Properties of the glomerular endothelial cell surface layer in vitro and in vivo

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University of Gothenburg 2011

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in manuscript).

Tack Mormor!

Abstract

A healthy kidney produces final urine that is practically devoid of proteins and other physiologically important solutes. Tremendous amounts of fluid are filtered every day through the glomerular filtration barrier which is the actual sieving site in the kidney. Failure of the filtering function leads to proteinuria, which is a feature common to nearly all kidney disease. In spite of this pivotal role, the central mechanisms behind proteinuria are still unexplained. The filtration barrier is a complex biological membrane composed of four different structures: the podocytes (epithelial cells), the glomerular basement membrane, the glomerular endothelium and the glomerular endothelial cell surface layer (ESL). During the last decade the focus for understanding the regulation of this selective sieve has rested heavily on the study of the podocytes, whereas the glomerular endothelium and the glomerular ESL has been more or less neglected as contributors to the permselectivity of the barrier. However, it is of fundamental importance to investigate all components of the filtration barrier in order to understand the pathophysiology of proteinuric kidney disease. The molecular structure of the glomerular ESL is largely unexplored, and available data about its constituents so far is only based on *in vitro* studies.

The aim of this thesis was to identify molecules located in the glomerular ESL with a functional significance for normal glomerular filtration *in vivo*, and to examine whether a disease-emulating milieu damages major structural glomerular ESL components and thus increases the glomerular permeability to proteins. We have developed a method for qualitative and quantitative assessment of the glomerular ESL in rats, which includes a brief injection of hypertonic sodium chloride into the renal artery. This displaces and elutes non-covalently bound components of the glomerular ESL which are then subsequently collected for further characterization with liquid chromatography-mass spectrometry. Morphological as well as functional effects have been characterized by electron microscopy and by universal methods analyzing charge- and size selectivity of biological membranes. A conditionally immortalized human glomerular endothelial cell line was used to study the effects of hyperglycemia on glomerular ESL proteoglycans. Functional alterations were analyzed in terms of protein restriction by measuring the passage of albumin across a human glomerular endothelial cell monolayer.

In conclusion, we have identified molecules from the glomerular ESL in rats that are essential for maintaining a normal glomerular barrier function. Further, we found that hyperglycemia was associated with an alteration of glomerular ESL proteoglycans which lead to an increased permeability for albumin. Overall, the observations in this thesis emphasize the importance of the glomerular ESL for the restriction of proteins in the glomerular filtration barrier.

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **High glucose causes dysfunction of the human glomerular endothelial glycocalyx**[1]
Singh A, <u>Fridén V</u>, Dasgupta I, Foster R.R, Welsh G.I, Tooke J.E, Haraldsson B, Mathieson P.W and Satchell S.C
Am J Physiol Renal Physiol 300:F40-F48 2011

II. **The glomerular endothelial cell coat is essential for glomerular filtration**[2]
<u>Fridén V</u>, Oveland E, Tenstad O, Ebefors K, Nyström J, Nilsson U.A and Haraldsson B
Kidney International, advance online publication*,* 16 March 2011;
doi:10.1038/ki.2011.58

III. **Further identification of components of the renal capillary endothelial cell coat**
<u>Fridén V</u>, Oveland E, Tenstad O, Björnson-Granquist A, Nyström J, Nilsson U and Haraldsson B
Manuscript

[1] *The Am Physiol Soc*, used with kind permission.

[2] Used with kind permission from *Kidney International.*

Abbreviations

Å	Ångström (1 Å =0.1 nm)
CD44	Cluster of differentiation 44
CS	Chondroitin sulfate
DS	Dermatan sulfate
EC	Endothelial cell
ECC	Endothelial cell coat
ELISA	Enzyme-linked immunosorbent assay
ESL	Endothelial cell surface layer
EXT	Exostoses
EXTL	Exostoses-like
FITC	Fluorescein isothiocyanate
GAGs	Glycosaminoglycans
GalNAc	N-acetyl-galactosamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
GlcA	Glucuronic acid
GlcNAc	N-actyl-glucosamine
GPI	Glycosyl-phosphatidylinositol
HA	Hyaluronic acid, hyaluronan
HCL	Hydrochloric acid
HO	High osmolarity
HPLC	High performance liquid chromatography
HS	Heparan sulfate
HS	High salt
IDF	International Diabetes Federation
IdoA	Iduronic acid
kDa	kilo Dalton
KS	Keratan sulfate
LC-MS	Liquid chromatography-mass spectrometry
M	Molar
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NDST	N-deacetylase/N-sulfotransferases
NS	Normal salt
PAPS	3´phosphoadenosine 5´-phosphosulfate
PG	Proteoglycan
RHAMM	Receptor for hyaluronic acid mediated motility
rRNA	Ribosomal ribonucleic acid

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPLPO	Large ribosomal protein
RT-PCR	Reverse transcriptase-polymerase chain reaction
SLRPs	Small leucine-rich repeat proteoglycans
TEER	Transendothelial electrical resistance
UDP	Uridine diphosphate
Xyl	Xylose

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1 Introduction

The kidney is a vital organ with several functions such as the regulation of fluid and electrolytes, maintenance of acid-base balance and the production of hormones which regulates calcium balance, blood pressure and the production of red blood cells. However, one of the major functions is to filter the blood and thereby removing waste products and excess fluid from the body via the urine. This process occurs in a large number of small functional units within the kidney, known as nephrons. A nephron consists of a tuft of glomerular capillary loops which is surrounded by Bowman's capsule attached to a convoluted tubule.

The blood is filtered under high pressure across the glomerular capillary walls. Under normal conditions proteins and blood cells are retained in the blood whereas water and small solutes can pass freely and continue into the tubule, where complex steps of excretion and reabsorption takes place before the final urine is produced. The rate of filtration is approximately 125 ml/min; since the human body contains roughly 5 liters of blood the entire blood volume is filtered 36 times per day.

The accumulation of proteins in the urine, that is to say proteinuria, is a primary feature of renal disease. Proteinuria is the result of an impaired glomerular capillary wall, synonymous with the glomerular filtration barrier. Although a key aspect of renal disease, the underlying mechanism behind proteinuria is still largely unknown. It is therefore essential to obtain more information about the filtration barrier under normal as well as pathological conditions. Added knowledge in this field will allow us to identify targets for a better and more specific treatment of many nephrological disorders.

1.1 The glomerular filtration barrier

In contrast to other capillaries, glomerular capillaries have a high permeability to water (hydraulic conductivity). Nonetheless they are able to remain relatively impermeable relative to macromolecules, similar to other capillaries. This is due to the unique structure of the glomerular filtration barrier, which is a complex biological membrane composed of four distinct layers that fluid and solutes must pass to reach the urinary space. The layers appears in the following order from the capillary lumen to the urinary space (Figure 1): 1) the glomerular endothelial cell surface layer (ESL), 2) the fenestrated glomerular endothelium, 3) the glomerular basement membrane (GBM), and 4) the podocyte [1, 2].

In terms of permselectivity the glomerular endothelium and the glomerular ESL have been only modestly investigated compared to the GBM and the podocytes. The major reason for this is likely the fact that glomerular endothelial cells possess transcellular pores (fenestrae) which were thought of as empty and therefore
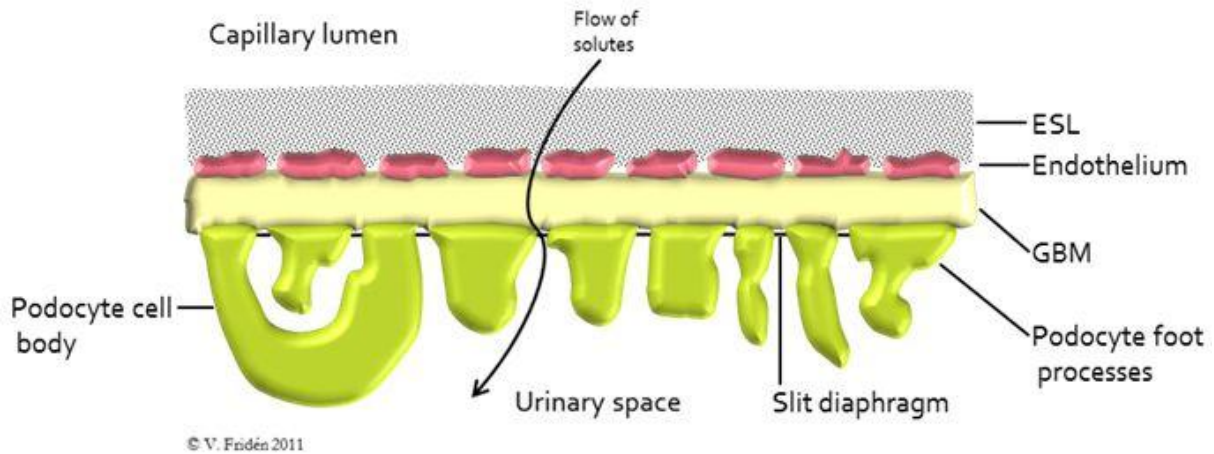
Figure 1. A representation of the glomerular filtration barrier showing the four-layer structure consisting of the glomerular endothelial cell surface layer (ESL), the fenestrated glomerular endothelium, the glomerular basement membrane (GBM) and podocyte cell body. Also shown are the podocyte foot processes with their slit diaphragms.

providing minute resistance to the passage of solutes. In addition, the glomerular ESL was for a long time impossible to visualize because of its intricate structure. However, there is a growing interest of the glomerular endothelium and its cell surface layer, and evidence is accumulating concerning their critical contribution to the permselective properties of the glomerular filtration barrier.

1.1.1 Podocytes

Podocytes are highly specialized epithelial cells covering the outside of the glomerular capillaries [3]. The podocyte has an extraordinary morphology consisting of a cell body that forms branches that extend into long thin foot processes. The basal surface of the foot processes is firmly attached to the underlying GBM via various adhesion molecules such as integrins and dystroglycans, whereas the apical surface faces the Bowman's capsule and the primary urine [4-7]. The foot processes of neighboring podocytes regularly interdigitate, forming a filtration slit that is bridged by a cell-cell junction, termed the slit diaphragm (Figure 1) [8]. The slit diaphragm is a dynamic multiprotein complex important for the permselectivity in the barrier, and it contains proteins that are not generally found elsewhere in the body [9].

The knowledge of the molecular structure of the slit diaphragm has increased considerably during the last decade. Several proteins, including nephrin, Cd2ap, Neph1-3, podocin, Trpc6, Fat1, and P-cadherin have all been shown to be associated with the slit diaphragm complex, and some of these proteins are crucial for normal glomerular filtration [9-17]. Moreover, the apical surface of the podocyte is covered by a negatively charged layer, rich in sialylated proteins like podocalyxin, α-dystroglycan and sulfated molecules such as proteoglycans [6, 18-

21]. The negative charge is important for the maintenance of the podocyte architecture and to prevent adhesion of neighboring podocytes or parietal epithelial cells [22, 23].

The podocytes are important for the structural integrity of the glomerular capillary wall, and the absence of specific components of the slit diaphragm, or any type of podocyte injury, result in a disrupted filtration barrier [24, 25].

1.1.2 Glomerular basement membrane

The GBM is an acellular, extracellular matrix layer forming the structural foundation of the glomerular capillary to which endothelial cells and podocytes are anchored on both sides [26]. The GBM accounts for 50% of the restriction of fluid flux (hydraulic resistance) in the glomerular filtration barrier [1, 27] and like all basement membranes, the GBM is composed mainly of type IV collagen, laminins, nidogen and various proteoglycans [28, 29]. The interconnection of type IV collagen and laminins with nidogen creates a scaffold that provides structural support and multiple binding sites for other components in the GBM. In addition, laminins and collagen are involved in the adhesion of endothelial cells and podocytes to the GBM [30]. Mutations in type IV collagen genes result in Alport's syndrome, a disease that manifests as hematuria and progressive renal disease but only mild proteinuria [31]. Moreover, both laminin β2 chain deficient mice and patients with laminin β2 gene mutation exhibit massive proteinuria [32, 33].

The GBM contains large amounts of heparan sulfate proteoglycans, predominantly agrin and perlecan, and their negative charge has for a long time been considered important for the charge selective filtration in the barrier [30, 34]. However, the primary role of heparan sulfate in the GBM regarding renal permselectivity has recently been questioned [35]. Genetically engineered mice lacking podocyte-derived agrin and perlecan heparan sulfate side chains do not develop proteinuria [36]. Podocyte-specific EXT1-deficient mice, lacking heparan sulfate polymerase and mice overexpressing the heparan sulfate degrading enzyme heparanase, also do not develop proteinuria, despite a reduction of anionic sites in the glomerular basement membrane [37]. Together this suggests that the majority of the effective charge selectivity in the glomerular filtration barrier lies in the endothelial and the epithelial layer.

1.1.3 Glomerular endothelial cells

Endothelial cells line the interior surface of all blood vessels in the body, thus forming the boundary between intra- and extravascular compartments. There is a large heterogeneity of endothelial behavior, structure and biochemical composition among endothelial cells derived from different species, organs and capillary beds. The endothelial cell is the principal regulator of vascular permeability, in addition to

its other important physiological functions such as control of vasomotor tone, hemostasis, leukocyte adhesion and trafficking, inflammatory responses and the formation of new blood vessels [38, 39]. There are three major phenotypes of endothelium recognized in the body; continuous (lacking fenestrations), fenestrated and discontinuous (lacking a continuous basal lamina). Here, the focus will be on the specific structure and function of the glomerular endothelium, which belongs to the fenestrated phenotype [40, 41].
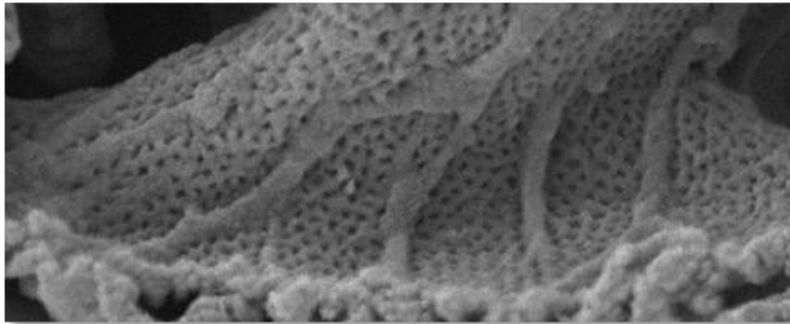
Figure 2. Scanning electron micrograph of fenestrated glomerular endothelium from mouse. By Dr. M. Jeansson.
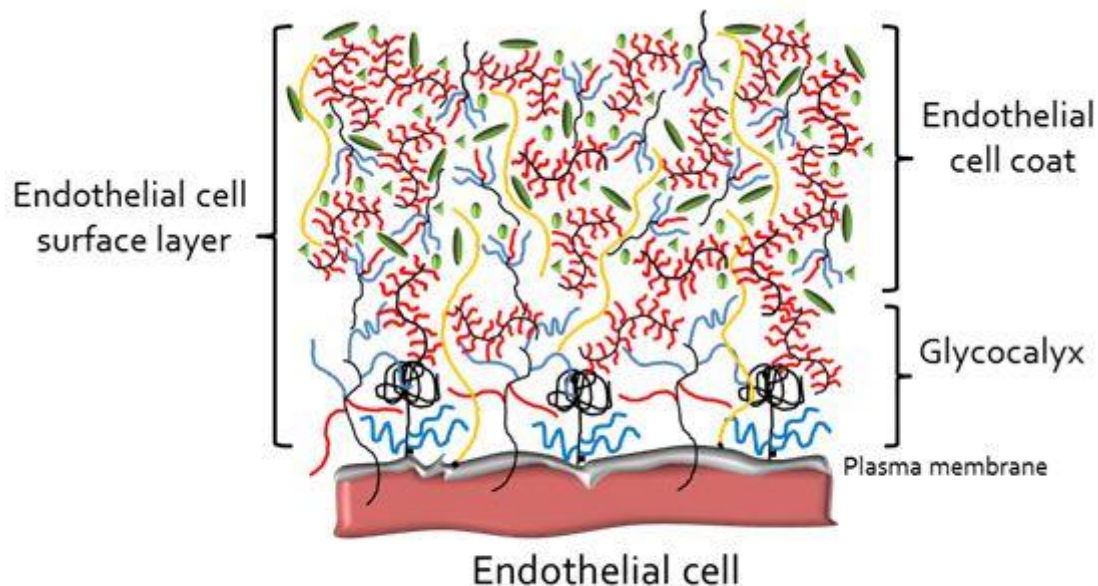
The glomerular endothelial cells are unique in many aspects compared to most other endothelial cells in the body. They have numerous round transcytoplasmic pores (fenestrations), which do not possess a diaphragm in the adult glomerular capillary (Figure 2). They are extremely flat, especially around the capillary loops periphery [42, 43]. The pores are 60-80 nm in diameter and constitute 20-50% of the total glomerular capillary surface area [2]. The unusually high density of pores is thought to be a prerequisite for the high permeability of glomerular capillaries to water and small solutes (hydraulic conductivity) [1]. There is a substantial body of evidence that glomerular endothelial dysfunction is a major determinant for the pathogenesis of preeclampsia, which is characterized by a reduced glomerular filtration area, glomerular capillary endotheliosis, loss of glomerular endothelial pores and mild proteinuria [44, 45].

Primary cultures of glomerular endothelial cells have been little studied in culture because of their poor replicative potential leading to phenotype alterations and early senescence. This issue was addressed by Satchell *et al.* a few years ago by generating a conditionally immortalized human glomerular endothelial cell line. These cells are a resource for the study of glomerular endothelial cells and their roles in glomerular filtration, glomerular disease, and response to glomerular injury [46]. Historically, glomerular endothelial cells have received little attention when it comes to permselectivity of the glomerular barrier and glomerular endothelial pores was actually for a long time believed to be wide open holes that allow free passage

of large proteins. However, the fenestrated glomerular endothelium, together with the dynamic negatively charged glomerular ESL reviewed in the next section, contributes indeed to the permselectivity of the barrier [47-49].

1.1.4 Glomerular endothelial cell surface layer

The luminal side of all endothelia is covered with a highly negatively charged gel-like mesh, the endothelial cell surface layer, consisting of two components: the glycocalyx and the endothelial cell coat (Figure 3). In this thesis the term glycocalyx refers to the cell membrane-bound part of the layer and the endothelial cell coat corresponds to the thicker, more loosely associated part of the layer [2]. It has been shown that the endothelial cell surface layer is involved in blood coagulation, modulation of angiogenesis, capillary rheology, and capillary barrier function [48-53].

Figure 3. Hypothetical drawing of the glomerular endothelial cell surface layer (ESL) which is composed of glycocalyx and the endothelial cell coat. Glycocalyx is the" backbone" of ESL and is dominated by cell membrane bound molecules such as glycoproteins and proteoglycans. Onto glycocalyx, the much thicker endothelial cell coat (ECC) is attached, which consists of an intricate arrangement of soluble molecules such as proteoglycans (PG), glycosaminoglycans (GAG), and proteins derived from the endothelium and/or the plasma.

The glycocalyx was visualized already 45 years ago with electron microscopy and has been suggested to be approximately 50-100 nm thick, and consist of a large variety of membrane-bound molecules. These include glycolipids, glycoproteins and membrane-bound proteoglycans [41, 54-57]. Since standard electron microscopy did not allow the visualization of the very delicate and highly aqueous

structure of the endothelial cell coat, the effective thickness of this layer had to be estimated using other approaches.

The thickness of the endothelial cell coat is approximately 200-400 nm depending on the plasma composition and local hemodynamic conditions since this layer is in dynamic equilibrium with the flowing plasma [58]. The endothelial cell coat consists of soluble molecules embedded in the glycocalyx, that is to say, secreted proteoglycans, glycosaminoglycans (hyaluronic acid) and proteins derived from the endothelium or the circulating plasma [57, 59, 60]. It is important to keep in mind that the endothelial cell surface layer is not a static structure. Instead, it's a very dynamic structure, defined by a balance between biosynthesis and shedding [61]. The composition of the endothelial cell surface layer differs widely between different types of vasculature and endothelial cells seem to adapt the expression of components depending on their location and functional requirements [62, 63].
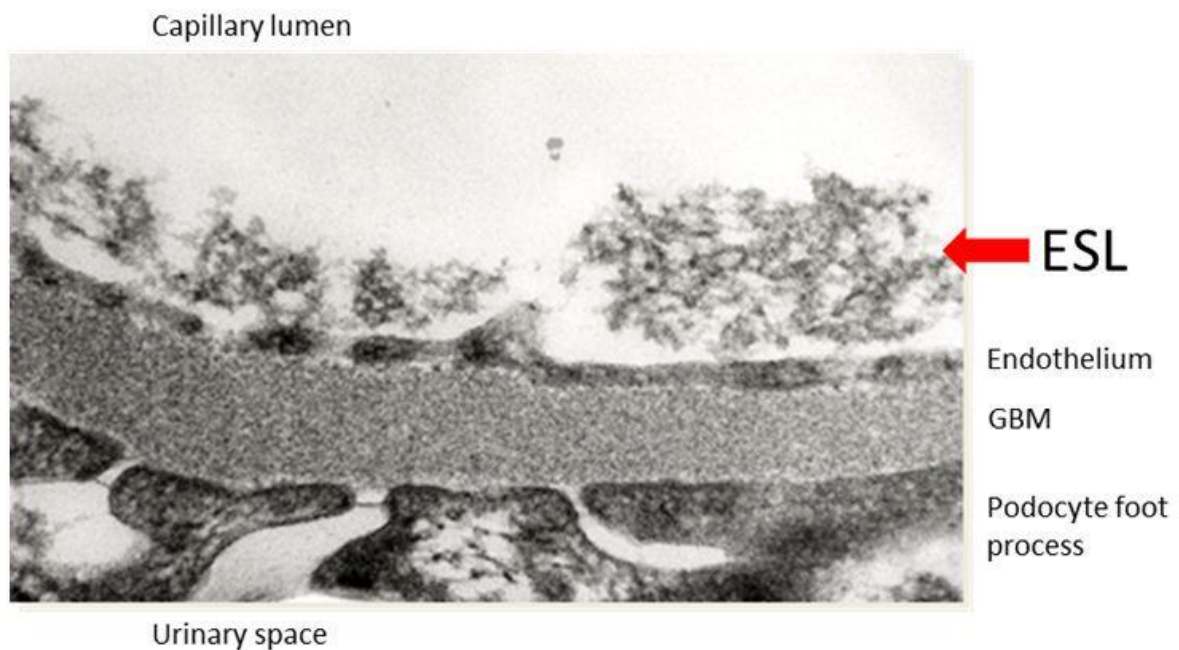
Figure 4. Transmission electron micrograph of a cross-section through the glomerular barrier from rat, where the glomerular endothelial cell surface layer (ESL) is visualized with Cupromeronic Blue staining. GBM is the glomerular basement membrane. By Dr. C. Hjalmarsson.

The composition and the physiological properties of the glomerular ESL have been sparsely investigated compared to the other layers in the glomerular filtration barrier, most likely because this delicate and fragile structure is difficult to preserve and visualize (Figure 4). However, it has been reported that the glomerular ESL covers the entire endothelium including the pores (fenestrae), and the glomerular endothelial cell coat has been suggested to be thicker than in other vessels [55, 64, 65].

Proteoglycans are an important constituent of the glomerular ESL, and they are partly responsible for its anionic properties that help retain macromolecules. Human glomerular endothelial cells express several types of proteoglycans: syndecans, glypicans (membrane-bound) and versican, perlecan, decorin and biglycan (secreted) [66]. There is also indirect evidence that hyaluronic acid (glycosaminoglycan) is present in the glomerular ESL [48]. Two circulating plasma proteins, albumin and orosomucoid are suggested to be included in the glomerular ESL, and these proteins are thought to contribute to a denser and more anionic structure, and they seem to be important for the permselectivity of the glomerular barrier [2, 67-69]. Likely mechanisms through which albumin and orosomucoid acts are by increasing the negative charge, physically reducing the space and acting as organizing molecules in the glomerular ESL [70-72].

Several studies suggest a role and provide evidence for the importance of the endothelial cell surface layer in glomerular permselectivity [73-77]. Infusion of enzymes (hyaluronidase, heparinase, and chondroitinase) that degrades glycosaminoglycans in mice reduced the thickness of the glomerular ESL. This morphological modification was accompanied by an increase in the flux of albumin across the glomerular filtration barrier [48]. In a mouse model of adriamycin-induced proteinuria, an apparent decrease of the thickness of the glomerular ESL was seen compared with control, maybe due to a down-regulated proteoglycan synthesis. In addition, the glomerular charge- and size selectivity was also altered [78]. The enzymatic removal of heparan sulfate glycosaminoglycans from the endothelial cell surface layer of cultured human glomerular endothelial cells lead to the disruption of its albumin-restricting ability in an established *in vitro* model [79]. The glomerular ESL undoubtedly contributes to the restriction of solutes. However, more information is required about this relatively uninvestigated layer of the glomerular filtration barrier regarding its composition and stability.

1.1.5 Glomerular filtration

The filtration pathway across the glomerular filtration barrier is extracellular; water and filtered solutes pass through the endothelial pores or between cells [75, 80]. It is generally accepted that the filtration barrier restricts the passage of solutes, depending on their size, charge and shape [76, 81-83]. Solutes with a Stokes-Einstein radius below 18 Å are filtered freely, while solutes that exceed 42 Å are close to completely restricted in the barrier. The transport of solutes across the glomerular filtration barrier occurs by a combination of convection (solutes gets a ride by the pressure driven filtration of water) and diffusion (random molecular motion) [1, 84]. According to models where macromolecular filtering properties of the glomerular filtration barrier has been correlated with the properties and structure of its components the filtration barrier should be seen as a single unit because each layer makes a significant contribution to selective permeability. Thus

proteinuria will occur regardless of which layer is affected by disease [1, 47, 75, 85]. It is significant to consider and investigate all layers in order to solve the conundrum of proteinuria in different renal pathological conditions.

1.2 Proteoglycans

The study of proteoglycans (PG) was initiated already in the late 19th century when chondroitin sulfate was isolated from cartilage tissue [86]. PGs are a very heterogeneous group of molecules that are found widely among vertebrates and invertebrates, suggesting essential functions in animal physiology [87, 88]. PGs are an important component of the extracellular matrix and they are involved in a large number of biological activities, such as cell signaling, adhesion, tissue organization, and they regulate the activity of growth factors, proliferation, migration and the movement of molecules through matrix [89]. A dual function for these negatively charged molecules in kidney physiology has been suggested: maintaining a fixed negative charge in the glomerular filtration barrier, and binding and storing cytokines essential for renal development and function.

1.2.1 Glycosaminoglycans

PGs consists of one or more long, linear and highly negatively charged polysaccharide chains called glycosaminoglycans (GAG), covalently attached to a core protein through a link region. The link region is a specific trisaccharide composed of one xylose (Xyl) O-linked to a serine residue in the core protein, followed by two galactose (Gal) residues [90]. The link region serves as an attachment site for the elongation of the GAG chain which consists of repeating disaccharide units. The repeating units are composed of a hexuronic acid, either glucuronic acid (GlcA) or iduronic acid (IdoA) and amino sugar N-acetyl-glucosamine (GlcNAc) or N-acetyl-galactosamine (GalNAc). Sulfation in various positions of the disaccharide unit determines the magnitude of the negative charge in the GAG.

There are five main groups of GAGs, classified according to the structure of the disaccharide unit: 1) chondroitin sulfate (CS) consisting of GlcA-GalNAc, 2) dermatan sulfate (DS) consisting of IdoA-GalNAc, 3) heparan sulfate (HS) consisting of GlcA or IdoA -GlcNAc, 4) keratan sulfate (KS) consisting of Gal-GlcNAc and 5) hyaluronic acid (HA) consisting of GlcA-GlcNAc [89, 91].

KS and HA differs from the other GAGs in some aspects. KS is also referred to as polylactosamine and is not as common as CS, DS and HS [92]. KS contains a galactose instead of an uronic acid as the residue that alternates with GlcNAc in the disaccharide unit, and it is N-linked to asparagine residues or O-linked to threonine residues with another type of link region [93]. Moreover, HA is not attached to a core protein and its disaccharide chain is unsulfated in contrast to all the other

GAGs [94]. HA is synthesized at the inner surface of the plasma membrane by membrane-bound synthases, while all other GAGs are synthesized in the Golgi and, because of its mode of synthesis, HA is extruded directly into the extracellular space. HA can form large aggregates and be attached to the cell surface via receptors such as CD44 and, RHAMM (Receptor for Hyaluronic Acid Mediated motility). HA can also be retained at the cell surface by sustained transmembrane interactions with its synthases. Both types of retention can generate a large pericellular matrix, which could incorporate several other hyaluronan-binding molecules. Its disaccharide units are identical and the chain length can be enormous (up to 25,000 disaccharides) [95-97].

1.2.2 The biosynthesis of chondroitin sulfate and heparan sulfate

The building blocks for GAG synthesis, monosaccharides and sulfate are metabolized by cells, converted in the cytosol to uridine diphosphate (UDP)-sugars and 3´phosphoadenosine 5´-phosphosulfate (PAPS) respectively, and translocated into the endoplasmatic reticulum (ER) and Golgi apparatus where the synthesis of GAG chains occur [91, 98]. PAPS is the universal donor of sulfate to all sulfotransferases, both in the Golgi and the cytosol. The biosynthesis of CS and DS is carried out by four glycosyltransferases (chondroitin synthase, chondroitin GalNAc transferase-1 and -2 and chondroitin GlcA transferase) those are responsible for the initiation and the elongation of the chains [99-103]. Specific sulfotransferases catalyze the sulfation of position 6 and 4, respectively, of the GalNAc residues. The C5 epimerase convert GlcA units to IdoA to form DS and an O-sulfotransferase can catalyze the sulfation of position 2 of the IdoA residue [91]. The biosynthesis of HS involves a large number of enzymes. The main enzymes responsible for the initiation and polymerization are the exostoses 1 and 2 (EXT 1 and 2) [104]. There are other enzymes that have been suggested to be involved in the polymerization of the HS chain namely exostoses-like 1-3 (EXTL 1-3). Less is known about them, although EXTL2 has been suggested to play a critical role in the initiation of HS synthesis [105, 106]. During the HS polymerization, a series of modifications can occur, there are four isoforms of N-deacetylase/N-sulfotranseferase (NDST) that can remove acetyl groups from GlcNAc residues, and add sulfate to the free amino groups. NDST-1 and NDST-2 are widely expressed in all investigated tissues [107], while NDST-3 and NDST-4 show a more restricted distribution and are mainly expressed during embryonic stages [108]. Moreover, the C5 epimerase might convert GlcA to IdoA and a group of sulfotransferases can catalyze the sulfation of position 6 and 3, respectively, of the GlcNAc residue and position 2 of the GlcA or the IdoA. PGs can be classified according to several parameters [89] but cited PGs in this thesis are only separated into two groups, cell membrane-bound and secreted. They are also related somehow to the included papers and to the glomerular filtration barrier. However,

there are proteoglycans associated with the glomerular filtration barrier not mentioned in this thesis.

1.2.3 Cell membrane-bound proteoglycans

Syndecans are a family of four members (syndecan-1, -2, -3 and -4), consisting of a type I transmembrane core protein and they are therefore able to transduce signals from the extracellular matrix to the interior of the cell and interact with the cytoskeleton [109, 110]. The core protein size ranges from 20-40 kDa and contains a short cytoplasmic domain, a transmembrane domain, and an extracellular domain with attachment sites for three to five GAG chains. The extracellular domain of syndecan core protein is believed to be an elongated structure based on the high proline content. HS is the primary GAG present in all four syndecans, although syndecan-1 and -4 may also be substituted with CS.

The syndecans are involved in many biological activities, binding of growth factors, adhesion, binding of extracellular matrix components, cell signaling. Syndecan-1 is the major syndecan of epithelial cells including the vascular endothelium. Syndecan-2 is present mostly in cells of mesenchymal origin, whereas syndecan-3 is found primarily in neuronal tissues and syndecan-4 is ubiquitously expressed. Syndecan-1 is an important regulator of cell–cell and cell–extracellular matrix interactions, and syndecan-4 is involved in the modulation of cytoskeletal rearrangements, which is important in regulation of cell adhesion and spreading [109, 111-113].

Glypicans are a family of six members (glypican-1, -2, -3, -4, -5 and -6), which are bound to the plasma membrane through a GPI-anchor (glycosyl-phosphatidylinositol). The size of the core protein of glypican is similar (60–70 kDa), and it is suggested to be folded into a disulfide-bonded globular protein, due to the presence of a large number of cysteine residues. In contrast to syndecans, glypicans are only substituted with HS chains, and these three HS chains are located close to the C-terminus end of the core protein. The main functions of glypicans are suggested to be interaction with other cell-surface molecules, growth factor regulation, morphogenesis and signaling. Glypican-1 is expressed ubiquitously, whereas glypican-2 does not appear to be expressed in the adult human. Glypican -3, -4 and -5 expression is limited to the nervous system in the adult whereas glypican-6 is less restricted and has been detected in heart, liver, kidney, ovary and intestine [114-116].

1.2.4 Secreted proteoglycans

Versican is a large extracellular matrix PG that is present in a variety of tissues. Versican occurs in four isoforms (V0, V1, V2 and V3), and the size of the core proteins and the number of CS chains is dependent on alternative splicing of the

versican mRNA transcript. The number of potential CS GAG attachment sites in human versican is 17–23 for V0, 12–15 for V1, 5–8 for V2, and 0 for V3 and the size of the core proteins range from 72-370 kDa (V3-V0) [117]. The precise distribution and function of the isoforms are not fully elucidated. However, versican is known to form enormous networks together with hyaluronic acid within the extracellular matrix for example in blood vessels [89]. The highly negative nature of versican makes it very interactive and versatile. Versican plays an important role as a structural molecule, creating loose and hydrated matrices. It interacts directly with cells, or indirectly with molecules that associate with cells, to partly regulate cell adhesion and survival, cell proliferation, cell migration and extracellular matrix assembly [53, 118].

Perlecan has a core protein molecular weight of 470 kDa. It can be substituted with four GAG chains; predominately HS but, it may also carry CS chains. Together with the GAG chains and numerous O-linked oligosaccharides, perlecan can reach a total molecular weight of 800 kDa. Perlecan is an important component of the vascular extracellular matrix, connective tissues and nearly all basement membranes. The core protein of perlecan and its HS chains are involved in a large number of different molecular interactions. It can bind and regulate several extracellular matrix components, including growth factors, and it is involved in maintaining endothelial barrier function [89, 104, 119, 120].

Lumican belongs to the structurally related members of the SLRPs (small leucine-rich repeat proteoglycans) and has a molecular weight of approximately 40 kDa [121]. The protein has a negatively charged N-terminal domain due to sulfated tyrosine and disulfide bonds, followed by a unique tandem leucine-rich repeat domain adapted for protein–protein interactions and a C-terminal end containing two conserved cysteines. Lumican has four potential sites for the substitution of KS chains within the leucine–rich repeat domain, and KS can be sulfated in position 6 of both residues of the disaccharide unit [122, 123]. The expression of lumican has been established in several tissues, including the kidney [124]. All SLRPs are extracellular matrix organizers; the leucine-rich repeat enables lumican to interact with collagen fibrils and regulate their diameter and assembly while the negatively charged GAGs are suggested to interact with several growth factors and regulate interfibrillar spacings. Lumican is also suggested to participate in cellular functions including cell proliferation, migration, and adhesion [123]. A common putative structure for all SLRPs has been suggested in the form of an arch where GAG chains extend from the convex surface and the concave surface is free to bind collagen fibers [125, 126].

1.3 Diabetes

Diabetes is a disease associated with high levels of blood glucose caused by a deficient insulin production, impaired effectiveness of insulin action or both. Diabetes occurs in three different forms; type-1 diabetes, type-2 diabetes and gestational diabetes. In type-1 diabetes insulin producing cells are destroyed due to an autoimmune reaction, type-1 diabetes can affect people of any age, but usually occurs in children or young adults. The underlying mechanism is not fully understood. Approximately 5% with diabetes have this form of the disease while type-2 diabetes accounts for the major part of all diagnosed cases of diabetes in the United States [127] .

The development of type-2 diabetes proceeds during several years and is initiated as insulin resistance, a disorder in which the cells do not utilize insulin properly [128]. The cellular insulin signal is reduced and glucose cannot be sufficiently eliminated from the blood, leading to an increased production of insulin. This overproduction compensates for the reduced insulin sensitivity, and can mask the pathological condition for years. Eventually though, the insulin producing cells become unable to compensate for the reduced insulin response, and glucose concentration will increase in the blood. At this point type-2 diabetes has developed and can be diagnosed [129]. Several factors are associated with the development of type-2 diabetes including obesity, physical inactivity, increasing age and family history of diabetes. Gestational diabetes is triggered by an insufficient production of insulin in some women during pregnancy [130]. The world is actually facing a pandemic of type-2 diabetes, the prevalence of diagnosed people among those aged 20-79 years increase dramatically worldwide according to IDF (international diabetes federation). Diabetes can affect many parts of the body, and the major chronic complications of diabetes include cardiovascular disease, neuropathy, retinopathy and nephropathy.

1.3.1 Diabetic nephropathy and microalbuminuria

Diabetic nephropathy is the leading cause of chronic renal failure and diabetes is responsible for 30-40% of all end-stage renal disease worldwide. One of the initial detectable alterations in diabetic nephropathy is the leakage of small amounts of albumin into the urine (microalbuminuria). The exact cause of diabetic nephropathy is not yet fully understood and various mechanisms have been suggested including the direct toxic effects of high glucose concentration in the blood, causing hyperfiltration and renal injury [131]. The presence of microalbuminuria suggests an impaired glomerular filtration barrier.

Early structural changes associated with diabetic microalbuminuria are increased glomerular size, mesangial expansion and GBM thickening and extending of podocyte foot processes [132-134]. No evaluation exists of any type of structural

change in the glomerular endothelium or the glomerular endothelial cell surface layer in relation to diabetic microalbuminuria although there is evidence for a reduction of the systemic endothelial cell surface layer due to high glucose concentrations in humans [135]. In type-1 diabetes patients the presence of microalbuminuria correlates with a decreased systemic endothelial cell surface layer volume [136]. Recent studies unseal an interesting research arena that definitely needs further attention.

2 Aims

The general aim of this thesis was to identify molecules located in the glomerular endothelial cell surface layer (ESL) with a functional significance for normal glomerular filtration *in vivo* in rats, and to examine whether a disease-like milieu damage major structural glomerular ESL components and thus increase the glomerular permeability to proteins.

The specific aims of the different studies were:

I.   To investigate whether hyperglycemia, which is an important factor in the pathogenesis of diabetic nephropathy, mediates an alteration of proteoglycans in the glomerular ESL *in vitro* and to assess associated functional implications in terms of decreased protein restriction.

II. To develop a subtle *in vivo* model that allows the elution, identification and evaluation of non-covalently bound glomerular ESL molecules important for normal charge- and size selectivity in the glomerular filtration barrier.

III. To identify the entire size-range of non-covalently bound glomerular ESL molecules by using the previously developed *in vivo* model but involving a more extensive elution approach.

3 Methodological considerations

A more detailed description or original references for each experimental procedure used in this thesis are described in individual papers, while this section contains a more general discussion of selected methods.

3.1 RNA isolation and real time PCR

RNA was isolated from human glomerular endothelial cells using a commercial kit. The utilization of intact RNA is a key element for successful RT-PCR analyses. Therefore, it is important to analyze the RNA quality of samples, which was done with an Agilent 2100 Bioanalyzer (Agilent Technologies). In order to save time and exclude errors we used pre-designed primers and probes that were already tested and validated by Applied Biosystems. Furthermore, to compare gene expression levels between samples, it is necessary to normalize the expression of the gene of interest, against an endogenous control, which has to be stable and unaffected by treatments. Several endogenous controls such as 18S rRNA, GAPDH and RPLPO were validated but the most stable endogenous control in the glomerular endothelial cells was human β-actin [137].

3.2 Metabolic labeling and isolation of PG

Proteoglycans were metabolically labeled by adding [^{35}S]-sulfate and D-[6-^{3}H]-glucosamine to the cell culture media. In order to avoid exclusion of certain synthesized GAGs i.e. unsulfated hyaluronic acid, it is important to double-label GAGs. There are several methods to isolate PG from cells, such as ethanol precipitation [138]. Here we use an anion exchange column with a protocol that is relatively fast and gives a clean end product. The major reason for filtering the cell lysate/cell supernatant before applying the lysate onto the column is to remove cell debris. The different wash steps in the protocol remove acidic proteins, nucleic acids, basic proteins and free radioactive precursors. The PGs are eluted in 4 M guanidine-HCl which facilitates further separation of PG/GAG on a size exclusion column.

3.3 Determination of size selectivity

Ficolls are inert sucrose polymers that are neutral and considered to have a spherical shape. These molecules have been suggested to be the most suitable type of solute for studying permeability of membranes and are frequently used in glomerular permeability studies. Ficolls (12-72 Å) are filtered freely across the glomerular barrier with no tubular secretion or reabsorption. Therefore, fluorescein isothiocyanate (FITC)–labeled Ficolls (12-72 Å) were used as reference tracers to estimate alterations of the size selectivity in the glomerular filtration barrier. The analysis of fractional clearance for Ficoll of different radii was determined by

measuring the amount in plasma and urine samples with gel filtration using a HPLC system and a fluorescence detector.

3.4 Determination of charge selectivity

Alteration of the charge selectivity in the glomerular filtration barrier was estimated by measuring the leakage of albumin into the final urine. It is important to emphasize that albumin clearance herein is underestimated due to tubular reabsorption of proteins from the primary urine. Enzyme-linked immunosorbent assay (ELISA) was used to detect the amount of albumin in plasma and urine samples, and these numbers were subsequently used for the calculation of fractional albumin clearance. The ELISA is a reliable and relatively fast method where the sample throughput is high when using 96 well plates.

3.5 Estimation of glomerular ESL thickness

Intralipid® droplets were used as indirect markers to estimate the thickness of the ESL and by using this method one can overcome the problem with dehydration following alternative methods involving tissue fixation [64]. The negatively charged lipid particles were used as tracers and are predicted to be repelled from the negatively glomerular ESL. Intralipid® is a fat emulsion that contains a chylomicron-like suspension of purified soybean oil, egg-yolk phospholipids, glycerol and water. In order to obtain an enriched fraction of floating lipid droplets to inject into the circulation, the Intralipid® solution was stored in 15 ml tubes in 4°C for 24 h and the top lipid layer was discarded. The remaining solution was subjected to centrifugation at 3000g for 10 minutes, and the enriched floating lipid fraction were kept for experiments [78]. The major purpose of the estimations of the glomerular ESL was to detect differences between treated and control animals, rather than measuring the absolute thickness of this layer. The flow-arrest eliminates the issue with a flow dependent distribution of Intralipid® droplets. It can be argued that a potential increase of the intrarenal pressure due to the subcapsular injection of fixative could change the morphology of glomerular capillaries. However, Bowman's capsule should protect the capillaries from such injury.

3.6 Surgical procedures

All animal experiments were performed in female rats but could have been performed in male rats as well. The major benefits of using female rats are their lower growth rate, smaller amount of visceral fat and the fact that they are less allergenic. The anesthesia was induced and maintained by inhalation of isoflurane, which enables real time adjustments, important for this type of animal experiments.

3.6.1 In vivo experiment in paper II

A prerequisite for the animal studies in this thesis was the establishment of an *in vivo* model where the following assumptions were made: high ionic strength solution (high concentration NaCl solution) displaces non-covalently bound components of the glomerular ESL according to the principle of ion exchange chromatography, and this will affect the glomerular filtration barrier negatively in terms of proteinuria. We developed a method where non-covalently bound components of the glomerular ESL could be displaced, eluted and collected by a short intrarenal perfusion with strong NaCl solution. In order to find the appropriate concentration of NaCl, three different concentrations (0.25, 0.5 and 1.0 M) were evaluated in a pilot study where the effect on charge selectivity of the barrier was analyzed by measuring the albumin clearance. We decided to use 1.0 M since no significant effects on albumin clearance were seen with the lower concentrations.

The method in brief: A catheter in the carotid artery was advanced through the abdominal aorta and fixed in the renal artery to restrict the perfusion to only include the kidney. A ligature around the renal vein in proximity of vena cava was ligated after a catheter was positioned for the collection of renal eluate. The renal vascular system was then perfused during 10-15 seconds with 1 ml of 1.0 or 0.15 M (control animals) NaCl. The catheter was subsequently withdrawn and the blood flow in the kidney was restored. Renal eluate was collected in a separate series of perfused animals since it was not possible, due to surgical circumstances, to perform functional measurements and collect eluate in the same animal. An important aspect in the procedure was to minimize the time between the fixation of catheters and the initiation of the perfusion. This was done in order to avoid ischemia which by itself can cause alterations of the glomerular barrier by [139]. In addition, animals were eviscerated in these experiments. It was not strictly necessary but facilitated the surgical procedure considerably, leading to lower strain on the animal.

3.6.2 In vivo experiment in paper III

The short intrarenal perfusion in paper II was relatively mild, as seen by a partly recovered fractional albumin clearance during the observation period. Consequently, the proteomic analysis of renal eluates did not reveal loss of any larger proteins. We believe that this depended on a too short displacement time for larger proteins in the glomerular ESL. Therefore, the major difference of the experimental procedure in this paper was extension of intrarenal perfusion from approximately 15 seconds to three minutes, with a perfusion volume of 12 ml. Furthermore, the collection of renal eluate was performed differently: The right kidney was disabled through ligation of the renal vein and artery, and a collecting catheter was transferred from the right renal vein, over the vena cava and into the

left renal vein. This allowed us to collect renal eluate and measure function in the same animal. However, the same salt concentrations were utilized, and to maintain a physiological milieu in the kidney during three minutes of perfusion, Tyrode's solution was used as a vehicle for the salt.

3.7 Proteomic analysis of renal eluates

We used various liquid chromatography-mass spectrometry (LC-MS) methods to perform qualitative and quantitative measurements of proteins in renal eluates. In a discovery-based approach LC-MS has several advantages over targeted protein identification methods such as Western blot or ELISA. LC-MS allowed us an efficient, sensitive, and accurate identification of the proteins in the complex samples, where concentration levels and number of proteins were unknown. Principally, the protein identification is based on the mass spectra of corresponding peptides and their fragments. Briefly, proteins are digested into peptides with trypsin to allow analysis by LC-MS. The peptides are separated in the LC-part based on hydrophobicity before they are charged by ionization and sprayed into the MS. In the MS the peptides are separated according to their mass to charge ratio (m/z) and fragmented (MSMS) to allow amino acid sequencing of the peptide subjects. To identify corresponding peptides, MSMS spectra (histograms of m/z ratios versus intensities) are compared to a database of pre-computed spectra from *in silico* digested proteins. The peptides are then matched to proteins, and a protein identity list is generated with information about relative protein quantity based on spectra intensities and/or spectral counts [140, 141]. Comparison of samples with LC-MS is dependent on reproducibility and it is important to treat each sample in exactly the same way. Furthermore, replicate LC-MS runs are important to ensure better statistics for the bioinformatics analysis of the data. There are different types of software and databases to process proteomics data in a trustworthy manner, and physiologists profit from cooperating with researchers that have in-depth knowledge of LC-MS in order to generate reliable proteomics data.

4 Results and discussion

4.1 Paper I

Acute hyperglycemia in humans has recently been linked to a damaged systemic endothelial cell surface layer [135]. Thus, a potential mechanism for diabetic microalbuminuria could be hyperglycemia-mediated disruption of the glomerular ESL, especially in the light of the importance of the glomerular ESL for the permselective properties of the filtration barrier that has been demonstrated by us and others [48, 49, 66, 76-79, 82, 83]. Therefore, the aim of this study was to investigate (A) structural effects of high glucose on the human glomerular ESL and (B) functional alterations of the filtration barrier in terms of protein permeability.

As an *in vitro* model we used a conditionally immortalized human glomerular endothelial cell line whose behavior and characteristics are comparable with primary cultures of glomerular endothelial cells, including fenestrations [46]. It is important to emphasize that in cell cultures the endothelial cell surface layer mainly consists of glycocalyx as previously described [79]. The absence of plasma flow and shear stress by definition makes it impossible to produce a proper endothelial cell coat, which has been a limiting factor in this study [2]. The major advantage of using an immortalized cell line instead of a primary culture is the absence of the poor replicative potential that leads to early senescence, which definitely is a limiting factor when primary cells are used.

PG/GAG biosynthesis was reduced in human glomerular endothelial cells exposed to high glucose. We analyzed this by measuring the quantity of incorporated D-[6-^{3}H]-glucosamine and [^{35}S]-sulfate into GAGs. These results are in line with a previous study where decreased synthesis of sulfated GAGs was observed when bovine glomerular endothelial cells were exposed to high glucose concentrations [142]. In addition, we found a 50% reduction of the amount of heparan sulfate (HS) GAGs on the surface of human glomerular endothelial cells exposed to high glucose compared with control cells, using a fluorescent anti-HS antibody. Similar results have been demonstrated in the GBM of patients with diabetes nephropathy [143]. These results are relevant to our findings, even if our study deals with the contribution of the glomerular endothelium.

The mechanism by which high glucose reduce the biosynthesis of PG/GAG of human glomerular endothelial might involve regulation, at the level of gene expression, of the battery of enzymes that are responsible for polymerization and elongation of GAGs [91]. To examine if this putative mechanism is in operation we analyzed the mRNA expression of four key biosynthetic enzymes: EXT-1, EXT-2, NDST-1 and chondroitin synthase 1. Surprisingly, we did not see any significant changes in the mRNA transcription of these enzymes in cells cultured in high

glucose compared with control cells. Still this does not necessarily rule out an impact of the biosynthetic enzymes. Even if the mRNA expression of these enzymes is unchanged, the activity of the existing enzymes could be affected by high glucose concentration and this matter needs to be further investigated. Furthermore, when we assessed the expression levels of PG core proteins for syndecan-1, syndecan-4, glypican-1, versican and perlecan with western blotting, we found no significant differences between cells cultured under normal- and high glucose conditions, respectively. This suggests that high glucose induce a post-transcriptional modulation of PG.

The endothelial cell surface layer is known to be a dynamic structure, defined by a balance between synthesis and degradation [61]. Therefore, we speculate that the loss of GAGs is due, in part, to increased GAG degradation mediated by an up-regulation of endogenous GAG-degrading enzymes such as chondroitinase, hyaluronidase or heparanase. This idea is supported by a recent study on diabetic nephropathy patients where loss of HS in the GBM was associated with an increased heparanase expression [144].

Another mechanism of GAG degradation could be related to the well-known fact that high glucose concentration *per se* induces mitochondrial overproduction of reactive oxygen species (ROS). The loss of GAGs may thus be secondary to a direct effect of oxygen radicals that damages the glomerular endothelial glycocalyx [145].

We also wanted to examine if the observed loss of GAGs from the glomerular endothelial glycocalyx was associated with a functional alteration in terms of protein restriction. This was done by analyzing the albumin permeability across glomerular endothelial monolayers. We found a significant increase of the passage of FITC-labeled albumin across monolayers exposed to high glucose compared with control cells. To confirm that this effect not depended primarily on the loss of endothelial junctional integrity, we measured the transendothelial electrical resistance (TEER) and analyzed the distribution of the key adhesion molecule VE-cadherin [146]. In confluent monolayers the TEER analysis may be used as a measure of monolayer integrity [46, 147]. The expression of VE-cadherin was analyzed in immunofluorescent images of glomerular endothelial monolayers and by western blotting. The TEER analysis showed no significant change in electrochemical difference, and there was a normal distribution of VE-cadherin in high glucose cultured cells. In addition, the high glucose concentration did not induce any adverse effects on cell survival and there were no morphological alterations. In summary, we demonstrate that high glucose modulate the composition of glycocalyx on human glomerular endothelial cells by altering the distribution of GAGs, particularly HS GAGs. This modulation was associated with

a functional alteration since protein restriction across the glomerular endothelial cell monolayer was impaired.

This paper provides additional evidence for the glomerular ESL as a significant contributor to the maintenance of macromolecular impermeability across the glomerular filtration barrier. Further, our results suggest a novel mechanism by which hyperglycemia in diabetes may cause a dysfunction of the filtration barrier, namely through modulation of major structural components in the glomerular ESL. Thus, our data leads to the conclusion that a damaged glomerular endothelial cell surface layer is one important reason for the development of microalbuminuria in diabetes.

4.2 Paper II

The glomerular ESL is generally accepted as a contributor to the permselectivity of the glomerular barrier. However, the molecular structure of this layer is largely unexplored, and available data about its constituents so far is based on *in vitro* studies [66, 79]. The aim of this study was to survey, *in vivo*, non-covalently bound glomerular ESL components of importance for normal glomerular barrier function.

We have developed a method that allows brief intrarenal perfusion of rats with a strong salt solution (1 M NaCl, "high salt"). In this way we can displace, elute and collect non-covalently bound components of the glomerular ESL according to the principles of ion-exchange chromatography. When restoring normal blood flow after perfusion of rats with high salt for just 15 seconds, we found a 12-fold increase of the fractional albumin clearance compared with control rats (0.15 M NaCl "normal salt"). The fractional albumin clearance was partially normalized during the "post salt" recovery period of 100 minutes, but remained significantly higher in the high salt animals than in the controls. The fractional clearance for Ficoll 35.5 Å, the neutral counterpart of albumin, was unchanged while there was a significant increase of the fractional clearance for Ficoll 55 Å.

From the above results it is clear that brief perfusion with high salt brings about a major deterioration of charge selectivity in the filtration barrier, but only a minor impairment of the size selectivity in terms of increased number of large pores. It should be noted that the *in vivo* albumin clearance is actually underestimated due to tubular reabsorption of proteins from the primary urine [148], thus sharpening the contrast between charge and size effects even further.

We speculate that the partial normalization of the fractional albumin clearance depends on readsorption of plasma-derived molecules to the glomerular ESL. The absence of total recovery during the time-frame of the observation period may depend upon that some components lost from the ECC need to be re-synthesized, preferentially by the endothelium, a process that usually requires several hours.

GFR data collected during the experiments showed a significant reduction of the GFR when measured 10 and 20 minutes after high salt perfusion compared with the pre-perfusion mean, but after 40 minutes the GFR had returned to the pre-perfusion level. Normal salt perfusion had no effect on GFR. We suggest that the transient reduction of GFR during the first 20 minutes after high salt perfusion depends on a vasoconstriction of afferent arterioles secondary to the high salt concentration. This concept is in accordance with a previously published review clarifying the association between salt and blood pressure regulation [149]. It could be argued that reduced GFR would increase albumin clearance *per se*, by allowing more time for albumin to diffuse across the barrier. The influence of the transiently reduced GFR on albumin clearance in this study is insignificant, however, because this phenomenon has been studied with neutral Ficoll in the same size as albumin and the clearance only increased approximately 10% [150, 151]. For anionic albumin, this effect would be even smaller.

Quantitative measurements of GBM thickness, podocyte foot process width and width of filtration slits was done to confirm that no abnormal morphological rearrangements were associated with the high salt perfusion that might increase the permeability of the filtration barrier. We found a 3% increase in GBM thickness and a 5% reduction in podocyte filtration slit width. These morphological alterations actually suggest a more impermeable barrier, associated with lower fractional albumin clearance. An explanation to these results could be that high salt solution induced a contraction of podocytes and/or affected the GBM *per se*. Since we hypothesize that the high salt perfusion should displace and elute molecules in the glomerular ESL, one could expect that the thickness of the glomerular ESL would change in a similar fashion as in a recent publication from our lab [48]. However, the evaluation of the ESL thickness revealed no significant differences between the two groups, and this is an indication of a subtle elution of molecules with the high salt solution.

To further assess functional alterations of the filtration barrier due to high salt perfusion, two mathematical models were included in this study, namely the gel membrane model and the more advanced charged fiber model. These models describe the size and charge selectivity of the glomerular barrier using fractional clearance data for Ficoll sizes (12-70 Å) [48, 76]. We found similar results with both models after perfusion with high salt. There was a reduction of the glomerular charge density and the size selectivity was unaffected except for the largest solutes, since there was an increase in the number of large pores in the filtration barrier.

Proteins displaced from the glomerular ESL in eluates from high salt and control groups, were analyzed with liquid chromatography-mass spectrometry. We identified 17 non-covalently bound proteins normally present in the glomerular ECC and in the molecular weight range from 5-190 kDa. Interestingly,

orosomucoid was included in the group of proteins that were found only in high salt eluate and plasma. The importance of this protein for maintaining a normal glomerular filtration is supported by earlier permeability studies in peripheral capillaries [68-70, 152, 153]. This protein is the primary candidate among plasma-derived molecules that might be readsorbed in the glomerular ECC and contribute to the observed recovery of the fractional albumin clearance mentioned earlier. Moreover, the PG lumican together with four other proteins was identified in significantly higher concentrations in high salt eluate compared with normal salt eluate, but not existing in plasma. This novel component of the glomerular ECC has been described as being present in the tubular interstitium [124] and is recognized as an important organizer of extracellular matrices [123]. An interesting point is that lumican could actually be one of the components lost from the ECC that needs to be re-synthesized by glomerular endothelial cells in order to attain a complete recovery of the fractional albumin clearance in high salt rats. In addition, lumican was indeed present in the glomerular capillary endothelium, as demonstrated by a clear co-localization of a lumican antibody and the endothelial-specific marker Ulex europaeus agglutinin I.

An important issue of this study is the origin of the eluted proteins, which we consider to be the ESL of the glomerular capillaries. The coincidence of increased albumin leakage with the elution of certain proteins, and the co-localization of lumican within the glomerular endothelium, strongly support that we study effects specific for the glomerular ESL. However, due to the nature of the intervention, that is, perfusion of the entire renal vasculature with high salt solution, one cannot exclude that some of the eluted proteins could be of peritubular origin, downstream of the glomerular capillaries.

In summary, this study contributes further with evidence for the glomerular ESL as an important determinant of the permselectivity in the glomerular filtration barrier. In addition, we have also identified novel molecules present in the glomerular ESL and proved that one or more of these molecules are essential for preserving an intact glomerular barrier. Our data also clearly show that morphologically subtle alterations of the glomerular ESL can cause clinically significant proteinuria.

4.3 Paper III

The perfusion procedure with 1 M NaCl, described in paper II was rather mild as shown by the reversion of the albuminuria, and it most likely exerted elution effects through a combination of electrostatic and osmotic forces. In order to elute and identify additional molecules, perhaps larger ones, we assumed that a more extensive perfusion was necessary. The aim of this study was therefore to continue the examination of components in the glomerular ESL, using a prolonged perfusion (3 minutes instead of 15 seconds) with high salt solution. In addition, we

wanted to assess morphological as well as functional alterations, and evaluate the presence of other noncovalent forces besides charge-charge interactions, by including perfusions with high osmolarity solution (1 M mannitol, an uncharged osmotic agent).

We found no structural alterations of the overall morphology of the glomerular filtration barrier in rats perfused for 3 minutes with normal salt (NS), and the fractional clearance also remained normal throughout the observation time. These results confirm that the longer perfusion does not induce barrier damage *per se*. The thickness of the glomerular ESL in high salt (HS) perfused rats was reduced by 28% compared with NS perfused rats. This was accompanied by a 25-fold increase of the fractional albumin clearance and a dramatic reduction of the GFR relative to NS when measured 10 minutes post perfusion. After 40 minutes the animals were anuric. We speculate that this was due to a hemodynamic effect of preglomerular vasoconstriction, causing a drop in glomerular hydrostatic pressure. Alternatively, a physical disturbance of the tubular system by detached tubular cells could be responsible. Further examinations of electron microscopy sections of HS perfused kidneys may shed further light on the mechanisms behind the anuria observed. In addition, the observed anuric state may be transient, which could be explored further by functional measurements possibly after 48h post perfusion [154].

The GFR in high osmolarity (HO) perfused rats was reduced by 50% compared to control rats and remained at that level during the observation period. Fractional albumin clearance in HO animals increased approximately 5 times, but no changes were seen in glomerular ESL thickness. A common point for the HS and HO rats is that morphological alterations of the filtration barrier, in terms of changes in the endothelium, GBM and podocytes, are not sufficient to readily explain the profound effects on urine flow.

Mass spectrometry analysis of renal eluates identified several molecules most likely lost from the glomerular ESL and of potential importance for normal glomerular permselectivity. In addition, we also analyzed the amount of hyaluronic acid in renal eluates with ELISA and found that the concentration of hyaluronic acid was 6 times higher in HS and HO rats compared to NS rats. Some of the identified molecules could actually be vital for the structural and the functional integrity of the glomerular filtration barrier. They are: Thrombospondin-1, Ficolin-1, Inter-alpha-inhibitor H4 heavy chain, Beta-2-glycoprotein-1, Syndecan-4 and Uromodulin and Hyaluronic acid. These molecules except for hyaluronic acid were not identified in NS eluate or plasma, and their elution caused a reduced glomerular permselectivity according to albumin clearance data for HS and HO. Syndecan-4 was confirmed by immunohistochemical analysis to be present in the glomerular capillary endothelium. Interestingly, Thrombospondin-1, Ficolin-1 and Fibrinogen alpha chain were only present in eluate from HS rats, which indicate that these

molecules definitely were displaced according to the principle of ion-exchange chromatography since they were not released with HO.

In paper II we raised the subject of the origin of the eluted molecules, and the same reasoning applies in this study, i.e., the identified molecules may be present in glomerular or peritubular capillary endothelium, or both.

An interesting point is that HO gave the same drastic release of hyaluronic acid and syndecan-4 as HS since both of these molecules are rather negatively charged. However, mannitol is known to be used in osmotheraphy to decrease acutely raised intracranial pressure i.e. induce dehydration by reducing the amount of accumulated fluid in the brain [155]. Perhaps this mechanism is in play also during HO perfusion, that is to say, mannitol drains water from the tissue, and molecules from the glomerular ESL travel with the water.

All in all, this paper demonstrates that the ESL is of importance for the restriction of proteins in the glomerular barrier. Our results also show that osmotic forces also can elute molecules from the glomerular ESL. In addition, several novel components of potential importance for maintaining an intact barrier function were identified.

5 Concluding remarks and future perspectives

This thesis provides the first actual study of the molecular composition of the glomerular ESL *in vivo*, since the current information about its constituents is only established on *in vitro* studies. We have identified a number of molecules present in the glomerular ESL and some of these are essential for retaining a normal glomerular filtration. Our data also demonstrate that minor morphological alterations in terms of loss of molecules in the glomerular ESL can cause clinically significant proteinuria. This thesis also describes relatively unknown mechanisms by which hyperglycemia causes dysfunction of the glomerular permselectivity to macromolecules, i.e., through modulation of GAGs, which are major structural components of the glomerular ESL. This interestingly implies that ESL loss can be involved also in the pathogenesis of other vascular diseases of micro- and macrovascular nature.

Overall, this thesis demonstrates that the glomerular ESL is indeed contributing to the permselectivity of the glomerular filtration barrier, and it reveals a novel mechanism by which disease milieu may increase glomerular permeability to proteins and hence contribute to the pathogenesis of proteinuria in clinical diseases, such as diabetes. Further opening of the "black-box" of the glomerular ESL and the study of its contribution to glomerular filtration, is of great importance to expand our knowledge about the mechanisms leading to proteinuria and vital for the development of novel therapies in the future.

One interesting future perspective would be to investigate whether the enzyme activity of GAG polymerases in human glomerular endothelial cells are affected by high glucose, in particular EXT-1 and EXT-2 because of the detected loss of heparan sulfate GAGs. It would also be interesting to explore whether the mRNA expression of endogenous GAG-degrading enzymes such as chondroitinase, hyaluronidase and heparanase are up-regulated in human glomerular endothelial cells upon high glucose stimuli. These experiments could present the full picture of the effects that hyperglycemia mediates on glomerular endothelial PG/GAGs. Moreover, a more detailed examination of human glomerular endothelial GAG alterations upon different stimuli related to kidney disease would also be a significant task. Since small changes in the GAG biosynthesis with respect to sulfation patterns might generate major functional implications, in terms of decreased binding capacity for plasma proteins, hormones and cytokines etc.

Furthermore, it would be interesting to see, in a clinical trial, if any of the proteins located in the glomerular ESL, and that we found to be important for normal glomerular filtration in rats, could be detected in higher amounts in urine or plasma in patients with kidney disease, either with or without proteinuria, compared to healthy subjects. Such clinical studies could pave the way for the establishment of a

specific glomerular ESL damage marker, and also generate valuable information for future development of specific therapies aiming at restoring the glomerular ESL and thus reduce proteinuria.

The relatively extensive loss of glomerular ESL that caused a dramatic increase of the fractional albumin clearance in paper III deserves further attention. An interesting approach could be an evaluation of the function of the barrier in these animals 2-3 days post perfusion by analyzing the charge selectivity in terms of fractional albumin clearance. In addition, it would be worthwhile to isolate glomeruli and glomerular endothelial cells for preparation of RNA and analyze if the gene expression of certain proteins/PGs, recognized to be expressed by glomerular endothelial cells *in vitro*, is up-regulated during the recovery time compared to control animals. This would generate valuable information concerning recovery of glomerular ESL components and further identification and/or verification of glomerular ESL molecules.

6 Acknowledgements

Till slut blev det en avhandling även för mig. De som är initierade vet att det tog en stund innan den blev färdig. Jag har träffat och lärt känna många trevliga personer här på Wallenberglaboratoriet under åren som gått. Jag skulle vilja tacka er alla som har hjälpt mig på vägen och som har skapat en trevlig atmosfär, bra gemenskap och gjort det extra roligt att gå till jobbet.

Ett särskilt tack skulle jag dock vilja rikta till:

Min huvudhandledare Börje Haraldsson för att du gav mig möjligheten att fortsätta min forskarutbildning i din grupp, vid en tidpunkt då saker och ting såg allt annat än ljust ut. Jag skulle vilja tacka för din entusiasm, bra handledning och ditt positiva tänkande. Vill också tacka för att du trott på mig och stöttat mig.

Vidare så vill jag tacka mina biträdande handledare. Ulf Nilsson, för dina goda idéer och all språkgranskning av manuskript och avhandling. Jenny Nyström för all stöttning och dina kunskaper om njurforskning.

Tack Johan Lindh för att du en gång i tiden introducerade mig för forskning.

I also would like to thank my co-author, Anurag Singh for giving me the opportunity for collaboration. We had a lot of fun, both during some intense working weeks here in Gothenburg and at different scientific meetings around the world. Hopefully we can work together again sometime with another project.

Likaså vill jag tacka mina samarbetspartners i Bergen, Eystein Oveland och Olav Tenstad, tusen tack för all hjälp med mass spektrometrin. Utan er hade det inte varit möjligt att generera denna avhandling. Tack också för en mycket trevlig vistelse i Bergen, hälsar gärna på fler gånger!

Tack alla glada och härliga människor som är eller har varit verksamma i Njurgruppen/Njurcentrum för all hjälp, uppmuntran, gemenskap, stöttning, omtanke och "abuse" ☺!!!!

Slutligen skulle jag vilja tacka alla nära och kära som alltid funnits där för mig. Vill tacka min fru Annica som har varit med på denna resa ända från början och min son Viking som har gjort de senaste 2,5 åren till de hittills bästa i mitt liv.

7 Additional publications

I. **Macrophages exposed to hypoxia secrete proteoglycans for which LDL has higher affinity.**
Asplund A, <u>Fridén V</u>, Stillemark-Billton P, Camejo G, Bondjers G.
Atherosclerosis. 2011 Mar;215(1):77-81. Epub 2010 Dec 23.

II. **Heparan sulfate in perlecan promotes mouse atherosclerosis: roles in lipid permeability, lipid retention, and smooth muscle cell proliferation.**
Tran-Lundmark K, Tran PK, Paulsson-Berne G, <u>Fridén V</u>, Soininen R, Tryggvason K, Wight TN, Kinsella MG, Borén J, Hedin U.
Circ Res. 2008 Jul 3;103(1):43-52. Erratum in: Circ Res. 2009 Jan 30;104(2):e24.

III. **Retention of low-density lipoprotein in atherosclerotic lesions of the mouse: evidence for a role of lipoprotein lipase.**
Gustafsson M, Levin M, Skålén K, Perman J, <u>Fridén V</u>, Jirholt P, Olofsson SO, Fazio S, Linton MF, Semenkovich CF, Olivecrona G, Borén J.
Circ Res. 2007 Oct 12;101(8):777-83. Epub 2007 Aug 30.

IV. **IFNgamma regulates PDGF-receptor alpha expression in macrophages, THP-1 cells, and arterial smooth muscle cells.**
Morelli PI, Martinsson S, Ostergren-Lundén G, <u>Fridén V</u>, Moses J, Bondjers G, Krettek A, Lustig F.
Atherosclerosis. 2006 Jan;184(1):39-47.

8 References

1. Deen, W.M., M.J. Lazzara, and B.D. Myers, *Structural determinants of glomerular permeability.* American journal of physiology. Renal physiology, 2001. **281**(4): p. F579-96.
2. Haraldsson, B., J. Nystrom, and W.M. Deen, *Properties of the glomerular barrier and mechanisms of proteinuria.* Physiological reviews, 2008. **88**(2): p. 451-87.
3. Faul, C., et al., *Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton.* Trends in cell biology, 2007. **17**(9): p. 428-37.
4. Adler, S., *Characterization of glomerular epithelial cell matrix receptors.* Am J Pathol, 1992. **141**(3): p. 571-8.
5. Mundel, P. and W. Kriz, *Structure and function of podocytes: an update.* Anat Embryol (Berl), 1995. **192**(5): p. 385-97.
6. Raats, C.J., et al., *Expression of agrin, dystroglycan, and utrophin in normal renal tissue and in experimental glomerulopathies.* Am J Pathol, 2000. **156**(5): p. 1749-65.
7. Regele, H.M., et al., *Glomerular expression of dystroglycans is reduced in minimal change nephrosis but not in focal segmental glomerulosclerosis.* J Am Soc Nephrol, 2000. **11**(3): p. 403-12.
8. Rodewald, R. and M.J. Karnovsky, *Porous substructure of the glomerular slit diaphragm in the rat and mouse.* The Journal of cell biology, 1974. **60**(2): p. 423-33.
9. Patrakka, J. and K. Tryggvason, *New insights into the role of podocytes in proteinuria.* Nature reviews. Nephrology, 2009. **5**(8): p. 463-8.
10. Barletta, G.M., et al., *Nephrin and Neph1 co-localize at the podocyte foot process intercellular junction and form cis hetero-oligomers.* The Journal of biological chemistry, 2003. **278**(21): p. 19266-71.
11. Boute, N., et al., *NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome.* Nat Genet, 2000. **24**(4): p. 349-54.
12. Donoviel, D.B., et al., *Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN.* Mol Cell Biol, 2001. **21**(14): p. 4829-36.
13. Inoue, T., et al., *FAT is a component of glomerular slit diaphragms.* Kidney Int, 2001. **59**(3): p. 1003-12.
14. Kestila, M., et al., *Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome.* Mol Cell, 1998. **1**(4): p. 575-82.
15. Reiser, J., et al., *TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function.* Nature genetics, 2005. **37**(7): p. 739-44.
16. Sellin, L., et al., *NEPH1 defines a novel family of podocin interacting proteins.* The FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2003. **17**(1): p. 115-7.
17. Shih, N.Y., et al., *Congenital nephrotic syndrome in mice lacking CD2-associated protein.* Science, 1999. **286**(5438): p. 312-5.
18. Kerjaschki, D., D.J. Sharkey, and M.G. Farquhar, *Identification and characterization of podocalyxin--the major sialoprotein of the renal glomerular epithelial cell.* J Cell Biol, 1984. **98**(4): p. 1591-6.
19. Pavenstadt, H., *The charge for going by foot: modifying the surface of podocytes.* Experimental nephrology, 1998. **6**(2): p. 98-103.
20. Roth, J., D. Brown, and L. Orci, *Regional distribution of N-acetyl-D-galactosamine residues in the glycocalyx of glomerular podocytes.* The Journal of cell biology, 1983. **96**(5): p. 1189-96.
21. Stow, J.L., H. Sawada, and M.G. Farquhar, *Basement membrane heparan sulfate proteoglycans are concentrated in the laminae rarae and in podocytes of the rat renal glomerulus.* Proceedings of the National Academy of Sciences of the United States of America, 1985. **82**(10): p. 3296-300.
22. Doyonnas, R., et al., *Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin.* The Journal of experimental medicine, 2001. **194**(1): p. 13-27.
23. Takeda, T., et al., *Loss of glomerular foot processes is associated with uncoupling of podocalyxin from the actin cytoskeleton.* The Journal of clinical investigation, 2001. **108**(2): p. 289-301.
24. Shankland, S.J., *The podocyte's response to injury: role in proteinuria and glomerulosclerosis.* Kidney Int, 2006. **69**(12): p. 2131-47.

25.   Tryggvason, K. and J. Wartiovaara, *How does the kidney filter plasma?* Physiology (Bethesda), 2005. **20**: p. 96-101.

26.   Abrahamson, D.R., *Structure and development of the glomerular capillary wall and basement membrane.* The American journal of physiology, 1987. **253**(5 Pt 2): p. F783-94.

27.   Daniels, B.S., et al., *Glomerular basement membrane: in vitro studies of water and protein permeability.* The American journal of physiology, 1992. **262**(6 Pt 2): p. F919-26.

28.   Chung, A.E. and M.E. Durkin, *Entactin: structure and function.* American journal of respiratory cell and molecular biology, 1990. **3**(4): p. 275-82.

29.   Paulsson, M., *Basement membrane proteins: structure, assembly, and cellular interactions.* Critical reviews in biochemistry and molecular biology, 1992. **27**(1-2): p. 93-127.

30.   Groffen, A.J., et al., *Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane.* The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society, 1998. **46**(1): p. 19-27.

31.   Hudson, B.G., et al., *Alport's syndrome, Goodpasture's syndrome, and type IV collagen.* The New England journal of medicine, 2003. **348**(25): p. 2543-56.

32.   Noakes, P.G., et al., *The renal glomerulus of mice lacking s-laminin/laminin beta 2: nephrosis despite molecular compensation by laminin beta 1.* Nature genetics, 1995. **10**(4): p. 400-6.

33.   Zenker, M., et al., *Human laminin beta2 deficiency causes congenital nephrosis with mesangial sclerosis and distinct eye abnormalities.* Human molecular genetics, 2004. **13**(21): p. 2625-32.

34.   Kanwar, Y.S., A. Linker, and M.G. Farquhar, *Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion.* The Journal of cell biology, 1980. **86**(2): p. 688-93.

35.   van den Hoven, M.J., et al., *Reduction of anionic sites in the glomerular basement membrane by heparanase does not lead to proteinuria.* Kidney international, 2008. **73**(3): p. 278-87.

36.   Goldberg, S., et al., *Glomerular filtration is normal in the absence of both agrin and perlecan-heparan sulfate from the glomerular basement membrane.* Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 2009. **24**(7): p. 2044-51.

37.   Chen, S., et al., *Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria.* Kidney international, 2008. **74**(3): p. 289-99.

38.   Aird, W.C., *Spatial and temporal dynamics of the endothelium.* Journal of thrombosis and haemostasis : JTH, 2005. **3**(7): p. 1392-406.

39.   Ghitescu, L. and M. Robert, *Diversity in unity: the biochemical composition of the endothelial cell surface varies between the vascular beds.* Microscopy research and technique, 2002. **57**(5): p. 381-9.

40.   Segal, M.S., C. Baylis, and R.J. Johnson, *Endothelial health and diversity in the kidney.* Journal of the American Society of Nephrology : JASN, 2006. **17**(2): p. 323-4.

41.   Risau, W., *Development and differentiation of endothelium.* Kidney international. Supplement, 1998. **67**: p. S3-6.

42.   Kriz, W., *Fenestrated glomerular capillaries are unique.* Journal of the American Society of Nephrology : JASN, 2008. **19**(8): p. 1439-40.

43.   Satchell, S.C. and F. Braet, *Glomerular endothelial cell fenestrations: an integral component of the glomerular filtration barrier.* American journal of physiology. Renal physiology, 2009. **296**(5): p. F947-56.

44.   Maynard, S.E., et al., *Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia.* The Journal of clinical investigation, 2003. **111**(5): p. 649-58.

45.   Stillman, I.E. and S.A. Karumanchi, *The glomerular injury of preeclampsia.* Journal of the American Society of Nephrology : JASN, 2007. **18**(8): p. 2281-4.

46.   Satchell, S.C., et al., *Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF.* Kidney international, 2006. **69**(9): p. 1633-40.

47.   Haraldsson, B. and J. Sorensson, *Why do we not all have proteinuria? An update of our current understanding of the glomerular barrier.* News Physiol Sci, 2004. **19**: p. 7-10.

48.   Jeansson, M. and B. Haraldsson, *Glomerular size and charge selectivity in the mouse after exposure to glucosaminoglycan-degrading enzymes.* Journal of the American Society of Nephrology : JASN, 2003. **14**(7): p. 1756-65.

49. Jeansson, M. and B. Haraldsson, *Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier.* American journal of physiology. Renal physiology, 2006. **290**(1): p. F111-6.

50. Brown, M.D., et al., *Appearance of the capillary endothelial glycocalyx in chronically stimulated rat skeletal muscles in relation to angiogenesis.* Experimental physiology, 1996. **81**(6): p. 1043-6.

51. Damiano, E.R., *The effect of the endothelial-cell glycocalyx on the motion of red blood cells through capillaries.* Microvascular research, 1998. **55**(1): p. 77-91.

52. Huxley, V.H. and D.A. Williams, *Role of a glycocalyx on coronary arteriole permeability to proteins: evidence from enzyme treatments.* American journal of physiology. Heart and circulatory physiology, 2000. **278**(4): p. H1177-85.

53. Kjellen, L. and U. Lindahl, *Proteoglycans: structures and interactions.* Annual review of biochemistry, 1991. **60**: p. 443-75.

54. Luft, J.H., *Fine structures of capillary and endocapillary layer as revealed by ruthenium red.* Federation proceedings, 1966. **25**(6): p. 1773-83.

55. Rostgaard, J. and K. Qvortrup, *Electron microscopic demonstrations of filamentous molecular sieve plugs in capillary fenestrae.* Microvascular research, 1997. **53**(1): p. 1-13.

56. Siegel, G. and M. Malmsten, *The role of the endothelium in inflammation and tumor metastasis.* International journal of microcirculation, clinical and experimental / sponsored by the European Society for Microcirculation, 1997. **17**(5): p. 257-72.

57. Reitsma, S., et al., *The endothelial glycocalyx: composition, functions, and visualization.* Pflugers Archiv : European journal of physiology, 2007. **454**(3): p. 345-59.

58. Vink, H. and B.R. Duling, *Identification of distinct luminal domains for macromolecules, erythrocytes, and leukocytes within mammalian capillaries.* Circulation research, 1996. **79**(3): p. 581-9.

59. Buonassisi, V., *Sulfated mucopolysaccharide synthesis and secretion in endothelial cell cultures.* Experimental cell research, 1973. **76**(2): p. 363-8.

60. Pries, A.R., et al., *Microvascular blood flow resistance: role of endothelial surface layer.* The American journal of physiology, 1997. **273**(5 Pt 2): p. H2272-9.

61. Lipowsky, H.H., *Microvascular rheology and hemodynamics.* Microcirculation, 2005. **12**(1): p. 5-15.

62. Brouland, J.P., et al., *Macro and microheterogeneity in normal endothelial cells: differential composition of luminal glycocalyx and functional implications.* Endothelium : journal of endothelial cell research, 1999. **6**(3): p. 251-62.

63. Haldenby, K.A., et al., *Focal and regional variations in the composition of the glycocalyx of large vessel endothelium.* Journal of vascular research, 1994. **31**(1): p. 2-9.

64. Hjalmarsson, C., B.R. Johansson, and B. Haraldsson, *Electron microscopic evaluation of the endothelial surface layer of glomerular capillaries.* Microvascular research, 2004. **67**(1): p. 9-17.

65. Rostgaard, J. and K. Qvortrup, *Sieve plugs in fenestrae of glomerular capillaries--site of the filtration barrier?* Cells, tissues, organs, 2002. **170**(2-3): p. 132-8.

66. Bjornson, A., et al., *Primary human glomerular endothelial cells produce proteoglycans, and puromycin affects their posttranslational modification.* American journal of physiology. Renal physiology, 2005. **288**(4): p. F748-56.

67. Fujihara, C.K., et al., *Enhanced glomerular permeability to macromolecules in the Nagase analbuminemic rat.* American journal of physiology. Renal physiology, 2002. **282**(1): p. F45-50.

68. Haraldsson, B.S., E.K. Johnsson, and B. Rippe, *Glomerular permselectivity is dependent on adequate serum concentrations of orosomucoid.* Kidney international, 1992. **41**(2): p. 310-6.

69. Johnsson, E. and B. Haraldsson, *Addition of purified orosomucoid preserves the glomerular permeability for albumin in isolated perfused rat kidneys.* Acta physiologica Scandinavica, 1993. **147**(1): p. 1-8.

70. Curry, F.E., J.C. Rutledge, and J.F. Lenz, *Modulation of microvessel wall charge by plasma glycoprotein orosomucoid.* The American journal of physiology, 1989. **257**(5 Pt 2): p. H1354-9.

71. Curry, F.R., *Effect of albumin on the structure of the molecular filter at the capillary wall.* Federation proceedings, 1985. **44**(10): p. 2610-3.

72. Michel, C.C., M.E. Phillips, and M.R. Turner, *The effects of native and modified bovine serum albumin on the permeability of frog mesenteric capillaries.* The Journal of physiology, 1985. **360**: p. 333-46.

73. Avasthi, P.S. and V. Koshy, *Glomerular endothelial glycocalyx.* Contributions to nephrology, 1988. **68**: p. 104-13.

74. Ciarimboli, G., et al., *Dynamic alterations of glomerular charge density in fixed rat kidneys suggest involvement of endothelial cell coat.* American journal of physiology. Renal physiology, 2003. **285**(4): p. F722-30.

75. Deen, W.M., *What determines glomerular capillary permeability?* The Journal of clinical investigation, 2004. **114**(10): p. 1412-4.

76. Ohlson, M., J. Sorensson, and B. Haraldsson, *A gel-membrane model of glomerular charge and size selectivity in series.* Am J Physiol Renal Physiol, 2001. **280**(3): p. F396-405.

77. Sorensson, J., et al., *Glomerular charge selectivity for horseradish peroxidase and albumin at low and normal ionic strengths.* Acta physiologica Scandinavica, 1998. **163**(1): p. 83-91.

78. Jeansson, M., et al., *Adriamycin alters glomerular endothelium to induce proteinuria.* Journal of the American Society of Nephrology : JASN, 2009. **20**(1): p. 114-22.

79. Singh, A., et al., *Glomerular endothelial glycocalyx constitutes a barrier to protein permeability.* Journal of the American Society of Nephrology : JASN, 2007. **18**(11): p. 2885-93.

80. Drumond, M.C. and W.M. Deen, *Structural determinants of glomerular hydraulic permeability.* The American journal of physiology, 1994. **266**(1 Pt 2): p. F1-12.

81. Fu, B.M., B. Chen, and W. Chen, *An electrodiffusion model for effects of surface glycocalyx layer on microvessel permeability.* Am J Physiol Heart Circ Physiol, 2003. **284**(4): p. H1240-50.

82. Ohlson, M., J. Sorensson, and B. Haraldsson, *Glomerular size and charge selectivity in the rat as revealed by FITC-ficoll and albumin.* Am J Physiol Renal Physiol, 2000. **279**(1): p. F84-91.

83. Sorensson, J., M. Ohlson, and B. Haraldsson, *A quantitative analysis of the glomerular charge barrier in the rat.* Am J Physiol Renal Physiol, 2001. **280**(4): p. F646-56.

84. Haraldsson, B. and B.R. Johansson, *Changes in transcapillary exchange induced by perfusion fixation with glutaraldehyde, followed by measurements of capillary filtration coefficient, diffusion capacity and albumin clearance.* Acta physiologica Scandinavica, 1985. **124**(1): p. 99-106.

85. Kalluri, R., *Proteinuria with and without renal glomerular podocyte effacement.* J Am Soc Nephrol, 2006. **17**(9): p. 2383-9.

86. Yanagishita, M., *A brief history of proteoglycans.* Experientia, 1993. **49**(5): p. 366-8.

87. Cassaro, C.M. and C.P. Dietrich, *Distribution of sulfated mucopolysaccharides in invertebrates.* The Journal of biological chemistry, 1977. **252**(7): p. 2254-61.

88. Yamada, S., et al., *Demonstration of glycosaminoglycans in Caenorhabditis elegans.* FEBS letters, 1999. **459**(3): p. 327-31.

89. Iozzo, R.V., *Matrix proteoglycans: from molecular design to cellular function.* Annual review of biochemistry, 1998. **67**: p. 609-52.

90. Gandhi, N.S. and R.L. Mancera, *The structure of glycosaminoglycans and their interactions with proteins.* Chemical biology & drug design, 2008. **72**(6): p. 455-82.

91. Prydz, K. and K.T. Dalen, *Synthesis and sorting of proteoglycans.* Journal of cell science, 2000. **113 Pt 2**: p. 193-205.

92. Funderburgh, J.L., *Keratan sulfate biosynthesis.* IUBMB life, 2002. **54**(4): p. 187-94.

93. Funderburgh, J.L., *Keratan sulfate: structure, biosynthesis, and function.* Glycobiology, 2000. **10**(10): p. 951-8.

94. Laurent, T.C. and J.R. Fraser, *Hyaluronan.* The FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 1992. **6**(7): p. 2397-404.

95. Aruffo, A., et al., *CD44 is the principal cell surface receptor for hyaluronate.* Cell, 1990. **61**(7): p. 1303-13.

96. Turley, E.A., P.W. Noble, and L.Y. Bourguignon, *Signaling properties of hyaluronan receptors.* The Journal of biological chemistry, 2002. **277**(7): p. 4589-92.

97. Toole, B.P., *Hyaluronan: from extracellular glue to pericellular cue.* Nature reviews. Cancer, 2004. **4**(7): p. 528-39.

98. Sugahara, K. and H. Kitagawa, *Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans.* Current opinion in structural biology, 2000. **10**(5): p. 518-27.

99. Gotoh, M., et al., *Enzymatic synthesis of chondroitin with a novel chondroitin sulfate N-acetylgalactosaminyltransferase that transfers N-acetylgalactosamine to glucuronic acid in initiation and elongation of chondroitin sulfate synthesis.* The Journal of biological chemistry, 2002. **277**(41): p. 38189-96.

100. Gotoh, M., et al., *Molecular cloning and characterization of a novel chondroitin sulfate glucuronyltransferase that transfers glucuronic acid to N-acetylgalactosamine.* The Journal of biological chemistry, 2002. **277**(41): p. 38179-88.

101. Kitagawa, H., T. Uyama, and K. Sugahara, *Molecular cloning and expression of a human chondroitin synthase.* The Journal of biological chemistry, 2001. **276**(42): p. 38721-6.

102. Uyama, T., et al., *Molecular cloning and expression of human chondroitin N-acetylgalactosaminyltransferase: the key enzyme for chain initiation and elongation of chondroitin/dermatan sulfate on the protein linkage region tetrasaccharide shared by heparin/heparan sulfate.* The Journal of biological chemistry, 2002. **277**(11): p. 8841-6.

103. Uyama, T., et al., *Molecular cloning and expression of a second chondroitin N-acetylgalactosaminyltransferase involved in the initiation and elongation of chondroitin/dermatan sulfate.* The Journal of biological chemistry, 2003. **278**(5): p. 3072-8.

104. Whitelock, J.M. and R.V. Iozzo, *Heparan sulfate: a complex polymer charged with biological activity.* Chemical reviews, 2005. **105**(7): p. 2745-64.

105. Kim, B.T., et al., *Human tumor suppressor EXT gene family members EXTL1 and EXTL3 encode alpha 1,4- N-acetylglucosaminyltransferases that likely are involved in heparan sulfate/ heparin biosynthesis.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(13): p. 7176-81.

106. Kitagawa, H., H. Shimakawa, and K. Sugahara, *The tumor suppressor EXT-like gene EXTL2 encodes an alpha1, 4-N-acetylhexosaminyltransferase that transfers N-acetylgalactosamine and N-acetylglucosamine to the common glycosaminoglycan-protein linkage region. The key enzyme for the chain initiation of heparan sulfate.* The Journal of biological chemistry, 1999. **274**(20): p. 13933-7.

107. Grobe, K., et al., *Heparan sulfate and development: differential roles of the N-acetylglucosamine N-deacetylase/N-sulfotransferase isozymes.* Biochimica et biophysica acta, 2002. **1573**(3): p. 209-15.

108. Aikawa, J., et al., *Multiple isozymes of heparan sulfate/heparin GlcNAc N-deacetylase/GlcN N-sulfotransferase. Structure and activity of the fourth member, NDST4.* The Journal of biological chemistry, 2001. **276**(8): p. 5876-82.

109. Simons, M. and A. Horowitz, *Syndecan-4-mediated signalling.* Cellular signalling, 2001. **13**(12): p. 855-62.

110. Woods, A., *Syndecans: transmembrane modulators of adhesion and matrix assembly.* The Journal of clinical investigation, 2001. **107**(8): p. 935-41.

111. Alexopoulou, A.N., H.A. Multhaupt, and J.R. Couchman, *Syndecans in wound healing, inflammation and vascular biology.* The international journal of biochemistry & cell biology, 2007. **39**(3): p. 505-28.

112. Bernfield, M., et al., *Functions of cell surface heparan sulfate proteoglycans.* Annual review of biochemistry, 1999. **68**: p. 729-77.

113. Tkachenko, E., J.M. Rhodes, and M. Simons, *Syndecans: new kids on the signaling block.* Circulation research, 2005. **96**(5): p. 488-500.

114. Filmus, J., M. Capurro, and J. Rast, *Glypicans.* Genome biology, 2008. **9**(5): p. 224.

115. Filmus, J. and S.B. Selleck, *Glypicans: proteoglycans with a surprise.* The Journal of clinical investigation, 2001. **108**(4): p. 497-501.

116. Song, H.H. and J. Filmus, *The role of glypicans in mammalian development.* Biochimica et biophysica acta, 2002. **1573**(3): p. 241-6.

117. Wight, T.N., *Versican: a versatile extracellular matrix proteoglycan in cell biology.* Current opinion in cell biology, 2002. **14**(5): p. 617-23.

118. Jackson, R.L., S.J. Busch, and A.D. Cardin, *Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes.* Physiological reviews, 1991. **71**(2): p. 481-539.

119. Iozzo, R.V., *Perlecan: a gem of a proteoglycan.* Matrix biology : journal of the International Society for Matrix Biology, 1994. **14**(3): p. 203-8.

120. Knox, S.M. and J.M. Whitelock, *Perlecan: how does one molecule do so many things?* Cellular and molecular life sciences : CMLS, 2006. **63**(21): p. 2435-45.

121. Iozzo, R.V., *The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins.* The Journal of biological chemistry, 1999. **274**(27): p. 18843-6.

122. Kusche-Gullberg, M. and L. Kjellen, *Sulfotransferases in glycosaminoglycan biosynthesis.* Current opinion in structural biology, 2003. **13**(5): p. 605-11.

123. Nikitovic, D., et al., *Lumican, a small leucine-rich proteoglycan.* IUBMB life, 2008. **60**(12): p. 818-23.

124. Schaefer, L., et al., *Small proteoglycans in human diabetic nephropathy: discrepancy between glomerular expression and protein accumulation of decorin, biglycan, lumican, and fibromodulin.* The FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2001. **15**(3): p. 559-61.

125. Kajava, A.V., *Structural diversity of leucine-rich repeat proteins.* Journal of molecular biology, 1998. **277**(3): p. 519-27.

126. Weber, I.T., R.W. Harrison, and R.V. Iozzo, *Model structure of decorin and implications for collagen fibrillogenesis.* The Journal of biological chemistry, 1996. **271**(50): p. 31767-70.

127. Adeghate, E., P. Schattner, and E. Dunn, *An update on the etiology and epidemiology of diabetes mellitus.* Annals of the New York Academy of Sciences, 2006. **1084**: p. 1-29.

128. Petersen, K.F. and G.I. Shulman, *Etiology of insulin resistance.* The American journal of medicine, 2006. **119**(5 Suppl 1): p. S10-6.

129. Stumvoll, M., B.J. Goldstein, and T.W. van Haeften, *Type 2 diabetes: principles of pathogenesis and therapy.* Lancet, 2005. **365**(9467): p. 1333-46.

130. Waugh, N., et al., *Screening for hyperglycaemia in pregnancy: a rapid update for the National Screening Committee.* Health technology assessment, 2010. **14**(45): p. 1-183.

131. Steinke, J.M., *The natural progression of kidney injury in young type 1 diabetic patients.* Current diabetes reports, 2009. **9**(6): p. 473-9.

132. Dalla Vestra, M., et al., *Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes.* Diabetes, 2003. **52**(4): p. 1031-5.

133. Dalla Vestra, M., et al., *Structural involvement in type 1 and type 2 diabetic nephropathy.* Diabetes & metabolism, 2000. **26 Suppl 4**: p. 8-14.

134. Pagtalunan, M.E., et al., *Podocyte loss and progressive glomerular injury in type II diabetes.* The Journal of clinical investigation, 1997. **99**(2): p. 342-8.

135. Nieuwdorp, M., et al., *Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo.* Diabetes, 2006. **55**(2): p. 480-6.

136. Nieuwdorp, M., et al., *Endothelial glycocalyx damage coincides with microalbuminuria in type 1 diabetes.* Diabetes, 2006. **55**(4): p. 1127-32.

137. Schmid, H., et al., *Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies.* Kidney international, 2003. **64**(1): p. 356-60.

138. Fager, G., et al., *Binding of platelet-derived growth factor and low density lipoproteins to glycosaminoglycan species produced by human arterial smooth muscle cells.* Journal of cellular physiology, 1995. **163**(2): p. 380-92.

139. Andersson, M., et al., *Mild renal ischemia-reperfusion reduces charge and size selectivity of the glomerular barrier.* American journal of physiology. Renal physiology, 2007. **292**(6): p. F1802-9.

140. Conrotto, P. and S. Souchelnytskyi, *Proteomic approaches in biological and medical sciences: principles and applications.* Experimental oncology, 2008. **30**(3): p. 171-80.

141. Panchaud, A., et al., *Experimental and computational approaches to quantitative proteomics: status quo and outlook.* Journal of proteomics, 2008. **71**(1): p. 19-33.

142. Ha, T.S., et al., *Regulation of glomerular endothelial cell proteoglycans by glucose.* Journal of Korean medical science, 2004. **19**(2): p. 245-52.

143. Makino, H., et al., *Ultrastructural changes of extracellular matrices in diabetic nephropathy revealed by high resolution scanning and immunoelectron microscopy.* Laboratory investigation; a journal of technical methods and pathology, 1993. **68**(1): p. 45-55.

144. van den Hoven, M.J., et al., *Increased expression of heparanase in overt diabetic nephropathy.* Kidney international, 2006. **70**(12): p. 2100-8.

145. Brownlee, M., *Biochemistry and molecular cell biology of diabetic complications.* Nature, 2001. **414**(6865): p. 813-20.

146. Lampugnani, M.G. and E. Dejana, *Interendothelial junctions: structure, signalling and functional roles.* Current opinion in cell biology, 1997. **9**(5): p. 674-82.

147. Satchell, S.C., K.L. Anderson, and P.W. Mathieson, *Angiopoietin 1 and vascular endothelial growth factor modulate human glomerular endothelial cell barrier properties.* Journal of the American Society of Nephrology : JASN, 2004. **15**(3): p. 566-74.

148. Gekle, M., *Renal tubule albumin transport.* Annual review of physiology, 2005. **67**: p. 573-94.

149.   Oberleithner, H., K. Kusche-Vihrog, and H. Schillers, *Endothelial cells as vascular salt sensors.* Kidney international, 2010. **77**(6): p. 490-4.

150.   Ohlson, M., et al., *Effects of filtration rate on the glomerular barrier and clearance of four differently shaped molecules.* American journal of physiology. Renal physiology, 2001. **281**(1): p. F103-13.

151.   Rippe, C., et al., *Effects of glomerular filtration rate on Ficoll sieving coefficients (theta) in rats.* Kidney international, 2006. **69**(8): p. 1326-32.

152.   Haraldsson, B. and B. Rippe, *Serum factors other than albumin are needed for the maintenance of normal capillary permselectivity in rat hindlimb muscle.* Acta physiologica Scandinavica, 1985. **123**(4): p. 427-36.

153.   Haraldsson, B. and B. Rippe, *Orosomucoid as one of the serum components contributing to normal capillary permselectivity in rat skeletal muscle.* Acta physiologica Scandinavica, 1987. **129**(1): p. 127-35.

154.   Lindgren, D., et al., *Isolation and characterization of progenitor-like cells from human renal proximal tubules.* The American journal of pathology, 2011. **178**(2): p. 828-37.

155.   Harukuni, I., J.R. Kirsch, and A. Bhardwaj, *Cerebral resuscitation: role of osmotherapy.* Journal of anesthesia, 2002. **16**(3): p. 229-37.