three progressive IgAN patients were excluded because the biopsies were too small, and one non progressive sample was lost while preparing the tissue for proteomic analysis. For the third article, one progressive IgAN patient was excluded.

The inclusion criteria for the **non-progressive IgAN group** were:

- diagnosis of IgAN at kidney biopsy;
- eGFR >45 ml/min/1.73m^2;
- urinary protein > 1g/day;
- no development of kidney failure during a follow-up period of at least 10 years.

The inclusion criteria for the **progressive IgAN group** were:

- diagnosis of IgAN at kidney biopsy;
- eGFR >45 ml/min/1.73m^2;
- urinary protein < 3.5g/day
- development of kidney failure during the first 10 years after kidney biopsy.

The biopsies were performed between 1989 (one biopsy) and 2011. The clinical information such as GFR, creatinine, blood pressure and comorbidities were extracted from the Norwegian Kidney Biopsy Registry. Data on kidney failure were retrieved by

linking data with data from the Norwegian Renal Registry. The study was approved by the Regional Committee for Medical and Health Research Ethics (approval number 2013/553).

GFR measurement

Serum creatinine at the time of the biopsy was measured at the local hospital laboratories, using kinetic Jaffe method until 2005. Afterwards, the IDMS traceable enzymatic test was used instead. This methodological switch was done at slightly different time points at different hospitals. In our studies, creatinine values measured before 2005 ware recalculated based on a formula used by Hallan et al. to recalibrate creatinine to IDMS-traceble values [208]. We then calculated GFR based on the CKD-EPI equation [209]. For patients under 18 years old, we used the pediatric GFR calculator [210] based on Bedside Schwartz [211].

Urinary protein quantification

Urinary protein was quantified as g/day either from directly measured values, by calculation from reported urinary protein to creatinine ratio, or if only reported by urinary dipstick a negative dipstick was set to 0 g/24h, 1+ was set to 0.5 g/24h, 2+ was set to 1.0 g/24h and 3+ was set to 3.0 g/24h.

Laser capture microdissection

Microdissection allows the use of standard paraffin embedded tissue sections for separation of specific compartments from the surrounding area.

Using this technique, it is possible to separate the glomerular and tubular /tubulo-interstitial compartment, to analyse them separately. For the first two studies, eligible glomeruli (without global sclerosis, more than minimal focal sclerosis, crescents or fibrinoid necrosis) were laser micro-dissected (PALM MicroBeam, Zeiss) and pressure-catapulted into a tube cap (AdhesiveCap 500 clear, Zeiss). For each patient, we aimed to micro-dissect about 100 glomerular cross sections. For the third study, approximately 3 million μm^2 tubulointerstitial tissue were laser micro-dissected and

collected. Tubulointerstitial areas with tubular atrophy or areas with interstitial expansion were not microdissected.

Protein extraction and trypsin digestion

The protein extraction was done together with Kenneth Finne, who established the method and used it for investigation of glomerular and tubular damage in hypertensive rats [212]. In-gel protein digestion was done at PROBE Proteomic Unit at University of Bergen, using a local protocol. [213]

First, the micro-dissected FFPE tissue was suspended in lysis solution (0.1 M Tris pH 8, 0.1 M dithiothreitol (DTT), 4% sodium dodecyl sulphate). The suspended lysis solution was heated to 99 °C for 1h, and the resulting lysate was run on SDS-PAGE and further subjected to in-gel digestion (Trypsin Percine, Promega, Fitchburg, MO, USA) in a 1:20 enzyme/substrate ratio. Eluted peptides were desalted and cleaned using Oasis HLB peptides.

Liquid chromatography and tandem mass spectrometry

The samples were analyzed on a Q-Exactive HF (Thermo Scientific), connected to a Dionex Ultimate NCR-3500RS LC system. In short, the peptides were separated using reverse phase chromatography (PepMap RSLC, 25cm x 75 µm i.d. EASY-spray column, packed with 2 µm C18 beads) at a flow of 200 nL/min. The MS instrument was equipped with an EASY-spray ion source (Thermo Scientific) and was operated in data-dependent-acquisition mode. The 12 most intense eluting peptides were sequentially isolated, fragmented and analyzed in MS/MS-mode.

Label free quantification

The raw data were analyzed with the Progenesis LC-MS software (version 4.0, Nonlinear Dynamics, UK) using default settings. Features were exported from Progenesis and imported into Proteome Discoverer (version 1.4, Thermo Scientific), for protein identification using the SwissProt human database. For the third article, the MaxQuant software was used. The protein intensity data were imported to the Perseus software for statistical analysis.

Summary of main results

Paper I

Glomerular abundance of complement proteins characterized by proteomic analysis of laser-captured microdissected glomeruli associates with progressive disease in IgA nephropathy

In this paper, 16 patients with non-progressive IgAN, 9 patients with progressive IgAN and 15 control patients were included. We quantified 2018 proteins with 2 or more unique peptides. Of these, 231 proteins had statistically significant different abundance between progressive and non-progressive IgAN: 18 of these belong to the complement system; 17 complement proteins were more abundant and one (complement receptor 1, CR1) were less abundant. The complement components that had significantly increased abundance were C1q, C1r, C1s, C3, C4-B, C5, C6, C7, C8 and C9. Complement regulators also had increased abundance, including clusterin, factor H, factor H-related proteins 2, 5 and C4b-binding protein alpha chain.

Linear regression statistics between abundance of complement proteins and clinical variables in patients with IgAN showed that C1r, C1s, C5, C6, C8, C9 and clusterin had higher abundance with lower eGFR, and there were increased abundances of the C1r, C1s, C4, C5, C8, C9, factor H, factor H-related protein 3 and C4b binding protein alpha with increasing systolic blood pressure. The complement score was calculated based on the abundance of complement-related proteins.

In ROC analyses testing discriminatory ability for progressive vs non-progressive IgAN, AUC values were 0.91 ($p=0.001$) for the complement score using all significant proteins; 0.91 ($p=0.001$) for the complement score including complement components C5, C6, C7, C8 and C9 and 0.90 ($p=0.001$) when only including protein abundance of complement factor C7. In comparison, AUC value for the clinical variables systolic blood pressure was 0.580 ($p=0.5$) and for the variable 1/eGFR it was 0.74 ($p=0.054$).

Paper II

Characterization of glomerular extracellular matrix in IgA nephropathy by proteomic analysis of laser-captured microdissected glomeruli

In this study we investigated the glomerular extracellular matrix (ECM) proteome in IgAN (n=25) compared to control patients (n=15).

We identified a total of 2018 proteins and, by comparing our findings with earlier published studies (ref 1, ref 2), we found 179 ECM proteins which we further categorized in: glomerular basement membrane (GBM) proteins; other structural ECM proteins and ECM-associated proteins.

We quantified 25 basement membrane proteins, 31 structural ECM proteins and 123 ECM associated proteins. We analyzed staining patterns in the human Protein Atlas [214] and found that most of the proteins previously described as basement membrane proteins also showed positive staining in the glomerular mesangium. Most of the ECM-associated proteins were related to inflammation and immune response (e.g. azurocidin, myeloperoxidase, neutrophil elastase, cathepsin G, annexin A1, Protein S100-A9 etc), epithelial-mesenchymal transformation (Protein-glutamine gamma-glutamyltransferase 2, Protein S100-A4) and collagen synthesis (serpin H1, MMP2 and MMP9).

Periostin was the protein with the higher abundance both between IgAN and controls, but also between progressive vs non-progressive IgAN, suggesting it might be an important marker of glomerular damage in IgAN.

We concluded that IgAN is characterized by widespread proteome changes of the extracellular matrix and that proteins related to inflammation and fibrosis play an important role in the disease.

Paper III

Proteomic signature of tubulointerstitial tissue predicts prognosis in IgAN

In this study, we microdissected areas with mild tubulointerstitial changes and compared the tubulointerstitial proteome between progressive vs non-progressive IgAN patients.

Proteomic analysis quantified 2562 proteins with 2 or more unique peptides. Of these, 150 proteins had statistically significant different abundance between progressive and non-progressive IgAN patients: 67 were more abundant and 83 less abundant. Periostin was the protein with the highest fold change between progressive and non-progressive IgAN (fc 8.75, $p<0.05$).

Pathway analysis revealed that the most affected pathways were the immune system (23 significantly different proteins) and signaling by Rho GTPases (10 significantly different proteins), while less abundantly changed pathways were mitochondrial translation (10 significantly proteins) and metabolism of proteins (23 proteins). ROC analysis of the first top 7 proteins and of periostin, could classify progressors from non-progressors with an area under ROC curve of 0.91.

Discussion

As described in the aim section, the scope of this thesis was to 1) Identify novel tissue biomarkers for progressive IgAN that may be used for prognostication and to improve understanding of underlying mechanisms and to 2) Separately investigate glomerular and tubular biomarkers in IgAN

In paper I and II we focused on investigating the proteome of the glomerular compartment and made several interesting findings. In the first paper we found that a higher glomerular abundance of complement proteins was associated with a progressive clinical course in IgAN. In the second article, we found that glomeruli from patients with IgAN had significantly higher abundance of extracellular matrix structural proteins as compared to controls and Periostin was the protein with the highest abundance both between IgAN and controls but also progressive vs non-progressive IgAN.

In paper III we microdissected tubulointerstitial tissue from patients with IgAN and found that proteins related to inflammation and signaling by Rho GTPases were higher in progressive IgAN, while proteins related to mitochondrial translation, were less abundant. Periostin was the protein with the highest abundance between progressive and non-progressive IgAN both in the glomeruli and tubulointerstitial tissue and could also classify patients into progressive vs non-progressive IgAN.

Methodological considerations

Study design

The three studies were retrospective studies, where the patient groups were retrieved from the Norwegian Renal Biopsy Registry. This registry was established in 1988 and it has been run by the Renal unit at Haukeland University Hospital. The aim of the registry was, first of all, to provide a platform for development of expertise and improvement of quality, second to have a material available for research. In 2012, the registry was acknowledged a national medical quality registry. From 2012, the registry has been building a digital slide archive of kidney biopsies. In 2021 the registry had

collected clinical and pathological data of 14600 non-neoplastic kidney biopsies (www.nephro.no). Formalin-fixed paraffin embedded (FFPE) tissue have been stored for almost all these biopsies and in about 50% of cases the biopsies are stored at Haukeland University Hospital. Selection of patients for inclusion might however be tricky as about 20-30% of kidney biopsies shows out to have little remaining tissue with too few glomeruli or too scarce suitable tubulointerstitial area for microdissection. Very important for the present project was the possibility to use the 11-digit national identity number to link with the Norwegian Nephrology Registry, this enabled us to identify subgroups that did or did not progress to kidney failure. The advantage of a retrospective design is that the kidney tissue was already stored, the clinical course was known and the number of eligible patients was relatively large.

Selection of control group

For the control group we used FFPE kidney biopsies from the Norwegian Renal Biopsy Register that were classified as "normal tissue or slightly unspecific changes" (diagnosis number 29 in the registry). However, one has to have in mind that these biopsies were taken with a specific indication, most common hematuria, proteinuria or unexplained low glomerular filtration rate. Thus, while some of the biopsies might indeed have been normal biopsies from a pathologist point of view, one cannot exclude that at a proteomic level, changes related to initial biopsy indication might have had occurred.

For the first two studies we used the same control cohort of 15 patients. The indication for biopsy were in 11 of the 15 control patients hematuria and in two of the control patients proteinuria. In two of the patients the indication for biopsy was not registered. None of the control patients had known hypertension, diabetes or malignancy. All the control patients, except one, had eGFR above 90 ml/min/1.73m^2. At the time of biopsy one control was using both ACE inhibitor/ARBs and beta-blocker. We chose to use normal biopsies as control, as they were easily available and in our opinion a good option for comparing to IgAN. In retrospect we think that an extra control group could have added more information about which proteomic changes are specific to IgAN and which are specific to CKD. It would however have been hard to decide which

particularly disease to choose in order to compare, as patients with hypertensive nephropathy, FSGS or lupus nephritis, will also differ from control in several aspects and would have specific changes related to the disease. As mentioned above, an extra control group could have added more information regarding which changes are specific to CKD. As IgAN is a chronic renal disease we believe that there are indeed many similarities with CKD in general and that IgAN could work as a model disease for CKD. The benefit of using IgAN patients as a model disease of CKD progression is that these patients are often young with few other comorbidities.

Selection of IgAN group

When planning the studies, the aim was to compare clinically relevant groups in IgA nephropathy, defined by preserved eGFR (above 45 ml/min/1.73m^2) and proteinuria between 1 and 3 grams per 24h. This group is difficult to predict prognosis for [215] and it is uncertain which treatment should be chosen. Furthermore, we wanted to compare a subgroup with progressive course to a subgroup with non-progressive course; these two subgroups were defined by development of kidney failure (in our study defined as need for renal replacement therapy) during follow-up, or not. Using these criteria, we selected all available patients, but the number of patients was too low. We therefore chose to expand the criteria somewhat by including three patients with proteinuria less than 1 gram and one patient with proteinuria more than 3.5 gram. These criteria yielded two comparable groups that were very similar in most respects with the exception that some patients develop kidney failure, and some do not. In our opinion this is a good approach for investigating novel markers of progression, and also elucidating mechanisms. The initial cohort of IgAN consisted of 29 patients (18 non progressive and 11 progressive) but, in the first two studies, four patients were excluded (either because there was not enough tissue for microdissection or due to loss of tissue in the preparation procedure) so we ended up with 16 non progressive patients and 9 progressive. In the last study, 2 progressive patients were excluded due to lack of enough tissue for microdissection, ending up with 18 non progressive patients and 9 progressive. The IgAN patients were not matched with the controls (due

to low number of patients) but we tried to chose patients with as similar characteristics as possible.

It is worth mentioning that the term "end stage renal disease" (ESRD), defined as GFR <15 ml/min per 1.73 m^2 or treatment by dialysis is a term often used in the literature as well as in our articles, but according to KDIGO Consensus Conference [216], the preferred term is "kidney failure".

Formalin-fixed paraffin-embedded tissue

The use of FFPE tissue in proteomics allows studying biopsy material from large biobanks such as the Norwegian Kidney Biopsy Registry. The advantage of using FFPE tissue is that it is readily available (from diagnostic biopsies), low cost (compared with frozen tissue due to storage costs) and the ability of being stored for decades or more at room temperature while retaining cellular morphology [207].

For many years FFPE tissue could not be used due to the aldehyde induced protein cross-linking that would complicate the extraction of proteins. Newer techniques based on heat mediated treatment have allowed the use of FFPE tissue for proteomic research. Studies have been carried out with the aim of demonstrating similarity between proteomic data retrieved from FFPE and fresh frozen tissues using shotgun proteomics [217]. These studies suggest that the analysis of FFPE tissues provides reliable biological information and while most recently developed extraction protocols appear to achieve nearly equivalent protein yields for FFPE and fresh frozen samples, formalin reversal may not be completely achievable [217]. Parameters that could influence the protein extraction from FFPE tissues include ischemic time (time between sample collection and formalin fixation), fixation time (time the sample was left in formalin solution), and storage duration (time from tissue fixation until protein extraction and analysis). It has been shown that prolonged fixation (> 48 h) correlates with lower protein extraction [218]. In our study, the biopsies came from different hospitals and we did not have information such as fixation time. Storage time up to 10 years does not affect the proteomic results [219] and this was also previously tested by our group in a pilot study - data not published. Our group has also previously tested

the use of FFPE tissue for proteomic studies and established a protocol [212]. Although there are advantages of using FFPE tissue for proteomics in research setting, the routine use in clinics for diagnosis or prognostic has not yet been established. Other sources for the quantification of proteins are blood and urine, which are easily obtained in a relatively non-invasive manner. Although many studies have been investigating biomarkers from blood and urine in CKD [220] and IgAN [117,221-224] the results have not been transferred /applied into clinical practice. Unfortunately, we did not have access to blood or urine from our patients.

Laser microdissection

Laser capture microdissection is a good tool that allows for isolation of cells or tissues from tissue sections. The area of interest is visualized in a special program on a PC screen and a guiding line is manually drew around it. The laser beam will then cut the selected tissue and this will be catapulted into the lid of an Eppendorf tube. The most important advantage of this technique is that it allows the selection of different compartments such as glomeruli and tubules. This is vital when investigating changes in different tissue compartments, for example glomerular changes. The main disadvantage is that it is time consuming. Another disadvantage is that the tissue morphology is quite difficult to see in microdissection microscope and we therefore had to use digitally scanned parallel PAS stained tissue sections to determine which areas to include. While theoretically it is possible to select just tubular tissue, and even separate areas of tubules (e.g. proximal, distal), in practice this would be very time consuming and difficult to execute. For our third paper we therefore microdissected areas of tubulointerstitial tissue. Although trying to be as precise as possible when selecting areas of interest (e.g. glomeruli), it is possible that very small amounts of surrounding tissue such as periglomerular areas have also been included. We believe that this amount would be so small that it wouldn't influence the final proteomic data.

For the first two studies we first aimed to microdissect 100 glomeruli per patient but unfortunately this was not possible for every patient. We microdissected an average of 86 glomeruli per patient with a minimum of 28 glomeruli (for a non- progressive IgAN patient) and a maximum of 140 glomeruli (for an progressive IgAN patient). A total of

3472 glomeruli were microdissected for the first two studies. For the third study we microdissected an average of 312,2167 micrometers of tubulointerstitial tissue per patient.

Proteomic workflow

In the three studies in this thesis we used an previously established protocol for an in-gel trypsinization as described in the methods. Our lab (Kenneth Finne) has previously tested several other protocols such as filter aided sample preparation (FASP) and in-solution digestion but the “in-gel” protocol had produced the most reliable results at the time of tissue preparation and were used for these studies (data not published).

Protein quantification

In our studies we used a general shotgun/discovery proteomic approach and while this approach allows the identification of thousands of proteins from relatively low amount of tissue, it also has the disadvantage of lower sensitivity and reproducibility than targeted proteomics [225] - where the molecules of interest are defined prior to acquisition [226].

In contrast to DNA and RNA, proteins cannot be amplified by copying. Following the hypothesis of “one gene = one protein,” there should be at least ~20,000 non modified (canonical) human proteins [227]. Most of these proteins are expressed at relatively low levels in the cell [184]. When using shotgun proteomics, one is only able to identify the most abundant proteins, missing interesting proteins that have too low abundance to be identified. In the first two papers, a total of 3274 proteins were identified, of which 2,018 were identified with 2 or more unique peptides. In the third study a total of 2,609 proteins were identified of which 2,562 with 2 or more unique peptides. This represents about 10-13% of the total protein-coding genes present in the human genome, thus the number of proteins in our studies are not very high. Reasons for this is the relatively low tissue volumes, the use of FFPE tissue and the inability of current methods to detect low abundant proteins. This is one of the main limitation in proteomic research. One has also to keep in mind that mass spectrometry gives a "snapshot" of a proteome, at that moment of time.

Software for data analysis

As technology advances and data from high throughput proteomics becomes more complex, so does bioinformatics and the software offered for data analysis. There are as of December 2021 registered 702 biological pathway related resources and molecular interaction related resources on http://pathguide.org/ and thus choosing "the best" software for data analysis can be difficult. Because different softwares have different algorithms for pathway detection, most probably different programs will give slightly different answers and it will not be clear which is more correct.

In the studies, the software for data analysis (Progenesis, Maxquant, Perseus) were used with standard settings and following specific tutorials available for free from the developers. Some software such as Reactome and String are online based. The advantage of using free online software is that these are user friendly, easily accessible and available for the entire scientific community. For the pathway analysis we used Reactome database. This is a relatively large database (the total number of curated human proteins including isoforms is 11,118) and has the advantage of very short average response time (seconds), easy graphical map of pathways and easy interface where the user can ‘click through’ to find detailed information on components and their relations [228].

As is very well underlined in the article written by Fabregat et al. [229] "pathway analysis methods should never be taken as black boxes from where experimental data goes in, and true statements come out, but perhaps more as metal detectors in haystacks helping researchers to find biologically meaningful needles"

Statistical analysis

When testing high numbers of proteins between groups, one can use correction for multiple comparison in order to decrease the possibility of Type I error. A Type I error occurs when a protein without differential abundance between groups is incorrectly considered to be significantly different. In our studies we mostly did not correct for

multiple comparison as we wanted to investigate pathways rather than single proteins and we also wanted to analyze subgroups of proteins (for example basement protein etc) where the number of proteins were significantly smaller than the total number of identified proteins.

Discussion of the main results

By using a modern proteomic approach to study the glomeruli and tubulointerstitium of IgAN patients we found significant changes both in IgAN patients as compared to controls but also in progressive vs. non progressive IgAN patients.

Differences in patient characteristics at baseline

When comparing the clinical characteristics of the control group with the IgAN group (in all three studies) we found a significant difference in serum creatinine (but not eGFR) and proteinuria. With the significantly different proteinuria, one can ask if this can influence the proteomic changes between groups. The primary role of the glomerular filtration barrier is to prevent sieving of proteins from the blood to the urine, but once proteins have reached the tubuli, the epithelial cells of the proximal tubuli are responsible for most of the reabsorption of this proteins. In our study, basement membrane proteins such as collagen, laminin, fibronectin, had lower abundance in IgAN patients as compared to controls (paper II). Proteins belonging to the slit diaphragm such as dendrin, nephrin, podocin tight junction protein ZO-1 had also lower abundance in IgAN patients as compared to controls as did proteins belonging to the podocyte foot process such as ezrin, synaptopodin, podocalyxin and alpha actin. As we only selected morphologically healthy glomeruli, we believe that these changes could represent an early sign of glomerular damage. The reabsorption of proteins from the tubular lumen is both nonspecific through absorptive endocytosis but also receptor-mediated [230]. Megalin and cubilin have been identified as receptors essential for the reabsorption of proteins [231]. Cubilin binds to either megalin or amnionless (AMN) for internalization of proteins [231]. In our study we found cubilin and AMN significantly more abundant in IgAN patients as compared to controls (fold change. 1.2, p value 0.004 respectively f.c. 1.3 p value 0.0021). This finding may

suggest that as more proteins are filtered through the glomeruli and arrive in the tubuli there will be an up-regulation of the receptors responsible for absorption.

The IgAN progressive patients had higher proportion of Oxford T lesions of 1 or 2 as compared to non-progressive IgAN patients. This was observed in retrospect, since patients were not selected based on MEST score but based of preserved eGFR. In our study we did however exclude tubuli with atrophy and areas with interstitial expansion but the findings suggest that progressive IgAN patients had more advanced disease at time of biopsy.

Immune system and IgAN

Immune complexes between galactose deficient IgA1 and autoantibodies are deposited in the glomerular mesangium, triggering a response which can eventually lead to fibrosis. By activating inflammatory and cellular signalling cascades, the immune complexes are contributing to local inflammation, mesangial matrix production, and mesangial cell proliferation [232]. This will eventually lead to glomerular injury such as mesangial hyperplasia, matrix expansion and interstitial fibrosis [233]. In the glomeruli of patients with IgAN (Paper II), we found a higher abundance of proteins related to the immune response and inflammation (e.g. azurocidin, myeloperoxidase, neutrophil elastase, cathepsin G, annexin A1, protein S100-A9 etc). Most of these proteins have been described as present in polymorphonuclear leukocytes [234-236], and some may also act as chemoattractants and activators of monocytes and macrophages [237].

As mentioned in the introduction, the mechanisms by which glomerular damage leads to tubular damage and interstitial fibrosis in CKD are not fully understood but is believed that glomerulotubular cross-talk [238], proteinuria [57], leakage of glomerular filtrate into the periglomerular space [58], or tubulointerstitial hypoxia and peritubular capillary regression [59] all play a role. It is believed that tubular epithelial cells (TEC) are not only victims in the context of kidney diseases, but also key inflammatory and fibrogenic cells that drive the progression from acute to chronic kidney disease [239] by actively participating in the tubulointerstitial damage. TEC

can synthesize inflammatory mediators and reactive oxygen species [240] that will attract even more inflammatory cells such as macrophages which will lead to a positive feedback loop of activation that may lead to the overproduction of extracellular matrix components, resulting in fibrosis and ultimately loss of kidney function [238]. Urinary proteins such as members of the complement cascade or cytokines may also activate specific cellular responses by binding to receptors on tubular apical membranes [240].

In paper III, we found that the immune system pathway was one of most abundant changed pathways between progressive vs non-progressive IgAN with 23 significantly different proteins. Some of these proteins belonging to the immune system were Cathepsin G, Cathepsin H and Neutrophil gelatinase-associated lipocalin (NGAL). NGAL is an acute-phase protein that is released by kidney tubular cells in response to various tissue injury. In a study of 70 IgAN patients [241], urinary NGAL levels and urinary NGAL/creatinine values were significantly higher in IgAN patients as compared to controls and correlated with histological severity (glomerular mesangial proliferation and tubulointerstitial injury). In another small study on 29 patients [242], NGAL-positive staining was higher in IgAN progressive vs IgAN non-progressive patients (89% vs. 55%, $p = 0.076$). Nickolas et al. found a significant correlation between NGAL monomer in the urine of CKD patients and glomerular filtration rate, interstitial fibrosis, and tubular atrophy [243]. Cathepsins are protease enzymes mainly found in acidic endo/lysosomal compartments where they play an important role in intracellular protein degradation, energy metabolism, and immune response [244]. There are currently 15 classes of known cathepsins in humans [245]. As a consequence of lysosomal injury (as for example from oxidative stress) cathepsins can be released into cytosol where they can promote apoptosis and inflammation [244]. Cathepsins can also be present in the extracellular space, playing an important physiological role in ECM degradation, or more commonly during pathological conditions, such as cancer and metabolic disorders [244]. Not much is known about cathepsins and kidney disorders, but cathepsin G has been shown to be involved in inflammation and tissue injury after reperfusion of ischemic kidneys [246]. Cathepsin D has also been shown to be up-regulated in damaged renal proximal tubular epithelial cell in a mouse model of

renal ischemia/reperfusion injury [247]. A small study showed significantly up-regulated Cathepsin S expression in the glomerular mesangium and tubular epithelial cells from IgAN patients, and higher levels of serum Cathepsin S were positively correlated proteinuria [248].

In response to injury, TEC may undergo a phenotypic switch, through the process of epithelial-to-mesenchymal transition (EMT) [249] and become myofibroblasts capable of producing ECM components [250]. In our Paper III we found 14 EMT related protein to be more abundant in tubulointerstitial tissue of progressive IgAN as compared to non-progressive, but this is a small number compared to the total number of genes which are known to be involved in EMT (1184 human Epithelial-Mesenchymal Transition genes in dbEMT 2.0. in January 2021). Also, just the presence of EMT related proteins cannot give us an insight in the pathological process that takes place in the tubulointerstitium.

Complement system and IgAN

The complement system plays an pivotal role in the pathogenesis of IgAN [173].
In the **glomeruli** of patients with IgAN we were able to quantify a total of 28 proteins belonging to the complement system, with 21 significantly different proteins (19 more abundant and two less abundant) between control patients and IgAN patients. These proteins belong to all three initiation pathways of complement as well as the terminal pathway. Between progressive vs non-progressive IgAN there were 18 complement proteins significantly different of which 17 proteins were more abundant and one (complement receptor 1, CR1) were less abundant. Although the role of complement in the pathology of IgAN has long been suspected, no other studies have used a proteomic approach from kidney biopsies in order to investigate its role in the progression of the disease.

In the **tubulointerstitial compartment**, complement C3, C5, C7, C8, C9, Factor H and Factor B had significantly higher abundance in IgAN patients as compared to controls (data not published). There was no statistically different change between progressive vs. non-progressive patients.

Classical pathway: Components of the classical pathway C1q, C1r and C1s, were significantly increased in the glomeruli of patients with progressive IgAN as compared to non-progressive IgAN, suggesting the involvement of the classical pathway in the progression of the diseases. This differs from most of the previous work that tend to find little evidence for classical pathway activation in IgAN. Studies done more than three decades ago (Berger, 1969 [251] and Evans et al., 1973 [252]) are still cited as proof for the involvement of the lectin pathway in the pathogenesis of the disease in the absence of C1. As one article points out, it has been accepted that, the glomerular presence of C3 alone implies alternative pathway activity and the presence of C4 and C3 in the absence of C1q implies lectin pathway activity [173]. When staining for C1q, we did not find a significant difference between groups (0.57±0.78 in controls, 0.43±0.64 in non-progressive IgAN and 0.38±0.52 in progressive IgAN). We are not sure why we could not reproduce this proteomic findings with immunohistochemistry staining. Quantitative proteomics is however a very sensitive and advanced method that gives us a fairly precise relative difference between groups, without knowing the exact amount of the protein in the sample. It could be that the difference between control and IgAN patients was not that strong enough to be detected by immunohistochemistry. Studies have shown that a minority of biopsies from IgAN patients have C1q mesangial positivity [172,253] but it is still unclear if and how the classical pathway is involved in IgAN.

Lectin pathway: We were not able to quantify proteins from the lectin pathway such as ficolins or MBL, but we found an increased abundance of complement C4 both in IgAN patients compared to controls and in progressive vs. non progressive IgAN. C4 can be activated by both classical and lectin pathway, and is further cleaved into C4b which is frequently identified in IgAN biopsies. C4d deposition has been shown to be an independent risk factor for the development of kidney failure in IgAN [254] and glomerular C4d staining has been associated with progressive disease [255].

In our study, also C4b-binding protein which is an inhibitor of C4b was significantly increased between progressive vs non progressive IgAN patients.

Alternative pathway: In our study C3 had significantly higher abundance in the glomeruli of progressive vs non progressive IgAN patients and IH staining scores were different between groups (0.14±0.38 for controls, 0.71±0.61for non progressive and 1.38±0.91 for progressive with p=0.055 for comparison progressive vs. non-progressive IgAN). A previous study has also shown that increased glomerular staining for C3b/iC3b/C3c, C3d was associated with progressive disease [255].

It has classically been believed that the alternative pathway is the main complement pathway activated in IgAN and is principally responsible for C3 deposition [173], although C3 can be activated also by C3 convertase (from classical and/or the lectin pathway). Complement factor H and complement factor H related protein, both inhibitors of the alternative pathway were also significantly increased in the glomeruli of progressive vs. non-progressive IgAN patients, while Complement receptor type 1 was less abundant.

Previous studies have shown that plasma levels of Factor H-related protein 1 (FHR1) were significantly higher in IgAN patients than controls with a negative correlation between FHR1 levels and eGFR [181,182]. Another study has shown glomerular staining for FHR5 and FHR1 associated with progressive disease [255].

In the tubulointerstitium, C3 and Factor H had higher abundance in IgAN patients as compared to controls, with no difference between progressive vs. non progressive disease. In IgAN, C3 gene was shown to be expressed primarily in proximal tubular cells [256] suggesting local production.

Terminal pathway: In accordance with other studies [255] we also found increased abundance of proteins of the terminal complement pathway in the glomeruli of IgAN patients. In our first article, members of the terminal pathway (complement factors C5-C9) that constitute the MAC, showed the strongest increase in progressive vs. non-progressive IgAN as well as in non-progressive IgAN vs. controls. In addition C5, C6, C8, C9 had higher abundance with lower eGFR. In order to differentiate local synthesis from local activation, we used antibody against a neoepitope in C9 that only

stains positive for the assembled complex, indicating activation of the complex and not just deposition of the native component [257].

In the tubulointerstitium, complement C3, C5, C7, C8, C9, had significantly increased abundance in IgAN patients as compared to controls (data not published) but there was no significant differences for these proteins between progressive vs. non progressive IgAN. Complement components can also be synthesized in the proximal tubular epithelium and it was suggested that such local production and activation, might also play a role in the mediation of tubular injury [258].

Over the last years, there have been more and more evidence of the important role of the complement system in IgAN. Our findings are in concordance with the latest literature, but our results must be interpreted cautiously since proteomic analysis cannot give us a full pathogenic overview of the clinical importance of the complement system in IgAN. Treatment options targeting the complement system are still under development and adverse effect such as susceptibility to infections by capsulated bacteria is still a worry [259]. To date, treatment with complement inhibitors has been limited to few published cases reporting the use of Eculizumab, a humanized monoclonal antibody that inhibits cleavage of C5 by C5 convertase, as rescue therapy [259]. Use of Eculizumab (Soliris, Alexion Pharmaceuticals) is associated with a 1,000-fold to 2,000-fold increased incidence of meningococcal disease and patients receiving the drug are recommended to receive meningococcal vaccines before beginning the treatment [260].

Given the heterogeneity of IgAN, there is a need for better biomarkers which will allow to select patients who would benefit from complement inhibitors.

Changes of the glomerular extracellular matrix of IgAN patients

The extracellular matrix (ECM) is composed of collagens, glycoproteins and elastin molecules which form a complex network interacting with each other and with the surrounding cells, forming a very dynamic, highly charged structure [3]. In the kidney, ECM is present both in the glomeruli (glomerular basement membrane, Bowman's capsule and mesangial ECM) and in the tubulointerstitium (tubular basement

membrane, peritubular capillary basement membrane and interstitial ECM) [3]. In the glomeruli, the glomerular basement membrane consists of laminin, collagen type IV, nidogen and heparan sulphate proteoglycans and its main function is as filtration barrier between the vascular system and the urinary space. Mesangial ECM consists of fibronectin, collagen type IV, collagen type V, laminin, chondroitin sulphate and heparan sulphate proteoglycans and nidogen and forms as a structural matrix surrounding mesangial cells and providing a scaffold for glomerular capillaries [261]. Mesangial matrix expansion has been regarded as a step towards glomerular sclerosis, as was suggested in a review paper by Fogo in 1999 [262], but the exact mechanism is not yet fully understood.

In our study, structural proteins such as collagen alpha-1 (IV) chain, fibronectin, laminin subunit beta-1, nidogen 1, vitronectin, extracellular matrix protein 1, fibulin-5 and fibrinogen were significantly more abundant in the glomeruli of IgAN patients than in controls. Since we did not microdissect glomeruli with more than minimal sclerosis, our findings are likely to illustrate the proteomic changes in mesangial expansion or early glomerular sclerosis.

Changes in tubulointerstitial compartment

The renal tubules and tubulointerstitium make up a significant portion of the kidney and are major sites in response to injuries [239]. Besides proteins belonging to the immune system that have already been mentioned, in the tubulointerstitial compartment we found that proteins belonging to the signaling by Rho GTPases pathway were significant more abundant in progressive IgAN patients as compared with non-progressive**.** Rho GTPases contribute to a wide range of cellular processes including organization of the actin and microtubule cytoskeletons, vesicle trafficking, cell cycle progression, cell morphogenesis, cell polarity and cell migration [263]. Rho GTPases have also been shown to be involved in numerous pathologies such as cancer development and progression [264], hypertension [265] and neurodegenerative diseases [266]. In the kidney, Rho-kinase has been shown to be involved in aldosterone-induced renal injury [267], diabetic renal disease [268] and to the

pathogenesis of dialysis-related peritoneal fibrosis through epithelial-mesenchymal transition (EMT) [269]. In a model of human renal proximal tubular epithelial cell line, it has been shown that Rho/ROCK signaling pathway plays a key role in the dissolution of tight junctions, an early and reversible event in EMT [270]. EMT is the process by which epithelial cells lose their epithelial proprieties and convert into mesenchymal cells. These mesenchymal cells are then able to migrate, secrete proinflammatory mediators and extracellular matrix (ECM) and thus promote fibrosis. In our third study, 14 EMT related protein wore more abundant in progressive IgAN as compared with non-progressive.

Proteins belonging to the mitochondrial translation pathway were less abundant in the tubulointerstitium of progressive IgAN as compared to non-progressive, indicating a mitochondrial dysfunction. Mitochondrial dysfunction is emerging as an important contributor to the development of CKD but the mechanism is not fully understood. It is believed that the proximal tubule is especially vulnerable to mitochondrial dysfunction as these tubuli depend mainly on aerobic metabolism for energy supply, with very little anaerobic capacity, as compared to distal tubule segments [271].

There is, to date, no study of mitochondrial dysfunction in IgAN, but its role has been investigated in diabetic nephropathy [272,273]. In a mouse model of type 2 diabetes, where renal cortex proteome was investigated, proteins related to beta-oxidation, fatty acid metabolism and tricarboxylic acid cycle were some of the most dysregulated proteins [274].

Novel markers of progression in IgAN

Complement

In Paper I, glomerular protein abundance of complement proteins could classify IgAN patients as progressive vs non-progressive. Unsupervised hierarchical clustering including the 18 significantly different abundant complement related proteins separated most patients with progressive vs non-progressive disease. We also calculated a complement score for each patient based on abundance of complement related proteins and found that patients with progressive IgAN had significantly higher

scores than patients with non-progressive IgAN. These scores were used to classify patients with progressive vs non-progressive IgAN. In ROC analyses, AUC values were 0.91 (p=0.001) for a complement score using all significant proteins, 0.91 (p=0.001) for the complement score including complement components C5, C6, C7, C8 and C9 and 0.90 (p=0.001) when only including protein abundance of complement factor C7, the rate limiting factor of the terminal pathway [275]. Other clinical or glomerular morphological variables could not be used to classify progressive from non-progressive IgAN.

Periostin

Periostin, a protein that has in recent years been shown to be involved the progression of renal disease [276] was the protein with the highest abundance between progressive vs non-progressive IgAN patients both in the glomerular and tubulointerstitial compartment.

Periostin is a matricellular protein which is expressed at low levels in adult kidney tissue, but upregulated during inflammation [277], cancer [278-280] and organ fibrosis [281-283]. Matricellular proteins are a group of non-structural ECM proteins that by binding to other ECM proteins, growth factor, and cytokines play a central role in the homeostasis of normal tissues regulating cell proliferation and differentiation [278]. Over the last several years, periostin has been recognized as a key factor of progression in kidney disease [276,284-286]. A previous study showed that periostin is induced by proinflammatory factors, mainly NFκB in a model of chronic renal disease, and that inhibition of periostin can be used as a therapeutic strategy to slow down renal disease progression [287].

In one study, glomerular gene expression profiles from patients with proteinuric diseases, including IgA nephropathy, were analyzed, identifying periostin as the most highly expressed protein [281]. Immunohistochemical staining of the biopsies showed that periostin was localized in areas of mesangial expansion, the tubulointerstitium and sites of fibrosis [288].

In Paper II, in the glomeruli, periostin was significantly more abundant in patients with IgAN as compared to controls (fold change 3.28, $p<0.01$) and also significantly more abundant in IgAN patients who progressed to kidney failure as compared to IgAN patients who did not progress (fold change 1.79, $p <0.05$). Immunohistochemistry for periostin showed stronger positivity for periostin in the glomeruli of IgAN as compared with controls (p-value = 0.003) but there was no significant difference in positivity between progressive and non-progressive IgAN.

In Paper III, in the tubulointerstitial tissue, periostin was the protein with the highest fold change between progressive and non-progressive IgAN (fold change 8.75, $p<0.05$) and periostin staining was also stronger in patients with progressive vs non-progressive IgAN. Receiver operating characteristic (ROC) showed that periostin had AUC values of 0.91 while AUC for 1/eGFR was 0.72.

Conclusion and further perspective

Conclusion

1. Investigation of the glomerular and tubulointerstitial proteome of patients with progressive IgAN, non-progressive IgAN and control patients have strengthened our understanding of pathophysiological changes in IgAN.

2. Microdissection of glomeruli and tubulointerstitial tissue allowed for investigation of mechanisms specific for the different compartments.

3. Abundance of periostin and complement proteins seem to be the most promising markers of progressive IgAN. Periostin abundance seems to associate with progression in both glomeruli and tubuli.

Further perspective

Use of novel biomarkers of progressive IgAN, such as periostin staining or complement C4d staining, could possibly improve prognostic accuracy and may also help targeting treatment for the patients who may benefit the most. Our studies have shown the prognostic importance of these morphological stainings. Ideally, serum or urine biomarkers would be easier to use. More studies that combine the analysis of tissue, blood and urine are therefore needed.

Our studies cannot discriminate whether periostin is only a marker of progression or whether targeting periostin may be a possible treatment strategy. Further studies should investigate the role of periostin in progressive kidney disease.

As we have shown in our studies, glomerular and tubular proteome changes seem to be common for several pathways in IgAN with complement system, immune system and mitochondrial metabolism being involved in the disease in both compartments. As such, further therapeutic strategies could address all these aspects.

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