INVESTIATION OF INITIAL AND ADVANCED STAGES OF GLOMERULOSCLEROSIS IN RODENT MODELS

PhD thesis

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Introduction

Chronic kidney disease (CKD) is a serious health and economic problem affecting 10% of the population worldwide. In human clinical practice, a significant proportion of CKDs are attributed to hypertension- or diabetes-related secondary glomerulopathies, as well as to primary glomerulonephritis, including focal segmental glomerulosclerosis (or FSGS), the most common one with increasing prevalence worldwide. Less frequently, CKD may be a late consequence of acute ischemic damage affecting mainly tubulointerstitial tissues, or of systemic sepsis, but it may also be due to autosomal dominant polycystic kidney disease, inflammation of various parts of the kidney, or certain urinary tract abnormalities. Regardless of disease origin, the final common pathway is renal fibrosis determining the progression of CKD, the pathomechanism of which is still insufficiently known, so there is no effective therapy to slow or reverse it. This motivated our experimental nephrology research.

In glomerulopathies, regardless of the initial damaging effect, the development and progression of fibrosis (or glomerulosclerosis) is determined by the various glomerular cells (podocytes, parietal epithelial, mesangial and endothelial cells) and changes in connective tissue structure (glomerular basement membrane (GBM), mesangial matrix, Bowman capsule) in pathological conditions. In recent years, the application of multiplex screening methods (RNA sequencing, mass spectrometry), which have become more and more widely available, have greatly increased our knowledge of genes and proteins expressed in the kidney glomeruli.

Regarding the composition of the glomerular extracellular matrix (ECM), which has been intensively studied in the last decade, it is slowly taking shape that the connective tissue structure providing mechanical

stability of the basement membrane contains many important, other than structural proteins, such as signaling molecules involved in the cell-cell and cell-matrix communication. Thus the glomerular ECM is where diverse, complex processes are also activated in response to tissue damage. Nowadays, we consider the network and system level analysis of the correlations among the above factors to be state-of-the-art. Better recognition of the glomerular ECM components and understanding key processes in glomerulosclerosis will possibly enable us to find novel targeted therapeutic approaches in the future, so we can expect to make a great advancement in the treatment of FSGS. In the near future, it is worth monitoring some of the following components: the enzyme system responsible for the turnover of structural ECM proteins, including matrix metalloproteinases, their inhibitory proteins (TIMPs), as wells as serine protease inhibitors (SERPINs) responsible for their regulation; the fibrinogen pathway, a long-known component of the scarring process, having many newly recognized roles beyond its function to form fibrin network; the complement system and the coagulation cascade as part of a strong acute phase response.

Objectives

The aim of the thesis was to study the pathomechanism of an initial and an advanced stage glomerulopathy of two different etiologies, in rodent experimental approaches. For this purpose, we have set the following sub-goals:

In Long-Evans rats made obese by feeding them a high fat diet and also characterized by a mild glucose metabolism disorder:

1. To characterize the renal damage caused by obesity and prediabetes, and to conclude from our observations the earliest events of its development;
2. To reveal hypothesized associations between renal and other obesity- and prediabetes-related organ damage.

In the miR-193a overexpression-induced FSGS model (**Figure 1**):

3. To identify extracellular matrix genes of potential relevance in advanced stage of FSGS by qualitative and quantitative analysis of the glomerular proteome;
4. To confirm and complete or results by comparing glomerular gene expression profiles in mouse and human FSGS, and to determine the correlation between the FSGS transcriptome and the protein results in our experimental FSGS glomeruli
5. To point out the key genes and pathophysiological processes, the targeting of which a more effective therapy of FSGS can be expected.

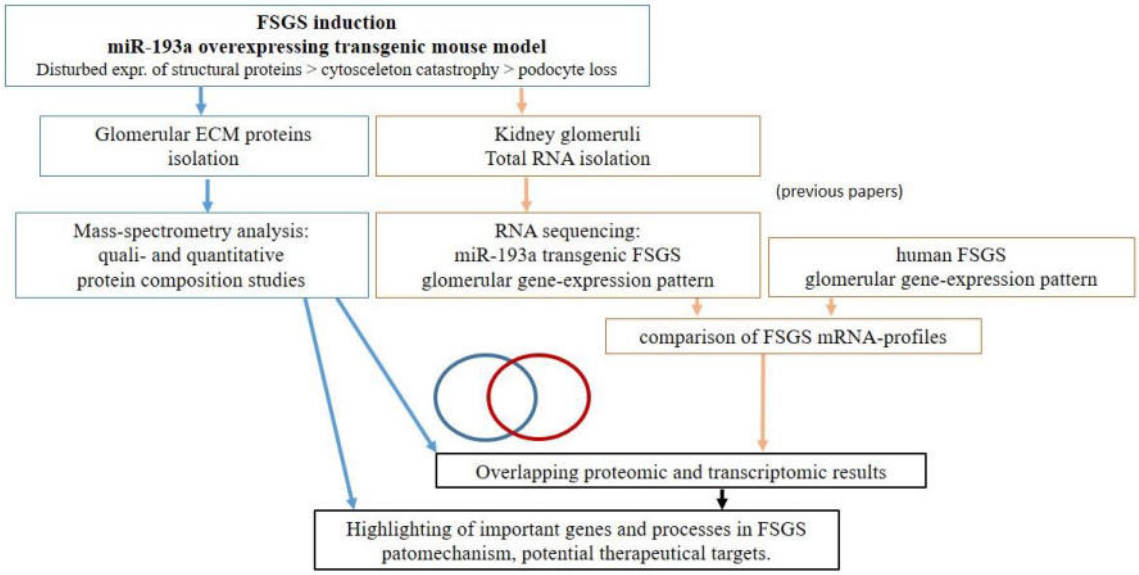

Figure 1. Objectives and pipeline of the miR-193a-induced FSGS study.

Renal and adipose tissue studies were performed in obese, prediabetic Long Evans rats at Semmelweis University (Budapest) in collaboration with the Cardiovascular and Metabolic Research Group of Institute of Pharmacology and Pharmacotherapy, and the cardiovascular results were published separately (referred to as *the obese, prediabetic Long Evans study*)

The identification of potentially important extracellular matrix genes in advanced FSGS in mice was performed at Medical University Vienna, Institute of Clinical Pathology in collaboration with the University of Veterinary Medicine Vienna, VetCore (Mass Spectrometry) facility and was published, in part, in a second separate publication (referred to as *the miR-193a-induced FSGS study)*

Methods

The obese, prediabetic Long Evans rat model

To induce obesity with mild glucose metabolism disorder, male Long Evans rats were fed a 40% lard supplemented diet for 20 weeks and injected with a pancreatic β-cell toxic agent (streptozotocin; STZ) at a single small dose (20mg/ bodyweight kg) at week 4, while the control group was fed normal chow and treated with vehicle only. During the feeding period body weight and blood glucose was monitored. At the end of study the cardiac function was extensively in a part of the animals in pentobarbital anesthesia, *in vivo* (published by Koncsos et al, 2016). In the rest of animals we collected urine and blood samples anticoagulated with EDTA. The kidneys were perfused with ice-cold saline via the aorta, and various organ samples were collected and weighed for routine formalin-fixed, paraffin-embedded (FFPE) histology, molecular biology and immunohistochemistry. Under macroscopic eye control the kidney cortex and medulla was separated using a surgical blade, and pieces of liver and adipose tissues of different localization were shock-frozen in liquid nitrogen and then stored at -80 ^{o}C.

Renal function parameters

We measured plasma and urine urea, creatinine and total urinary protein concentrations (and calculated proteinuria normalized to creatinine) using enzymatic, colorimetric assays in plasma and urine samples. Urinary concentration of neutrophil gelatinase associated lipocalin (NGAL), a sensitive marker of renal tubular cell damage, was measured by urine NGAL ELISA. Total protein and urine NGAL concentrations were estimated using four parameter logistic curve-fit.

RNA preparation

The kidney cortex, medulla and adipose tissue samples were treated with TRI-reagent, a chloroform-isopropanol method for extracting total RNA. The RNA concentration and purity was checked at 260 nm and by calculating 260/280 nm absorption ratios respectively. Integrity of RNA samples was checked on agarose gel-electrophoresis, and based on the ratio of 28S and 18S ribosomal RNA fractions.

Gene- and microRNA-expression analysis of kidney tissue

The mRNA and miRNA expression levels were performed after an intermediate reverse transcription step by a double-strand DNA (dsDNA)-specific dye (SYBR Green)-based quantitative real-time PCR (qRT-PCR) system. For miRNA expression analysis specific TaqMan probe kits were used. All samples were analyzed in duplicate and gene-expression results were counted applying the relative quantification (ΔΔCt) method. The qPCR efficacy was checked by standard curves.

The miR-193a overexpressing, FSGS-induced transgenic mouse model

The miR-193a-induced transgenic mouse was constructed according to tetracyclin-controlled transcriptional activation principle, providing doxycyclin in the drinking water induced miR-193a overexpression. Indeed, we applied doxycyclin in our study as an inducer rather than counting on its antibiotic or pharmacological properties. MiR-193a overexpression represses the expression of its target genes, including the master regulator Wilms' tumor 1 transcription factor, and via consequently reduced expression of podocyte cytoskeleton proteins it leads to severe loss of podocytes. The earliest histological signs of FSGS can be detected from 2nd week by electron microscopy, and from the 4th week by light microscopy. In our study we achieved significant degree of ECM accumulation at week 8 after induction, corresponding to advanced FSGS.

Isolation of mouse kidney glomeruli and glomerular ECM

At the end of the induction period we performed dissection of glomeruli in heparin anticoagulation and ketamine-xylasine anesthesia. The kidney glomeruli were isolated after direct renal perfusion of magnetic beads and a subsequent mechanic and enzymatic treatment method. Afterwards, applying a multistep fractionation method adapted from the literature we highly enriched glomerular ECM proteins in our samples. A series of different buffers, such as a detergent containing added proteinase and phosphatase inhibitors, alkylating agent-based and Dnase enzyme containing solutions were used.

Preparation of glomerular ECM for mass spectrometry analysis

After steps of dithiothreitol-reduction in urea buffer, alkylation with iodoacetamide and in-solution digestion with trypsin/LysC preparation, the glomerular ECM samples were analyzed and assessed by Nano-HPLC/Hybrid Quadrupole–Orbitrap mass spectrometry. For LC-MS data processing, protein identification and quantification was done by Proteome Discoverer software, based on UniProt database-registered *Mus musculus* and cRAP (common Repository of Adventitious Proteins: ftp://ftp.thegpm.org/fasta/cRAP/crap.fasta) approved collection of known proteins. The condition of our database search was tailored to the preparation-related potential modifications (trypsin digestion, alkylation of cysteine disulphide-bonds), so at the identification such dynamic variations were tolerated as oxidation on methionine or N-terminal acetylation. Minimal recognition criteria of a protein were set as detection of at least 2 peptide fragments in our samples.

Raw data were normalized to the same amount of peptide for quantification.

The value of the biological sample was given by the median of the measured values of the technical replicates. According to the experimental design, we performed a non-nested statistical analysis on four-four independent biological replicates of our two study groups (control vs. miR-193a), and pairwise comparison of normalized values were used.

Comprehensive data analysis of mouse and human FSGS glomerular transcriptome

We searched for at least 3-fold altered (increased or decreased) potentially important genes in the pathomechanism of FSGS by comparing glomerular gene expression patterns known form previous publications on our miR-193a-induced mouse model and human FSGS renal samples.

Histology and immunohistochemistry

Kidney samples were fixed in buffered formalin and embedded in paraffin after dehydration (FFPE) for histological and immunohistochemical analysis in both experiments. In the obese, prediabetic *Long Evans study* glomerulus diameter, matrix protein accumulation, brush-border of proximal tubular cells, indirect signs of lipid accumulation were assessed on periodic-acid Schiff (PAS) stained sections.

We performed collagen IV immunostaining to examine the extracellular matrix composition, αSMA to check for mesangial cell activation and desmin to detect possible podocyte stress. Lipid vacuoles were stained with oil-red-O on cryo-sections of liver and kidney tissue.

In the miR-193a-induced FSGS study we examined podocyte foot-process detachment and fusion, GBM widening and other ultrastructural changes by electron microscopy. Glomerulomegaly and accumulation of PAS-positive material in the glomerular matrix on PAS-stained sections, intracapillary cast formation on acid-fuchsin orange-G stained sections were detected using

light-microscopy. We confirmed the accumulation of fibrinogen/fibrin and collagen I using immunostaining.

Statistical analysis

The results are presented as mean±standard error of the mean (SEM). The two groups were compared using unpaired Student's t-test or two-way, repeated measurements ANOVA followed by Sidak's post hoc test. Logarithmic data transformation was performed if Bartlett-test indicated inhomogeneity of variances. The null hypothesis was rejected at 5 % significance level. GraphPad Prism6 software (GraphPad Software, La Jolla, California, USA) was used for data processing, statistical analysis and graphical imaging. Statistically significant differences are indicated with asterisks in all figures. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Results

Obese, prediabetic Long Evans rat study

Obese, prediabetic Long Evans model

In male Long Evans rats fed with a high-fat diet for 20 weeks and treated with STZ at a single, low-dose, body weight, relative amounts of adipose tissues, plasma leptin concentration increased, body fat distribution changed and as signs of mild glucose metabolism disorder, chronic hyperglycemia, subtle glucose intolerance and insulin resistance appeared compared to control.

Morpho-functional and molecular changes in the heart and liver

Based on *in vivo* and *ex vivo* cardiovascular examinations our colleagues described left ventricular diastolic dysfunction and hypertrophy intracellular lipid accumulation, increased subsarcolemmal mitochondrial oxidative stress and early changes of mitophagy in cardiomyocytes on the same groups of animals in another part of the same study. Moreover, histomorphological signs of hepatic steatosis were shown, but plasma liver enzyme and lipid concentrations, reflecting liver function did not change in relation to the treatment (Koncsos et al, 2016).

Adipose tissue remodeling without signs of inflammation

Despite sustained and marked elevation in plasma leptin concentration in parallel with the degree of obesity, we did not detect any signs of fibrotic remodeling, inflammation, increased oxidative stress or metabolic dysfunction of adipose tissue except an increase in TGF-β1 mRNA, gene-expression in the visceral adipose tissue in the obese, prediabetic animals.

Surprisingly plasma CRP concentration decreased to a small, but significant extent, which result pointed out a decreased inflammatory propensity of obese, prediabetic animal.

Mostly preserved renal morphology and kidney function

According to the literature signs of obesity-related glomerulopathy glomerulomegaly, glomerular hyperfiltration-induced, mechanical stress-mediated podocyte damage, connective tissue matrix accumulation subsequent to sustained hyperglycemia and decline in renal function with albuminuria were expected to be observed in obese, prediabetic kidneys. However, routine histology did not support such alterations, and renal function was preserved as shown by similar plasma creatinine concentration in both the prediabetic and control rats, and there was no albuminuria. The lower plasma urea concentration in obese, prediabetic rats may be explained by a known decrease in urea synthesis in cafeteria diet.

The earliest signs of renal impairment in prediabetic obesity

Upon further investigation of our kidney samples by specific immunostainings, we detected clearly enlarged proportion of collagen IV in glomeruli of the obese, prediabetic group despite otherwise unchanged amount of glomerular ECM. In the background of this phenomenon, decreased degradation is more likely than increased production of collagen IV regarding the lack of mesangial cell activation (see negative αSMA staining) (**Figure 2**). Although we did not find any difference in renal function, we report significantly increased Lcn2 mRNA expression in both kidney cortex and medulla of obese, prediabetic rats compared to controls that indicated a small change in tubular function, although urinary Lcn2 protein excretion has not increased in parallel.

It should be noted that the observed differences in Lcn2 expression were much smaller than those observed in sever ischemic, toxic or inflammation-related injuries of the kidney.

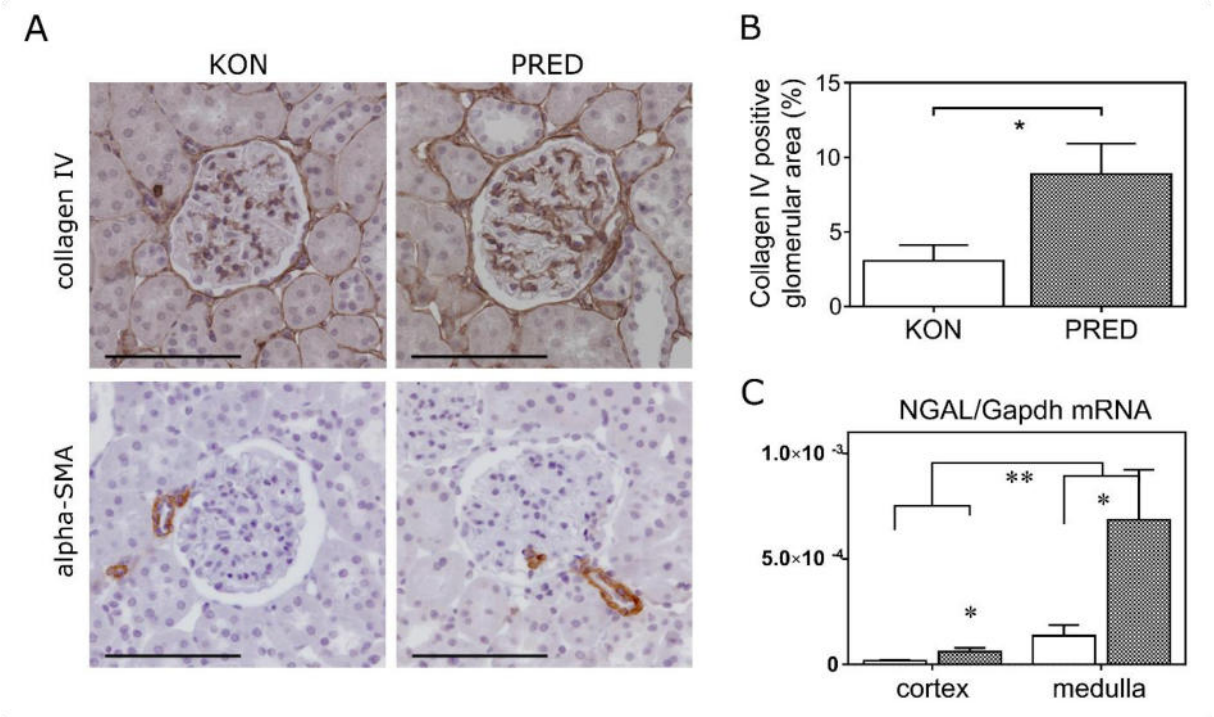

Figure 2. The earliest signs of renal impairment in prediabetic obesity. (A) Representative immunohistochemistry images show specific collagen IV (above) and alpha-SMA (below) staining in control (KON, left) and obese, prediabetic (PRED, right) rat kidney glomeruli. (B) Larger area of collagen IV-stained glomerular surface shows increased amounts of this protein in ECM of obese, prediabetic glomeruli, but no mesangial cell activation was evident as αSMA staining was non-detectable; (C) The tubular damage marker NGAL gene (Lcn2) mRNA-expression increased in obese, prediabetic kidneys. Data: mean ± SEM, n≥7/group, 2-way, repeated measure ANOVA; unpaired Student's t-test (B-E); *: $p<0.05$, **: $p<0.01$.

Lack of renal inflammation and activation of fibrosis pathways

Expression of TGF-β1 and short-form of leptin-receptor (Ob-Ra), which can mediate renal fibrosis by elevated plasma leptin, did not change. Similarly, neither the expression of inflammation indicator cytokines, IL-1β and TNFα, nor miRNAs known for fine-tuning of inflammatory and fibrotic processes showed any increase in the treatment groups.

Surprisingly – but in line with our above adipose tissue and systemic observations – expression of IL-1β mRNA even decreased in both the kidney cortex and medulla of obese, prediabetic rats compared to controls.

Overall we may conclude that the unexpectedly subtle renal effects are probably due to lack of dyslipidemia, systemic and local inflammation, which we have uniquely documented in our model contrary to published results.

The miR-193a-induced FSGS study

Advanced mouse FSGS induced by 8 weeks of miR-193a-overexpression

In miR-193a-overexpressing mice, we detected ultrastructural signs such as podocyte foot-process detachment and fusion, podocyte loss, widening of GBM; and glomerulomegaly, extracellular protein accumulation, intracapillary cast formation in electron and light microscopy respectively, together with severe albuminuria compared to healthy wild-type controls.

The glomerular extracellular matrix proteome in mouse FSGS

Our mass spectrometry analysis identified more than 1500 different proteins in the glomerular ECM, amongst which we detected 111 ECM-associated proteins, which corresponds to the extent of previously made observations on similar samples in healthy mice of different gender and genetic background.

Concerning the strongly significant quantitative concentration changes in glomerular ECM protein samples, 18 proteins increased, 2 proteins decreased and 4 proteins (CFP, ITIH1, PIGR, PSTD) were exclusively detected in the glomerular ECM of FSGS mice compared to healthy controls. The accumulation of fibrinogen/fibrin and collagen I were confirmed by specific immunostaining.

Pathophysiological changes observed in FSGS

Based on manual curation (Matrisome database and Pubmed search) and Ingenuity Pathway Analysis, the most significant phenomena in *miR-193a-overexpressing* glomerular ECM were extensive acute phase response activation, indicated by the increased presence of complement components, fibrinogen pathway, and coagulation cascade elements. At the same time, we found increased incidence of a few serine protease inhibitors that potentially contribute to adverse changes in ECM turnover.

Table 1. Significantly dysregulated genes in miR-193a-overexpressing glomeruli.

ID	Symbol	Gene Name	FC
P11680	CFP	Properidin	*
F8WJ05	ITIH1	Inter-alpha-trypsin inhibitor 1	*
O70570	PIGR	Polymeric Immunoglobulin Receptor	*
P50404	SFTPD	Pulmonary surfactant-associated protein D	*
Q566I6	C1R	Complement C1r	27.8
A8DUV3	HBA1	Hemoglobin subunit alpha 1	18.9
Q3UER8	FGG	Fibrinogen gamma chain	15.4
Q3TGR2	FGB	Fibrinogen beta chain	10.0
Q91X17	UMOD	Uromodulin	8.8
Q54AH9	HBB-B2	Hemoglobin subunit beta-2	8.7
Q542I3	CRP	C-reactive protein	7.6
A8DUK0	HBB-B1	Hemoglobin subunit beta-1	7.4
P01029	C4B	Complement C4-B	6.2
P07759	SERPINA3	Serine protease inhibitor A3K	6.1
A0A2P9DUN6	SERPINA1	Alpha-1-antitrypsin 1	5.6
Q00623	APOA1	Apolipoprotein A-I	5.3
P11087	COL1A1	Collagen alpha-1(I) chain	4.9
Q8VBX5	PPT1	Palmitoyl-protein thioesterase 1	4.8
Q9JMG7-2	HDGFL3	Hepatoma-derived growth factor-related protein 3	−5.2
O55186	CD59A	CD59A glycoprotein	−5.7

Differentially regulated genes assessed by pairwise ratio comparison of glomeruli in miR-193a-overexpressing and wild-type mice; * not detectable in

control samples; ID, protein accession number; FC, fold change miR-193a versus wild-type.

Typical glomerular ECM changes in FSGS

Comparing the mir-193a-FSGS glomerular gene-expression profile with that previously published in the same mouse model (Gebeshuber et al, 2013) and human FSGS glomeruli (Bennet et al. 2007), a central role of uromodulin (UMOD), SERPINA1 and the complement system can be suspected in the pathomechanism of FSGS. UMOD plays in inflammatory and immune regulatory roles in the kidney, while the serine protease inhibitor SERPINA1 is implicated in ECM homeostasis and coagulation cascade interactions as well. Recognition of complement system activation in FSGS draws attention to the mechanistic role of innate immunity in the process of progressive renal fibrosis.

Conclusions

Based on our results obtained in two different rodent models, we draw the following conclusions on the pathomechanism of glomerulopathy:

In obese Long Evans rat with mild glucose metabolism disorder due to 20 weeks of high-fat diet and a single, small dose streptozotocin injection

(1) Morphological and functional signs of organ damage due to obesity and prediabetes occur earlier in the heart and liver than in the kidney.

(2) The earliest signs of obesity-related renal impairment are increased proportion of collagen IV (as part of glomerular extracellular matrix remodeling), and increased mRNA expression of Lcn2 (NGAL), an indicator of tubular injury (which precedes the decline of renal function).

(3) Chronically high plasma leptin level alone does not cause obesity-related nephropathy, glomerulosclerosis or tubulointerstitial fibrosis.

(4) Despite significant obesity, the lack of dyslipidemia, adipose tissue and systemic inflammation – that we uniquely found in the literature – suggests that these factors are closely related to the development of kidney injury in obesity and/or mild glucose metabolism disorder. The Long Evans rat strain may be suitable for studying the mechanisms of relative resistance to nephropathy.

In advanced FSGS mouse model induced by miR-193a overexpression analysis of glomerular extracellular proteins and glomerular gene-expression profile:

(5) More than 60, partly known but many new, not yet related to FSGS, differentially expressed genes have been detected, furthermore

(6) Properdin (CFP), inter-α-trypsin inhibitor 1 (ITIH1), polymer immunoglobulin receptor (PIGR) and pulmonary surfactant-

associated protein D (SFTPD) were identified exclusively in FSGS glomeruli.

(7) Regarding the pathomechanism of FSGS the most significant changes affected the complement system, the fibrinogen pathway, some coagulation cascade elements and a few protease inhibitors potentially regulating ECM turnover. All the factors listed above deserve increased attention for primary FSGS therapy to be developed in the near future.

Candidate's List of Publication

Related to the theme of the PhD thesis

Bukosza EN, Kornauth C, Hummel K, Schachner H, Huttary N, Krieger S, Nöbauer K, Oszwald A, Razzazi Fazeli E, Kratochwill K, Aufricht C, Szénási G, Hamar P, Gebeshuber CA (2020). ECM Characterization Reveals a Massive Activation of Acute Phase Response during FSGS. Int J Mol Sci. 21(6). pii: E2095. **IF(2018/19): 4,183**

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Unrelated to the theme of the PhD thesis

Bukosza EN, Kratochwill K, Kornauth C, Schachner H, Aufricht C, Gebeshuber CA.(2020). Podocyte RNA sequencing reveals Wnt- and ECM-associated genes as central in FSGS. PLoS One. 15(4):e0231898. **IF(2018/19): 2,776**

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