The endothelial surface layer: a new target of research in kidney failure and peritoneal dialysis

Vlahu, C.A.

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CARMEN ANCA VLAHU

THE ENDOTHELIAL SURFACE LAYER:
A NEW TARGET OF RESEARCH
IN KIDNEY FAILURE AND PERITONEAL DIALYSIS

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The endothelial surface layer:
a new target of research in kidney failure and
peritoneal dialysis

Carmen Anca Vlahu
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peritoneal dialysis

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aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
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ten overstaan van een door het college voor promoties ingestelde
commissie, in het openbaar te verdedigen in de Agnietenkapel
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Faculteit der Geneeskunde
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Chapter 1

Introduction
Objectives and Outline of the Thesis
1. The endothelial glycocalyx

1.1 Structure and functions

The endothelial glycocalyx is a negatively charged mesh of glycoproteins, proteoglycans and glycosaminoglycans (GAGs) coating the luminal side of the endothelium (Fig. 1A).\(^1\) Together with associated plasma molecules it forms the endothelial surface layer, that can restrict the flow of plasma, and can exclude red blood cells and various macromolecules.\(^1\) Visualized for the first time in 1966 by Luft et al after ruthenium red staining,\(^2\) this layer is an important regulator of endothelial function in the systemic circulation and exerts various vasculoprotective effects.\(^3\) These include mechanosensing and shear-induced release of nitric oxide, regulation of coagulation and redox state, prevention of leucocyte adhesion, regulation of endothelial permeability, angiogenesis and protection of the vasculature against accelerated atherosclerosis.\(^1,4\) This complex and dynamic structure harbours various enzymes (e.g., extracellular super oxide dismutase), and binds several growth factors and therefore modulates their signaling capacity. In addition, the endothelial glycocalyx reversibly binds Na\(^+\) via the negatively charged GAGs and acts as a buffer for sodium at the endothelial level.\(^5\)

Heparan sulfate (HS) is the main glycosaminoglycan within the endothelial glycocalyx accounting for up to 60% of the total GAGs.\(^4,6\) Chondroitin sulfate (CS), dermatan sulfate and hyaluronan (HA) account for the rest. It has been shown that HS is distributed in the luminal part of the EG, more distal from the plasma membrane, whereas CS and HA are attached to the proteoglycans at sites closer to the membrane.\(^7\)

![Figure 1A. Staining of the mesenteric microvasculature with *Lycopersicon esculentum* lectin (red: the lectin binds to N-acetylglucosamine- (GlcNAc) 2-4 within the endothelial glycocalyx; green: autofluorescence of the erythrocytes present in the vascular lumen; blue: DAPI for the nuclei) in rats with normal kidney function. A continuous lining is visible at the luminal side of the endothelium.](image-url)
Hyaluronan is the only non-sulfated GAG and is not attached to a proteoglycan but to its receptors CD44 and RHAMM (receptor for hyaluronan mediated motility). Amongst the proteoglycans, syndecans and glypicans are the most common molecules on the endothelial surface (Fig. 1B). The glycocalyx dimension increases with the vascular diameter and is dependent upon the balance between biosynthesis and shedding of its components into the blood. A hydrodynamically relevant glycocalyx dimension of approximately 0.5 µm has been measured by Vink et al in hamster cremaster muscle capillaries using a fluorescent dye-exclusion technique, whereas in rat mesenteric small arteries its thickness reached 3 µm.

1.2 Consequences of glycocalyx damage
The endothelial glycocalyx is an important regulator of vascular homeostasis. Degradation of glycocalyx structures was found to occur after provocation with inflammatory and atherogenic stimuli such as ischemia-reperfusion, infusion of oxidized low-density lipoproteins, administration of tumor-necrosis factor-α and hyperglycemia. Consequences of glycocalyx damage include increased leucocyte and platelet adhesion, activation of coagulation, increased vascular permeability, impaired signal transduction followed by reduced release of nitric oxide in response to shear stress, and accelerated atherosclerosis. Pathologic loss of glycocalyx may therefore be associated with increased endothelial vulnerability and an impaired vascular wall protection throughout the circulatory system. Disruption of the endothelial glycocalyx may be the first step in the development of atherosclerosis.

1.3 Methods for the assessment of the endothelial glycocalyx in vivo
Given the role of the endothelial glycocalyx in vascular homeostasis, the development
of non-invasive methods that allow the assessment of this structure in vivo, has become of major importance. Although successfully used in experimental settings, intravital microscopy is not suitable for studies of the endothelial glycocalyx in humans. Nieuwdorp et al. reported for the first time measurements of the systemic glycocalyx volume in humans by comparing the distribution volume of dextran 40, a glycocalyx permeable tracer, with that of fluorescently labelled erythrocytes, which cannot penetrate into the glycocalyx. Using this method, the authors measured a systemic glycocalyx volume of 1.7 liters in healthy individuals. However, this method is invasive and cannot be easily implemented in clinical studies. Recently, a new imaging method, Sidestream Darkfield microscopy, has been developed based on the observation that in normal conditions the red blood cells (RBC) are largely excluded from the endothelial cell by the endothelial surface layer. Only the luminal part of the ESL transiently allows the access of erythrocytes. With this non-invasive method, movies of the sublingual microvasculature are recorded and measurement sites are placed automatically every 10 µm along each visible microvessel. An extensive description of the method is given in chapter 2 of this thesis. The RBC column width is measured at each site in consecutive frames. The position of the outer edge of the RBC perfused lumen at each measurement site (DPerf) is derived from the RBCW distribution, and the perfused boundary region is defined as the distance of the median RBCW to the outer edge of the perfused diameter. Importantly, this parameter reflects the erythrocyte permeable region of the endothelial surface layer and therefore, it is not a measure of its anatomic thickness.

In addition, determinations of plasma levels of glycocalyx constituents have been performed in various clinical conditions. The most widely used are hyaluronan and syndecan-1, but also heparan sulfate. Although most of these molecules, for instance hyaluronan, which is the main constituent of the extracellular matrix, are not specific for the endothelium, high plasma levels have been associated with alterations of the endothelium.

Figure 2. Schematic illustration of endothelial surface layer imaging method. RBC width = median red blood cell column width.* DPerf = perfused diameter (RBC perfused lumen). ** PBR = perfused boundary region (RBC permeable part of cell free layer including cell permeable glycocalyx). *** Cell free layer.
lial surface layer. However, the interpretation remains challenging, also because effects of kidney function on plasma concentrations have not been clearly established.

2. Chronic kidney failure

2.1 Endothelial surface layer in chronic kidney failure and after successful renal transplantation

Dialysis patients have increased morbidity and mortality, which is only partly caused by their increased risk for cardiovascular disease. Endothelial dysfunction, increased endothelial permeability, oxidative stress, inflammation, overhydration, and accelerated vascular disease are common features in patients with chronic renal failure. Therefore, considering its vasculoprotective effects, the endothelial glycocalyx may be relevant for the development of vascular complications in chronic kidney disease, and it is reasonable to hypothesize that changes in the endothelial glycocalyx may occur in this condition. Successful renal transplantation improves the survival of patients with end-stage renal disease but is accompanied by only a modest decrease in cardiovascular mortality. Successful transplantation solves the uremia related toxicity but cannot erase the pre-existent cardiovascular burden in these patients. In addition, the side effects of various immunosuppressive regimens add to this pre-existent burden. It is not known if successful kidney transplantation restores the endothelial glycocalyx and thereby improves endothelial function, contributing to an improved risk profile of transplanted patients.

2.2 The endothelial glycocalyx in peritoneal dialysis

Peritoneal dialysis (PD) makes use of peritoneal tissues as a dialysis membrane to remove waste products and an excess of fluid from the body. The capillary wall of peritoneal blood vessels represents the initial resistance to solute transport from the plasma through the interstitial tissues and the mesothelial layer to the dialysate in the peritoneal cavity. Low molecular weight solutes are removed from the blood through the endothelial small pore system, probably interendothelial clefts with radii of 40-55 Å. Circulating macromolecules pass through a limited number of so-called large pores, the anatomic substrate of which has not been established with certainty, but may be interendothelial gaps that can be present in the venular endothelium, especially in situations of vasodilation, and have radii >250 Å. The transport of macromolecules is size-dependent, but independent of electric charge. Transcapillary ultrafiltration of fluid occurs through the interendothelial pores and through the endothelial water channel aquaporin-1 (ultrasmall pore, radius<5Å) that is present in peritoneal endothelial cells, and allows the transport of water only. Whereas solute removal occurs mainly by diffusion through the pores, fluid removal is by filtration, either by hydrostatic gradients (small pores) or by crystalloid osmosis (both small and ultrasmall pores). The latter is generated by the extremely high glucose concentrations present in the conventional dialysis solutions.
Endothelial permeability is in part dependent on the endothelial glycocalyx\textsuperscript{4,16,35} Therefore, this structure is the primary barrier in transendothelial solute and water transport in the systemic circulation. The role of this delicate layer of polysaccharides in transcapillary transport has been extensively demonstrated in several studies which showed that various insults to the glycocalyx result in increased vascular permeability to solutes of different size\textsuperscript{36} or to water, followed by development of tissue edema\textsuperscript{37}. Therefore, it has been argued that the state of the peritoneal glycocalyx might also be of major importance to PD. This has led to new hypotheses concerning the factors that influence the transport characteristics of the peritoneal membrane\textsuperscript{38,39} however without any proof. The peritoneal tissues in patients treated with PD are chronically exposed to extremely high glucose concentrations present in PD fluids, which cause neoangiogenesis, and contribute to the development of peritoneal fibrosis\textsuperscript{40}. Similar to the situation for the over-all glycocalyx in diabetes mellitus\textsuperscript{15}, the peritoneal endothelial glycocalyx may be altered in patients treated with chronic peritoneal dialysis. Therefore, it can be hypothesized that the changes in the systemic endothelial glycocalyx in patients with diabetes mellitus due to hyperglycemia might also occur in the peritoneal microcirculation of PD patients due to the high dialysate glucose concentrations, although the exposure routes are different: in diabetes the endothelial exposure is luminal, while in PD it is from the basolateral side. Also, the glucose concentrations are of a different magnitude: from 20 mmol/L in blood in hyperglycemic diabetics to 200 mmol/L in the dialysate of a PD patient. In addition, neoangiogenesis occurs during PD and new vessel formation is associated with disrupted endothelial glycocalyx at the luminal side of a blood vessel\textsuperscript{41}. All this makes it likely that changes in the peritoneal endothelial glycocalyx occur during PD and they are distinct from the changes present in the systemic microcirculation.

Endothelial glycocalyx damage in PD may be of importance not only with regard to peritoneal transport. Peritoneal alterations, such as angiogenesis and interstitial damage,
are promoted by the release of growth factors, cytokines and various proteins into the peritoneal tissues, caused by damage to the endothelial glycocalyx. Therefore, defining the role of the glycocalyx in this condition will guide further strategies on its preservation.

3. Peritoneal dialysis - effects on the peritoneum

Long-term treatment with peritoneal dialysis can induce morphological and functional alterations of the peritoneal tissues. Structural changes include: loss and degeneration of the mesothelium, thickening of the submesothelial compact zone, fibrosis, and a variety of vascular abnormalities, like diabetiform reduplication of the basement membrane of peritoneal capillaries, subendothelial hyalinization with luminal narrowing or obliteration, deposition of type IV collagen within the arterial wall, neoangiogenesis and lymphangiogenesis. The extremely high concentrations of glucose in the dialysis fluids together with heat sterilization-induced glucose degradation products (GDPs), both resulting in peritoneal deposition of advanced-glycation end-products (AGEs), and the high lactate concentration are probably the most important factors responsible for these alterations. The aforementioned changes may lead to an increase in peritoneal solute transport and, in some patients, to ultrafiltration failure, which sometimes leads to cessation of this method of renal replacement therapy. Therefore, in order to reduce the PD-related peritoneal alterations and prolong the treatment with PD, new dialysis solutions with lower GDPs content, neutral pH or using different osmotic agents than glucose, have been developed. The effects of different solutions have been investigated mainly in experimental studies, and most of those studies reported favorable effects of the new solutions on the peritoneum, showing less fibrosis and less angiogenesis compared to the conventional solutions. Importantly, many of these studies either used animals with normal kidney function, or the follow-up period was very short. Surprisingly, the significant favorable effects on peritoneal morphology have not been confirmed by functional data in either patients or animals with renal failure and a long exposure duration. No significant favorable effect on peritoneal transport parameters was found in these studies and therefore, the question arose whether these models can actually mimic the human situation and many questions remained unanswered. The long-term peritoneal exposure in a rat model with chronic kidney failure, as developed by our group, allows to include both the effects of renal failure and those of long-term peritoneal exposure. Therefore we used this model for our further investigations.

Ultrafiltration failure may develop in some patients treated with PD, and is characterized by insufficient removal of excess of fluid from the body. Whereas the presence of a large vascular surface area, associated with high transport rates of small solutes and a rapid decrease in the osmotic gradient, is the major cause of ultrafiltration failure, another factor that can contribute to the development of this complication is a high
lymphatic reabsorption of intraperitoneally administered dialysis solutions, leading to a decrease in the intraperitoneal volume. Lymphatic drainage from the peritoneal cavity occurs mainly via the subdiaphragmatic lymphatics, but also by lymphatics that accompany the peritoneal interstitial vasculature. The lymphatic absorption rate can be assessed in patients during a standard peritoneal permeability analysis, by measurement of the disappearance rate of Dextran 70, which is used as volume marker, from the peritoneal cavity. Lymphangiogenesis has been described to develop in rats during peritoneal exposure and is related to peritoneal fibrosis. However in PD patients no effect of long term exposure to dialysis solutions on the lymphatic absorption rate from the peritoneal cavity has been found. Therefore, the relationship between the degree of lymphangiogenesis and the lymphatic reabsorption, as measured by a peritoneal function test, is unknown.

4. Objectives and outline of the thesis

The objectives of this thesis are to identify the alterations of the endothelial surface layer in the systemic microvasculature associated with end-stage renal disease, and to investigate the state of the endothelial glycocalyx in both the systemic and the peritoneal microcirculation during peritoneal dialysis, and explore its relationships with peritoneal transport. In addition, we do an extensive analysis of the peritoneal alterations induced by long-term exposure to glucose-based dialysis solutions.

The first part of the thesis addresses the state of the endothelial surface layer in patients with chronic kidney failure. In chapter 2 we assess the state of the endothelial surface layer in the systemic microcirculation in patients with end-stage renal disease, treated with hemodialysis or peritoneal dialysis, and compare the results with the data in healthy individuals. In all study participants, imaging of the sublingual microvasculature is performed, and plasma concentrations of circulating glycocalyx constituents are measured. The state of the endothelial surface layer in renal transplant recipients with stable renal function is investigated in chapter 3. Chapter 4 addresses the utility of hyaluronan plasma concentrations as marker for the endothelial glycocalyx in patients with chronic kidney disease. The second part of the thesis focuses on the importance of endothelial glycocalyx during treatment with peritoneal dialysis. In chapter 5 we test whether relationships are present between the state of the systemic microvascular glycocalyx as assessed by sidestream darkfield imaging of the sublingual microvasculature, and parameters of peritoneal transport in patients treated with peritoneal dialysis. In chapter 6 we assess the endothelial surface layer in the peritoneal microvasculature in rats with chronic kidney disease, with or without exposure to dialysis solutions and compare it to rats with normal renal function. For this, SDF imaging of the peritoneal microcirculation and immunohistochemistry of peritoneal specimens are performed.
In addition, relationships with peritoneal transport parameters are tested. The third part of the thesis focuses on the peritoneal membrane alterations induced by long-term exposure to dialysis solutions. In chapter 7 we investigate the effects of chronic kidney disease and long-term exposure to a conventional and biocompatible dialysis solutions on the peritoneum in an experimental rat model. The results of the measurement of the effective lymphatic absorption rate during a standard peritoneal permeability analysis in rats, and the relationship with lymphangiogenesis are presented in chapter 8. In chapter 9 all the above mentioned chapters are discussed and a summary of the main findings is formulated.
References

The Endothelial Glycocalyx in Chronic Kidney Failure
Chapter 2

Damage of the Endothelial Glycocalyx in Dialysis Patients

Carmen A. Vlahu, Bregtje A. Lemkes, Dirk G. Struijk, Marion G. Koopman, Raymond T. Krediet, Hans Vink

J Am Soc Nephrol 2012 Nov; 23(11):1900-8
Abstract

Damage to the endothelial glycocalyx, which helps maintain vascular homeostasis, heightens the sensitivity of the vasculature to atherogenic stimuli. Patients with renal failure have endothelial dysfunction and increased risk for cardiovascular morbidity and mortality, but the state of the endothelial glycocalyx in these patients is unknown. Here, we used Sidestream Darkfield imaging to detect changes in glycocalyx dimension in dialysis patients and healthy controls from \textit{in vivo} recordings of the sublingual microcirculation. Dialysis patients had increased perfused boundary region and perfused diameters, consistent with deeper penetration of erythrocytes into glycocalyx, indicating a loss of glycocalyx barrier properties. These patients also had higher serum levels of the glycocalyx constituents hyaluronan and syndecan-1 and increased hyaluronidase activity, suggesting the shedding of these components. Loss of residual renal function had no influence on the imaging parameters but did associate with greater shedding of hyaluronan in blood. Furthermore, patients with higher levels of inflammation had more significant damage to the glycocalyx barrier. In conclusion, these data suggest that dialysis patients have an impaired glycocalyx barrier and shed its constituents into blood, likely contributing to the sustained endothelial cell activation observed in ESRD.
Endothelial Glycocalyx in ESRD

Introduction

Patients with chronic renal failure have endothelial dysfunction and accelerated vascular disease leading to increased morbidity and mortality as a result of cardiovascular events. The mechanisms responsible are unclear, controversial, and presumed to be multifactorial. The vascular endothelium is coated on the luminal side by the glycocalyx, a negatively charged mesh of proteoglycans (PGs) and associated glycosaminoglycans. It is involved in mediating shear-induced release of nitric oxide and contributes to the endothelial permeability barrier, the regulation of redox state, and the inhibition of coagulation as well as leucocyte and platelet adhesion. Perturbation of glycocalyx occurs after provocation with inflammatory or atherogenic stimuli (such as ischemia reperfusion, infusion of oxidized LDL, administration of TNF-α or endotoxin, and during hyperglycemia) and after stimulation with thrombin, atrial natriuretic peptide, or abnormal blood shear stress. Consequences of glycocalyx perturbation include a wide range of vascular abnormalities in experimental models, including increased vascular permeability followed by generation of tissue edema, increased rolling and adhesion of leucocytes, and increased platelet adhesion. Therefore, disruption of the glycocalyx leads to enhanced sensitivity of vasculature to atherogenic stimuli. Based on these observations, the importance of integrity of the endothelial glycocalyx in vascular homeostasis has become evident.

Attempts to assess the impairment of endothelial function in vivo are a challenge given the multifunctional nature of endothelial cells and lack of standardized tools to non-invasively assess endothelial function in a patient friendly manner. We recently developed an imaging-based method to detect changes in glycocalyx dimension from in vivo recordings of the sublingual microcirculation, enabling us to assess the microvascular glycocalyx in vivo in patients. Previous studies have shown that, in healthy volunteers, the glycocalyx is disrupted by acute hyperglycaemia. Subsequently, a significant reduction in glycocalyx volume was found in patients with type 1 diabetes. This disruption may contribute to the known predisposition of these patients to vascular disease.

No data are available on the state of the endothelial glycocalyx in patients with chronic renal failure. However, it is reasonable to hypothesize that the endothelial glycocalyx is affected in these patients given their predisposition to endothelial dysfunction and vascular disease. A damaged glycocalyx may lead to increased vulnerability and susceptibility of endothelial cells to vascular risk factors present in uremia. Therefore, the objective of this study was to answer the following questions: (1) Is the microvascular endothelial glycocalyx damaged in patients with ESRD on both hemodialysis (HD) and peritoneal dialysis (PD) compared with age- and sex-matched healthy controls? (2) Do dialysis patients have increased serum concentrations of glycocalyx constituents reflecting increased shedding? (3) Do the changes in endothelial glycocalyx correlate with other serum markers of endothelial activation, like sE-selectin?
Results

Clinical Characteristics
Clinical characteristics of the healthy controls and dialysis patients are listed in Table 1. Dialysis patients had significantly higher systolic and diastolic BPs, a different lipid profile, and increased levels of C-reactive protein (CRP) and malondialdehyde (MDA) compared with healthy controls. Residual renal function was present in 35% of our dialysis group. IL-6 levels were available in 24 dialysis patients and had a median of 8.6 (4.8-10.0) pg/ml. We found a positive correlation between IL-6 and CRP levels ($r=0.58$, $p<0.01$).

Imaging of the Microcirculation
The perfused diameter (DPerf) and perfused boundary region (PBR) were increased in patients compared with healthy controls: DPerf, 17.7 (1.1) versus 16.4 (1.3) µm, $p<0.01$; PBR, 3.6 (0.5) versus 3.3 (0.4) µm, $p<0.01$ (Figure 1). No significant difference was found in red blood cell column width (RBCW): 10.5 (0.7) versus 10.1 (0.6) µm, $p=0.06$.

Table 1. Baseline characteristics of study participants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Controls (n=21)</th>
<th>Dialysis Patients (n=40)</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex male/female</td>
<td>12/9</td>
<td>25/15</td>
<td>0.78</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>44.1 (14.1)</td>
<td>44.1 (115.1)</td>
<td>0.99</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>22.6 (2.5)</td>
<td>23.0 (8.0)</td>
<td>0.43</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>121.7 (13.4)</td>
<td>147.7 (26.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75.0 (6.0)</td>
<td>91.5 (17.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Time on dialysis (mo)</td>
<td>-</td>
<td>50.6 (15.4-103.6)</td>
<td></td>
</tr>
<tr>
<td>Time on RRT (mo)</td>
<td>-</td>
<td>55.6 (17.4-167.4)</td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.9 (0.5)</td>
<td>5.2 (0.9)</td>
<td>0.20</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.1 (0.9)</td>
<td>4.3 (0.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.1 (0.8)</td>
<td>2.4 (0.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.6 (0.5)</td>
<td>1.1 (0.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.9 (0.7-1.0)</td>
<td>1.5 (1.1-2.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.0 (1.0-1.0)</td>
<td>2.3 (1.0-9.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ALAT (U/L)</td>
<td>22.0 (5.8)</td>
<td>19.4 (10)</td>
<td>0.27</td>
</tr>
<tr>
<td>MDA (µM/L)</td>
<td>1.8 (1.6-2.5)</td>
<td>3.9 (3.1-17.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>RRF present (%)</td>
<td></td>
<td>35%</td>
<td></td>
</tr>
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</table>

Results are expressed as mean (SD) except for time on dialysis, time on renal replacement therapy (RRT), triglycerides, CRP, and MDA, which are expressed as median (interquartile range). BMI, body mass index; ALAT, alanine aminotransferase.
To test whether both PD and HD patients behaved the same way, subgroup analysis was performed (Figure 2). Both PD and HD patients showed a significant increase in DPerf and PBR compared with healthy controls: Dperf, 16.4 (1.3) µm in controls, 17.3 (1.0) µm in PD ($p=0.03$), 18.1 (1.1) µm in HD ($p<0.01$); PBR: 3.3 (0.4) µm in controls, 3.6 (0.4) µm in PD ($p<0.01$), and 3.6 (0.5) µm in HD ($p=0.02$). We found no correlation between the imaging parameters and BP, parameters of lipid profile, CRP, time on renal replacement therapy (RRT), or time on dialysis (data not shown).

**Glycocalyx Constituents and Their Regulating Enzymes**

Serum levels of hyaluronan (HA), hyaluronidase activity, syndecan-1, and E-selectin were higher in patients compared to healthy controls (Table 2). We did not find any correlation between these parameters and the imaging parameters, CRP, or parameters of lipid profile (data not shown). In dialysis patients, HA levels positively correlated with...
total time on RRT (Figure 3) and time on dialysis ($r^2=0.24$, $p<0.01$) and hyaluronidase activity was correlated with MDA levels ($r=0.57$, $p<0.01$). There was no correlation between HA levels and hyaluronidase activity (data not shown).

**Effect of Residual Renal Function**

Within our dialysis group, the presence of residual renal function (RRF) seemed to have no influence on the degree of alterations in microcirculation as determined by Sidestream Darkfield (SDF) imaging. Patients without RRF had increased levels of HA and decreased hyaluronidase activity and sE-selectin compared with those patients with preserved RRF. Furthermore, this group of patients had a longer time on RRT (Table 3).

**Effect of Inflammation**

Patients were divided in two groups based on CRP levels higher or lower than 10mg/L. Patients with higher levels of CRP seemed to have a significant increase in PBR (3.91
Endothelial Glycocalyx in ESRD

[0.56] versus 3.55 [0.39] µm, \( p = 0.03 \)) and sE-selectin (55.4 [44.1-81.2] versus 45.6 [26.1-61.1] ng/ml, \( p = 0.04 \)). No significant differences were observed with regard to the other biochemical or imaging parameters (Table 4).

Cardiovascular Disease
Six patients (15%) had a history of documented cardiovascular disease (CVD): coronary artery disease, cerebrovascular disease, or peripheral vascular disease. These patients seemed to have a significant increase in PBR and CRP compared with those patients without CVD (PBR, 4.04 [3.6-4.4] versus 3.5 [3.3-3.9] µm, \( p = 0.04 \); CRP, 16.8 [4.5-34.5] mg/l versus 1.9 [1.0-4.9] mg/l, \( p = 0.02 \)). No significant differences were observed with regard to other biochemical or imaging parameters (Table 5).

Discussion
Analysis of the spatial and temporal variations of erythrocyte column width in the sublingual microvasculature reveals a significant increase in the dimension of the erythrocyte-permeable region bordering the red blood cell (RBC) column in dialysis patients compared with healthy controls. The increased PBR and the corresponding increased dimension of the erythrocyte DPerf are consistent with deeper penetration of RBCs into glycocalyx on the luminal surface of the endothelium (i.e., loss of glycocalyx barrier properties). Additionally, we found increased serum levels of HA and syndecan-1 in patients, consistent with shedding of these components from the vascular wall. To our

Table 3. Effect of RRF on outcome parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dialysis (RRF+)</th>
<th>Dialysis (RRF-)</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Imaging parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBCW (µm)</td>
<td>10.40 (0.74)</td>
<td>10.55 (0.69)</td>
<td>0.49</td>
</tr>
<tr>
<td>DPerf (µm)</td>
<td>17.71 (1.15)</td>
<td>17.75 (1.13)</td>
<td>0.90</td>
</tr>
<tr>
<td>PBR (µm)</td>
<td>3.69 (0.45)</td>
<td>3.58 (0.45)</td>
<td>0.44</td>
</tr>
<tr>
<td><strong>Biochemical parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaluronan (ng/ml)</td>
<td>19.2 (6.6-61.1)</td>
<td>44.7 (28.5-71.4)</td>
<td>0.04</td>
</tr>
<tr>
<td>Hyaluronidase activity (U/ml)</td>
<td>31.6 (26.4-38.9)</td>
<td>24.9 (23.4-31.7)</td>
<td>0.04</td>
</tr>
<tr>
<td>Syndecan-1 (ng/ml)</td>
<td>95.3 (68.1-159.5)</td>
<td>116.6 (90.7-152.8)</td>
<td>0.69</td>
</tr>
<tr>
<td>sE-selectin (ng/ml)</td>
<td>56.6 (12.9-154.0)</td>
<td>43.8 (23.5-53.2)</td>
<td>0.03</td>
</tr>
<tr>
<td>Time on RRT (mo)</td>
<td>17.9 (11.5-39.5)</td>
<td>137.5 (64.8-238.5)</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

Results of imaging parameters are presented as mean (SD). Results of biochemical parameters and time on RRT are presented as median (interquartile range). RRF+, RRF is present; RRF-, RRF is absent.
Table 4. Effect of inflammation on outcome parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dialysis, (CRP&lt;10mg/L)</th>
<th>Dialysis, (CRP&gt;10mg/L)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Imaging parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBCW (µm)</td>
<td>10.57 (0.75)</td>
<td>10.22 (0.45)</td>
<td>0.19</td>
</tr>
<tr>
<td>DPerf (µm)</td>
<td>17.68 (1.17)</td>
<td>17.92 (0.97)</td>
<td>0.48</td>
</tr>
<tr>
<td>PBR (µm)</td>
<td>3.55 (0.39)</td>
<td>3.91 (0.56)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Biochemical parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaluronan (ng/ml)</td>
<td>34.9 (15.0-69.3)</td>
<td>51.5 (7.2-84.1)</td>
<td>0.92</td>
</tr>
<tr>
<td>Hyaluronidase activity (U/ml)</td>
<td>26.3 (24.1-34.6)</td>
<td>26.4 (22.9-37.5)</td>
<td>0.71</td>
</tr>
<tr>
<td>Syndecan-1 (ng/ml)</td>
<td>111.0 (76.8-165.5)</td>
<td>107.0 (80.5-144.5)</td>
<td>0.74</td>
</tr>
<tr>
<td>sE-selectin (ng/ml)</td>
<td>45.8 (26.1-61.1)</td>
<td>55.4 (44.1-81.2)</td>
<td>0.04</td>
</tr>
<tr>
<td>Time on RRT (mo)</td>
<td>52.6 (16.7-147.8)</td>
<td>129.3 (15.2-281.4)</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Results of imaging parameters are presented as mean (SD). Results of biochemical parameters and time on RRT are presented as median (interquartile range).

Table 5. Effect of presence of CVD on outcome parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dialysis (CVD+)</th>
<th>Dialysis (CVD-)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Imaging parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBCW (µm)</td>
<td>10.19 (9.66-10.44)</td>
<td>10.50 (10.00-11.03)</td>
<td>0.14</td>
</tr>
<tr>
<td>DPerf (µm)</td>
<td>17.75 (17.35-18.24)</td>
<td>17.59 (17.15-18.24)</td>
<td>0.87</td>
</tr>
<tr>
<td>PBR (µm)</td>
<td>4.04 (3.57-4.40)</td>
<td>3.50 (3.32-3.87)</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Biochemical parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaluronan (ng/ml)</td>
<td>56.1 (3.8-107.6)</td>
<td>34.9 (15.02-69.18)</td>
<td>0.83</td>
</tr>
<tr>
<td>Hyaluronidase activity (U/ml)</td>
<td>33.1 (23.2-39.9)</td>
<td>27.8 (24.0-32.7)</td>
<td>0.45</td>
</tr>
<tr>
<td>Syndecan-1 (ng/ml)</td>
<td>101.2 (64.1-149.0)</td>
<td>116.5 (84.7-158.3)</td>
<td>0.52</td>
</tr>
<tr>
<td>sE-selectin (ng/ml)</td>
<td>51.8 (38.8-76.8)</td>
<td>45.9 (26.9-83.7)</td>
<td>0.38</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>16.8 (4.5-34.5)</td>
<td>1.9 (1.0-4.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>Time on RRT (mo)</td>
<td>119.4 (9.8-292.1)</td>
<td>53.6 (17.4-154.6)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Results are presented as median (interquartile range).
knowledge, this study is the first to examine the endothelial glycocalyx using non-invasive microvascular imaging and report increased serum levels of glycocalyx constituents and regulating enzyme in patients with ESRD.

The presence of glycocalyx prevents RBCs from approximating the endothelium very closely. Previous studies, in which microvascular glycocalyx was assessed in humans by SDF imaging of the sublingual microvasculature, together with previous experimental studies, have led to the concept that perturbation of the glycocalyx is associated with an impairment of its RBCs-excluding properties, affecting the temporal and spatial variations of the microvascular RBC column width. Similarly, recent experimental studies showed that enzymatic degradation of glycocalyx is followed by increases in PBR. The PBR reflects how far RBCs can penetrate into the erythrocyte-accessible part of the cell-free layer. The increase in PBR found in dialysis patients is consistent with increased penetration of RBCs into glycocalyx. Overall, our findings imply that analysis of dynamic microvascular RBCs column width variations can be used to detect pathophysiological changes near the microvascular wall in dialysis patients.

In the setting of CKD, several factors may contribute to the alteration of the endothelial glycocalyx; however, the exact mechanisms responsible still remain to be elucidated. The vascular endothelial dysfunction in CKD is associated with a chronic inflammatory state. In our study, patients with higher levels of inflammation as measured by CRP had an increased PBR compared with those patients with lower levels of CRP. This finding is indicative for a more pronounced alteration in glycocalyx barrier properties in this group. CKD is also associated with a chronic deficiency in antioxidant systems. Increased oxidative stress acts as a major contributor to severe atherosclerosis and cardiovascular morbidity and mortality found in these patients, and reactive oxygen species can mediate alterations of the endothelial glycocalyx. Overhydration is another common problem in dialysis patients, especially after loss of RRF; also, hypervolemia can alter the endothelial surface layer potentially through atrial natriuretic peptide, which causes shedding of glycocalyx constituents in blood. Loss of RRF is associated with progressive impairment of endothelial function and is a risk factor of cardiovascular mortality in dialysis patients. In the present study, we found significantly higher levels of HA in the anuric group compared with patients with RRF, but the SDF imaging of the microcirculation did not detect significant differences between the two groups.

One of the earliest changes in the endothelium upon activation is an alteration in the glycocalyx composition, with shedding of its constituents into the circulation. Here, we investigated the plasma levels of the glycocalyx constituents HA, syndecan-1, and the regulating enzyme hyaluronidase. HA, a high molecular weight polysaccharide, is an important component of the endothelial glycocalyx. It serves as a mechano-shear...
sensor, regulates NO release, and maintains vascular permeability. In vitro studies have shown that inflammatory cytokines, particularly IL-1, IL-6, and TNF-α, as well as reactive oxygen species are involved in the degradation of HA. High serum levels of HA have been reported in CKD and seem to be a risk predictor of poor survival in dialysis. However, the mechanisms underlying the increased levels have not been fully explained. They may reflect altered connective tissue metabolism in uremia. Shedding of HA from the endothelial glycocalyx on the vascular wall may be another cause. In the present study, the high HA levels found in patients were positively correlated with the duration of dialysis treatment, confirming previously reported data. Although the correlation was highly significant, the explained variance was small: only 24% of the variability of HA was explained by its relationship with the duration of dialysis treatment. This finding makes it likely that complex mechanisms are responsible for the perturbation of HA metabolism. In general, increased HA production and turnover are associated with increased hyaluronidase levels, and our results support these findings. The fact that other glycosaminoglycans than HA, like heparan sulfate (HS) or chondroitin sulfate, are bound by hyaluronidase may explain the lack of correlation between HA levels and hyaluronidase activity. Syndecans are a family of four transmembrane HSPGs that is the major source of cellular HS. Syndecan-1 can be shed through a proteolytic, or oxidative mechanism and functions as soluble HSPGs in the extracellular milieu. Syndecan shedding is stimulated by inflammatory factors and activated under inflammatory conditions. The increased heparanase activity in HD patients may contribute to shedding of syndecan-1 from the endothelial glycocalyx and may lead to acceleration of the atherosclerotic process. To our knowledge, this study is the first to specifically investigate the syndecan-1 levels in dialysis patients. Serum E-selectin, a surrogate marker of endothelial activation, is also increased in dialysis patients. Increased synthesis/release after cytokine stimulation and inadequate clearance because of renal failure can both be responsible for the high levels. We found similar increased levels of sE-selectin in our dialysis group but no relationship with the changes in imaging parameters or serum levels of glycocalyx constituents.

Several recent studies support the hypothesis that disturbance of the glycocalyx accelerates atherosclerosis. The inhibition of hyaluronan synthesis in a murine model of atherosclerosis led to enhanced inflammatory and thrombotic responses and increased atherosclerosis. In another murine model, the glycocalyx was decreased both after an atherogenic diet and at lesion-prone locations in the vascular tree, and this thin glycocalyx was accompanied by greater intima-to-media ratios. These findings support the hypothesis that perturbation of the glycocalyx interferes with its protective function and contributes to increased vascular vulnerability. This may be also true for CKD, where a damaged endothelial glycocalyx may increase the susceptibility of the endothelial cells to vascular risk factors present in uremia.
Interestingly, we found that patients with CVD had increased PBR compared with patients without CVD. These findings were not supported by a significant increase in plasma levels of HA or syndecan-1. This suggests that the alterations of the endothelial glycocalyx present in dialysis patients are already so severe that the presence of CVD is not accompanied by additional significant shedding of its constituents. Whether our imaging method is able to identify a more severe alteration of the glycocalyx in this group of patients needs to be confirmed by other studies. Here, the number of patients is too small to allow any definite conclusions.

One of the limitations of our study is the cross-sectional design. Future studies should focus on specific longitudinal effects of dialysis treatment on the glycocalyx. Furthermore, ESRD is associated with alterations in RBC mechanical properties and changes in rheology (haematocrit and plasma viscosity), and additional studies are needed to investigate their effect on PBR as measured by SDF imaging. Here, we did not find any correlation between the imaging parameters and the hemoglobin levels of the patients (results not shown). Another issue that needs additional investigation is the effect of local haematocrit in microvasculature on the imaging parameters. To answer this question, a stepwise controlled reduction in haematocrit independent of endothelial glycocalyx dimension should be obtained in a controlled experimental setting. A strong point of our study is the well documented phenotype of all dialysis patients, including measurement of RRF.

In conclusion, in this study, we show that dialysis patients have loss of glycocalyx barrier properties as estimated by analysis of the dynamic variations of erythrocyte column width in the sublingual microcirculation. Additionally, we found high levels of HA and syndecan-1 in blood, consistent with increased shedding of glycocalyx from the vascular wall. Impaired glycocalyx barrier properties, together with shedding of its constituents into blood, are consistent with sustained pathogenic endothelial cell activation in dialysis patients and probably contribute to the aggressive vascular pathology present in this group of patients.

The SDF imaging of the microcirculation provides a direct, noninvasive, and fast method for the assessment of the endothelial glycocalyx, whereas the plasma levels of glycocalyx constituents are only an indirect measure of its integrity. The state of endothelial glycocalyx and its circulating components show great promise as markers of endothelial dysfunction, and their measurement may be of value in the clinical setting. They could provide valuable tools to monitor vascular vulnerability, detect early stages of disease, evaluate risk, and judge the response of patients with kidney disease to treatment.
Concise methods

Study Design and Subjects
We performed a single-center cross-sectional observational study to determine the status of endothelial glycocalyx and plasma levels of glycocalyx constituents in 17 PD patients, 23 HD patients and 21 age- and sex-matched healthy controls. All patients were recruited from the Dialysis Center of the Academic Medical Center, University of Amsterdam, between August 2008 and May 2009. Patients were excluded if one of the following applied: diabetes mellitus, any acute inflammatory episode, use of antioxidants, use of statins 6 weeks before the measurements, or use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers on the day of the measurements. The study was carried out in accordance with the principles of the Declaration of Helsinki. Approval was obtained from the Committee of Medical Ethics of the Academic Medical Center, University of Amsterdam, and all participants gave written informed consent.

Blood Sampling and General Laboratory Measurements
Blood samples were drawn after an overnight fast from healthy volunteers and most of the patients. Patients who were scheduled for HD in the afternoon were asked to fast for at least 3 hours before the measurement, and blood was drawn before the dialysis session. BP was measured in triplicate, and the last two measurements were averaged to determine systolic and diastolic BPs.

All standard laboratory measurements were performed on a Hitachi P-800 (Roche Diagnostics, Mannheim, Germany). All reagents were provided by the same company. Glucose, creatinine, alanine aminotransferase, urea, and triglycerides were measured by standard enzymatic methods. Total cholesterol and HDL-cholesterol were measured with colorimetry. LDL-cholesterol was calculated using the Friedewald formula. CRP was measured using an immunoturbidimetric assay. IL-6 levels were measured with a commercially available ELISA (R&D Systems Europe Ltd., Abingdon, United Kingdom). For additional analysis, plasma aliquots were snap-frozen and stored at -80ºC.

RRF was expressed as the residual GFR, which was calculated as the mean of creatinine and urea clearance obtained from a 24-hour urine collection in dialysis patients. Presence of RRF was defined as residual GFR≥ 1ml/min.

Measurements of Glycocalyx Constituents and Regulating Enzyme
HA (Corgenix Inc., Broomfield, CO), hyaluronidase activity (ELISA previously described in the work by Nieuwdorp et al.20), and syndecan-1 (Diaclone; Gen-Probe Inc., CA) were determined in all participants by ELISAs.
Measurements of Endothelial Activation
Serum E-selectin was measured in all participants by a commercially available ELISA (Quantikine; R&D Systems, Ltd., United Kingdom).

Oxidative Stress
Plasma MDA concentrations were determined by HPLC using a previously described method\(^{57}\) with some adaptations (Supplemental data 1 has a detailed description).

Imaging of the Microcirculation
We performed intravital microscopic imaging of the sublingual microvasculature using a Sidestream DarkField (SDF) MicroScan videomicroscope (MicroVision Medical Inc., Wallingford, PA) (Supplemental data 2). In HD patients, the measurements were performed before the dialysis session. Images were collected with a 5x objective with a 0.2 NA providing a 325-fold magnification on screen, and were sized 720x576 pixels. The frame rate was 23/s. Video sequences of 2 seconds each were recorded using Streampix software (Norpix Inc. Montreal, Canada) in at least 10 areas close to the frenulum. Movies consisted of 40 consecutive frames of 950x700 µm sublingual tissue surface area. The first frame of each movie was used to automatically identify all available microvessels, and measurement lines perpendicular to the vessel direction were placed automatically every 10 µm along each visible microvessel. All vessels with a diameter of 50 µm and larger were excluded. Each line represented a measurement site; at each measurement site a total of 21 parallel (every ± 0.5 µm) intensity profiles was plotted using ImageJ (National Institutes of Health, Bethesda, MD) and RBCW (full width half maximum) was determined at each line for all 40 consecutive frames in a movie, revealing a total of 840 RBCW measurements at a measurement site (21 profiles x 40 frames). The RBCW showed considerable variation in these 40 frames. The associated (cumulative) distributions of the RBCWs for these 840 measurements were used to determine median RBCW (P50), as well as lower and upper percentiles of the RBCW distribution. To assess the position of the outer edge of the RBC perfused lumen at each measurement site, the RBC DPerf was derived from the RBCW distribution by linear extrapolation of all RBCW percentiles between P25 and P75. The PBR was defined as the distance of median (P50) RBCW to the outer edge of the extrapolated Dperf as shown in Figure 4. Approximately 100–300 measurement sites were indentified per video recording, giving approximately 1000–3000 measurements of median RBCW, PBR, and DPerf per patient.

Reproducibility data were acquired by performing SDF imaging on two separate days in 16 healthy volunteers (unpublished data) (Supplemental data 3). The observed changes in dialysis patients were significantly greater than the differences between visits of healthy volunteers.
Chapter 2

Outcome Measures
The primary outcome was the difference in sublingual endothelial glycocalyx dimension as determined by SDF imaging between the dialysis patients and healthy volunteers. Our study parameters were median RBCW, DPerf, and PBR. We also tested whether the treatment modality (HD or PD) had any influence on the degree of alterations of the imaging parameters. Secondary outcomes were the differences in plasma levels of glycocalyx constituents and a marker of endothelial activation between the dialysis patients and controls. We measured HA, syndecan-1, hyaluronidase activity, and sE-selectin. The correlations between the outcome parameters and total time on RRT and time on dialysis were tested in the dialysis patients. Additionally, we investigated the effect of RRF, inflammation, oxidative stress, and presence of CVD on the outcome parameters.

Statistical Methods
Results are expressed as means (SD) or median (interquartile range) depending on the distribution of the data. For baseline characteristics, continuous variables were compared with the use of either an unpaired t test or Mann-Whitney depending on the distribution of the data. Categorical variables were compared with the use of the chi-squared or Fisher exact test where appropriate. Analysis was performed using SPSS version 16.0 (Chicago, IL). A p value<0.05 was defined as statistically significant. The correlations between different parameters were tested using either Pearson or Spearman correlation.

Figure 4. Schematic illustration of endothelial glycocalyx imaging method in dialysis patients. Perturbation of glyocalyx allows the erythrocytes to approach the vessel wall, leading to increased DPerf and PBR compared with healthy controls. RBC width = median RBCW. *DPerf is the perfused diameter (RBC perfused lumen). ** PBR is the perfused boundary region (RBC-permeable part of the cell free layer including cell permeable glyco-calyx). *** Cell free layer.
test depending on the distribution of the data. Only correlations with a $p$ value $<0.01$ were considered significant.

**Acknowledgements**
The authors thank the Nephron Foundation for the financial support. Dr. J.A. Bijlsma, Dr. I. Keur, Dr. W. Smit and Dr. J.M.R. Willemsen are gratefully acknowledged for their contribution to the inclusion of patients. We also thank D. Lopes-Barreto, J. Sierts, S. L. Yong and D. R. de Waart for their excellent laboratory support.

**Disclosures**
None.
References


Endothelial Glycocalyx in ESRD


Supplemental data 1

Oxidative stress
Plasma malondialdehyde (MDA) concentrations were determined by high liquid performance chromatography (HPLC) using a previously described method (reference 57), with some adaptations. The derivatization method used thiobarbituric acid as agent. This method measures the total content of MDA in the sample. Five microliters of sample were applied to a Omnispher C18 HPLC column (internal diameter: 3 µm, column length: 10 cm; Agilent Technologies, Middelburg, The Netherlands) operated at 20°C. The starting eluent consisted of 1% acetic acid (pH 4.5), followed by linear gradient or isocratic elution with methanol at the indicated concentration: 0% (0.5 min), 80% (11 min), 80% (13 min) and 0% (30 min). The flow rate was 0.8 mL per minute. The chromatographic system consisted of an Ultimate 3000 pump, autosampler, RS variable wavelength detector and column compartment (Dionex Corporation, Sunnyvale, USA).

Supplemental data 2

Sidestream darkfield imaging of the sublingual microvasculature

Supplemental data 3

Imaging of the microcirculation using Sidestream Darkfield microscopy
Reproducibility data:
Reproducibility data was acquired by performing SDF imaging on two separate days (V01 and V02) in 16 male volunteers. Participants did not smoke, did not use any medication, and were free from any illness, including overt cardiovascular disease. All experiments were performed after an overnight fast. Group averages (SD) for RBCW, PBR and DPerf are RBCW_V01: 10.09 (1.23) µm, RBCW_V02: 10.05 (1.06) µm; PBR_V01: 2.72 (0.59) µm, PBR_V02: 2.59 (0.50) µm; DPerf_V01: 16.01 (1.19) µm, DPerf_V02: 15.67 (1.38) µm.
Chapter 3

The Endothelial Surface Layer after Successful Kidney Transplantation

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*the authors equally contributed to the article

Submitted for publication
Abstract

Background
Dialysis patients have alterations of the endothelial surface layer (ESL) and increased plasma levels of glycocalyx constituents. It is not known whether renal transplantation leads to restoration of the endothelial glycocalyx. Here, we investigated the state of ESL in stable renal transplant recipients (RTR), compared to healthy individuals (HI).

Methods
Investigations were performed in 15 stable RTR and 19 HI. Plasma levels of the glycocalyx constituents hyaluronic acid (HA) and syndecan-1, and hyaluronidase activity were measured by ELISA. The ESL was assessed by sidestream darkfield imaging of the sublingual microvasculature and the perfused boundary region (PBR), which reflects the erythrocyte permeable part of the ESL, was measured.

Results
The estimated glomerular filtration rate (eGFR) was 47 (39-67) ml/min/1.73m² in RTR and 98 (92.5-104) ml/min/1.73m² in HI (p<0.001). Plasma HA levels were similar in RTR and HI: 25.3 ± 11 ng/ml versus 20.5 ± 11 ng/ml, p=0.25; syndecan-1 concentration and hyaluronidase activity were slightly higher in patients compared to HI: 30.8 ± 15 versus 20.4 ± 12 ng/ml, and 25.8 (24.3-27.9) U/ml versus 23.6 (21.1-26.6) U/ml, p=0.06 for both. PBR was thicker in patients compared to HI (3.3 ± 0.4 vs 2.9 ± 0.5 µm, p=0.005). In addition, correlations were present between PBR and both eGFR (p=0.004, r=-0.51) and syndecan-1 (p=0.005, Spearman’s rho=0.49).

Conclusions
After successful renal transplantation no significant changes in plasma levels of HA, syndecan-1 and hyaluronidase activity were found as compared to HI. However, the thicker perfused boundary region suggests that alterations of the ESL may still be present in these patients and their magnitude is dependent on the graft function.
Introduction

Amongst the different renal replacement therapies, successful renal transplantation confers the highest survival benefit. However, mortality caused by cardiovascular disease remains increased in renal transplant recipients (RTR) as compared to healthy individuals (HI). This suggests the presence of endothelial dysfunction, which may be due to the preceding renal failure, but also to persistent cardiovascular disease and/or effects of the immunosuppressive treatment.

Essential for the vascular homeostasis is an unaltered endothelial glycocalyx (EG). This negatively charged, highly hydrated mesh of proteoglycans (PG), glycoproteins and glycosaminoglycans (GAGs) covers the vascular endothelium, and together with associated plasma molecules forms the endothelial surface layer (ESL). Under physiological conditions, the endothelial glycocalyx has several well defined functions aimed at preserving the integrity of the vessel wall. Thus, it is involved in the regulation of vascular permeability, lipid homeostasis, mechanotransduction of shear stress, redox regulation, leucocyte adhesion and (anti) coagulant responses. Several data indicate that disruption of the glycocalyx results in increased vascular vulnerability and accelerates atherosclerosis.

Recently, our group and others have shown that patients with end stage renal failure (ESRD) on dialysis have alterations of the ESL and high concentrations of glycocalyx constituents in plasma. Also, relationships were found between levels of circulating glycocalyx components and markers of endothelial activation and dysfunction. Here, we questioned whether successful renal transplantation is associated with restoration of the endothelial glycocalyx, contributing to the improved risk profile of this patient-group. The measurements of plasma levels of glycocalyx components represent a useful tool for the assessment of ESL in humans. Hyaluronic acid (HA), the only non-sulfated GAG within the endothelial glycocalyx, is essential for the maintenance of vascular integrity. It is involved in the regulation of vascular permeability, mechanotransduction of fluid shear stress, leucocyte adhesion. Syndecan-1 is a transmembrane heparan sulfate (HS) PG, which binds various ligands via its GAGs side chains, and subsequently modulates inflammation, angiogenesis, microbial attachment and entry, matrix remodeling, and carcinogenesis. In this study we investigated the state of the ESL in stable renal transplant recipients and compared the data to those of healthy individuals. HA, syndecan-1 and the activity of the regulating enzyme hyaluronidase, were measured in plasma. In addition, using sidestream darkfield (SDF) imaging of the sublingual microcirculation, we measured the perfused boundary region (PBR), which reflects the erythrocyte permeable part of the ESL.
Materials and methods

Study design and subjects
We performed a cross-sectional study and included 15 transplant recipients of a first renal allograft, with age between 18 and 70 years, who were treated with prednisolone in combination with cyclosporine. In addition, we studied 19 age and sex-matched healthy individuals. All patients received their kidney transplant between 2001 and 2008. Their immunosuppressive regimen included quadruple therapy (basiliximab, prednisolone, mycophenolate sodium and cyclosporin) in the first 6 months after transplantation, followed by double therapy with prednisolone and cyclosporine. All RTR had a stable renal allograft function (estimated glomerular filtration rate >30 ml/min/1.73m²), and no change in the immunosuppressive regimen in the previous 6 months. Exclusion criteria were: smoking, history of diabetes mellitus, any recent acute inflammatory episode, the use of angiotensin converting enzyme inhibitors or angiotensin receptor blockers on the day of the measurements, use of anti-oxidants. The study was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the Committee of Medical Ethics of the Academic Medical Center, University of Amsterdam. Written informed consent was obtained from all participants.

Laboratory measurements
Blood was drawn done after an overnight fast. Blood pressure, heart rate and body weight were also measured. All routine laboratory measurements were performed in the central laboratory of the Academic Medical Center on a Hitachi P-800 (Roche Diagnostics, Germany), with reagents provided by the same company. Glucose, creatinine, alanine aminotransferase, and urea were measured by enzymatic methods. The glycated hemoglobin (HbA1c) was measured in patients by HPLC, and C-reactive protein (CRP) by an immunoturbidimetric assay. Total cholesterol was measured by colorimetry and LDL cholesterol was calculated with the Friedewald formula. Estimated glomerular filtration rate (eGFR) was calculated using the abbreviated MDRD formula: GFR = 175 x (Pcr ÷ 88.4)^-1.154 x age^-0.203 (female: multiply result by 0.742, black: multiply result by 1.210).

Circulating glycocalyx constituents and regulating enzyme
Quantitative total plasma hyaluronic acid levels were measured by enzyme-linked immunosorbent assay (ELISA) (Corgenix Inc., Broomfield, Colorado, USA), as was syndecan-1 (Diaclone, Gen-Probe Inc., California, USA). Hyaluronidase activity was determined using an assay previously described,²⁰ it could not be determined in nine of the healthy individuals because of unavailability of blood samples.

Sidestream Darkfield (SDF) imaging of the microcirculation
Intravital microscopic imaging of the sublingual microvasculature was performed all study participants using a SDF MicroScan videomicroscope (MicroVision Medical
Inc., Wallingford, PA, USA). An extensive description of the method has been given previously.\textsuperscript{14} In short, movies of the sublingual microvasculature are recorded, and the red blood cell column width (RBCW) is measured in blood vessels with a diameter up to 50 µm. Images were collected with a 5x objective with a 0.2 NA providing a 325-fold magnification on screen and were sized 720x576 pixels. The frame rate was 23/s. Video sequences of 40 consecutive frames of 950x700 µm sublingual tissue surface area were recorded using Streampix software (Norpix Inc. Montreal, Canada) in at least 10 different areas. All visible vessels are automatically identified and measurements lines are placed every 10 µm, perpendicular to the vessel direction. At each measurement sites intensity profiles were obtained and RBCW was measured. A maximum of 3000 measurements were obtained per individual. By measuring the dynamic of RBCW near the vessel wall, the perfused boundary region (PBR) is calculated, which reflects the erythrocyte permeable part of the ESL (GlycoCheck B.V.).

\textbf{Statistical analysis}

Results are expressed as median and interquartile range or mean and standard deviation, depending on the distribution of the data. Between-group differences were analysed using the unpaired t-test, Mann Whitney U test, or $\chi^2$-test as appropriate. Bivariate correlations between different variables were assessed using Spearman or Pearson correlation test. For statistical analyses, we used SPSS 20.0 (Chicago, IL, USA).

\section*{Results}

\textbf{Patients}

Two patients were excluded from the analysis because of the presence of liver disease, which may interfere with the breakdown of the glycocalyx constituents.

\textbf{Clinical characteristics}

The baseline characteristics of the study participants are summarized in Table 1. As expected, RTR had a lower eGFR, higher systolic and diastolic blood pressure, and a different lipid profile compared with healthy individuals.

\textbf{Circulating glycocalyx constituents}

Hyaluronic acid levels were similar in RTR and HI: 25.3 ± 10.7 and 20.5 ± 11.3 ng/ml, $p=0.25$, as shown in Figure 1. Both syndecan-1 and hyaluronidase activity were moderately increased in RTR, but the difference did not reach statistical significance (syndecan-1: 30.7 ± 15.1 versus 20.4 ± 12 ng/ml, $p=0.06$, and hyaluronidase activity: 25.8 (24.3-27.9) versus 23.6 (21.1-26.6) U/ml in HI, $p=0.06$).
Table 1. Baseline characteristics of renal transplant recipients and healthy individuals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Renal transplant recipients (n=13)</th>
<th>Healthy individuals (n=19)</th>
</tr>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>48.2 ± 10.5</td>
<td>50.0 ± 9.9</td>
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<tr>
<td>Male gender n (%)</td>
<td>8 (61.5)</td>
<td>11 (57.9)</td>
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<tr>
<td>Time since transplantation (mo)</td>
<td>29 (23.5-83)</td>
<td>-</td>
</tr>
<tr>
<td>Time on dialysis (mo)</td>
<td>29.9 ± 26.4</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.4 ± 4.8</td>
<td>24.5 ± 3.6</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>142 ± 19</td>
<td>125 ± 16*</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>88 ± 11</td>
<td>76 ± 9*</td>
</tr>
<tr>
<td>eGFR (ml/min per 1.73m²)</td>
<td>47 (39-67)</td>
<td>98 (92.5-104)*</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>5.2 (4.8-5.6)</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.9 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>ALAT (U/l)</td>
<td>28 (20-51)</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>4.8 ± 0.7</td>
<td>5.5 ± 0.8*</td>
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<tr>
<td>LDL-C (mmol/l)</td>
<td>2.4 ± 0.5</td>
<td>3.3 ± 0.8*</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.7 (0.6-2.0)</td>
<td>1.0 (1.0-1.2)</td>
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<td>Statins (%)</td>
<td>75</td>
<td>-</td>
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<tr>
<td>Anthypertensives (%)</td>
<td>75</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation) or median (inter-quartile range) depending on the distribution. ALAT: alanine aminotransferase; BMI: body mass index; BP: blood pressure; TC: total cholesterol; eGFR estimated by the abbreviated MDRD. *p<0.05 Mann Whitney U test or t-test depending on the distribution of the data

Imaging of the microcirculation
PBR was significantly increased in patients compared to controls (3.3 ± 0.4 versus 2.9 ± 0.5 µm, p=0.005).

Relationships between glyocalyx parameters and other clinical and biochemical parameters
Relationships were present between the perfused boundary region and both eGFR (p=0.004, r=-0.51) and syndecan-1 (p=0.005, Spearman’s rho=0.49). Neither syndecan-1 nor HA was associated with renal function. A positive correlation was present between HA levels and age (p=0.004, Spearman’s rho=0.50). We found no other relationships between the parameters for ESL and other biochemical or clinical parameters, including the blood pressure, lipid profile, glucose, glycated haemoglobin, time on dialysis or time since transplantation (data not shown).
Measurements of circulating glycocalyx constituents in stable RTR with moderately reduced kidney function, showed normal or slightly increased plasma levels of HA, syndecan-1 and hyaluronidase activity compared to HI. Analysis of the ESL by SDF imaging of the sublingual microvasculature identified a significantly thicker perfused boundary region in patients compared to controls.

The direct assessment of the endothelial glycocalyx in vivo is challenging and hence plasma concentrations of its constituents have been used to assess the state of EG in humans. Although not specific for the endothelial cells, high plasma levels of HA and syndecan-1 have been associated with alterations of the endothelial glycocalyx in various conditions, e.g., kidney failure, ischemia-reperfusion, diabetes mellitus (DM), sepsis.\textsuperscript{14-16,20-23}

**Discussion**

Figure 1. Hyaluronan, syndecan-1 and hyaluronidase activity have similar levels in renal transplant recipients (RTR) and healthy individuals (HI). The perfused boundary region is significantly increased in RTR compared to HI. Mean with SEM are shown.
Also, robust correlations of their plasma concentrations with markers of endothelial dysfunction have been reported in kidney disease.\textsuperscript{16,24} HA is present in the extracellular matrix but is also an essential constituent of the EG, where it is critical for the vascular homeostasis. Renal excretion is of minor importance for the clearance of hyaluronan from circulation, and therefore, of no significance in patients with good renal function.\textsuperscript{25} Hyaluronidases but also inflammatory cytokines and reactive oxygen species are involved in the degradation of HA.\textsuperscript{17} Within the EG, hyaluronidase binds HA but also other GAGs, like HS and chondroitin sulfate (CS). Therefore, the plasma hyaluronidase activity is indicative for its glyocalyx degrading capacity, and increased values have been found in conditions associated with alterations of ESL in patients with type 1 and type 2 DM, and uremia.\textsuperscript{14,25-27} Syndecan-1 is a proteoglycan present primarily on epithelial and plasma cells, but also expressed by endothelial cells.\textsuperscript{7} It has attachment sites for both HS and CS,\textsuperscript{28} binds various chemokines, growth factors, cytokines, extracellular matrix components, and thus functions primarily as co-receptor.\textsuperscript{29} The syndecan-1 ectodomain is shed from the cell surface via various processes, including proteolytic or oxidative mechanisms stimulated by inflammation.\textsuperscript{18} In kidney disease, plasma levels of syndecan-1 are elevated in ESRD and chronic kidney disease stage 4, but normalize in RTR with good or reduced graft function.\textsuperscript{14-16} Also, high levels were found in microalbuminuric type 1 DM patients but not in matched patients with normoalbuminuria, suggesting that this proteoglycan may be important in the pathogenesis of diabetic nephropathy.\textsuperscript{30} A recent study excluded a role for renal accumulation in the high syndecan-1 levels reported in kidney disease,\textsuperscript{16} suggesting the importance of increased shedding in this setting. Although an association between renal function and plasma levels of syndecan-1 has been previously reported,\textsuperscript{15,16} we did not confirm this finding in our group of patients, possibly due to the limited number of patients.

The measurement of the erythrocyte permeable region of the ESL by non-invasive imaging of the sublingual microvasculature showed a thicker PBR in RTR compared to HI. This reflects increased accessibility to RBCs and is suggestive of a disrupted endothelial surface layer. Our data seems to be at odds with a study that reported similar thickness of PBR in RTR and HI.\textsuperscript{15} This discrepancy can be explained by the different renal function in the two patient groups, as our transplant recipients had lower eGFR compared to the other study. Importantly, in both studies, a thicker PBR was associated with a low eGFR, suggesting the important effect of reduced renal function on the ESL. Interestingly, in our study higher syndecan-1 levels were associated with a thicker PBR. The RBC excluding properties of the ESL are determined by the complex organization and the interactions between its components. To date little is known about the exact contribution of each component to the mechanical stability of this structure.
A limitation of our study is the cross-sectional design which does not allow for follow-up data. In addition, the direct effects of the immunosuppressive medication on the ESL were not addressed. Both calcineurin inhibitors and glucocorticoids have been associated with the development of hypertension, dyslipidemia and diabetes, and have been shown to promote the development of atherosclerosis. Taking into account the effects on the endothelium, we cannot rule out an effect of immunosuppression on the EG.

Although renal transplantation cannot erase the associated cardiovascular burden, it does solve the uremia related complications, leading to less oxidative stress and less inflammation. Whether this leads to restoration of the endothelial glycocalyx and reduced vascular vulnerability which may contribute to the improved prognosis of these patients, is not known. Here we show that successful renal transplantation is associated with normalization of plasma level of glycocalyx constituents and regulating enzyme, which is indicative of a normal turnover within this structure. However, disruption of the endothelial surface layer is still present and its magnitude is determined by the graft function.

Acknowledgements
The authors would like to acknowledge Si La Yong, Nelly van de Bom-Baylon, Gerrie Nieuwenhuizen and Jeroen Sierts for excellent technical assistance.
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Can Plasma Hyaluronan and Hyaluronidase be Used as Markers of the Endothelial Glycocalyx State in Patients with Kidney Disease?

Carmen A. Vlahu and Raymond T. Krediet

Adv Perit Dial. 2015;31:3-6
Abstract

Hyaluronan (HA) is widely spread in the body and is an important component of the extracellular matrix, including the endothelial glycocalyx (EG). Essential for its vasculoprotective function, HA is involved in vascular permeability and many other processes. In patients with kidney disease, plasma HA is higher than expected, but the extent to which plasma HA and its degrading enzyme hyaluronidase can be used as markers for the state of the EG has not yet been determined.

In the first part of this review we describe HA synthesis and degradation; we then focus on the involvement of the kidney in the process. In the second part, we summarize the available data on HA and hyaluronidase in patients with kidney failure. Plasma HA is somewhat elevated in kidney failure and predicts for poor survival in dialysis patients. The increased HA levels in kidney failure are probably a result of decreased excretion, but an upregulated turn-over cannot be ruled out with certainty in some patients. Hyaluronan might be involved in the regulation of peritoneal transport in PD.
Introduction

Hyaluronan (HA) is present in many tissues, including the extracellular matrix and the vascular endothelial glycocalyx (EG). The role of the kidney in the handling of hyaluronan and hyaluronidase has been investigated only to a limited extent. Studies on plasma concentrations in patients with kidney failure have not been performed on a large scale, and the extent to which plasma HA reflects the EG is unknown. The objective of the present review is to summarize and discuss the available published data on these matters.

Hyaluronan and hyaluronidase

Hyaluronan is an unsulfated glycosaminoglycan composed of a linear repeat of disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. It has a molecular weight in the order of 1000 kDa (HMWHA), and it is negatively charged, hydrophilic with hydrophobic patches, and not bound to a core protein. Hyaluronan is synthesized at the cytosolic site in the cell membrane by HA synthases. A small quantity of lower molecular weight HA (LMWHA) is present and can be synthesized either by a specific HA synthase or by degradation of HMWHA. In contrast to HMWHA, the lower molecular weight fragments are highly angiogenic; they are considered to cause tissue damage - for instance, by induction of cytokines and by increasing the synthesis of collagen I.2-4 Hyaluronan is an important constituent of the extracellular matrix. The highest concentrations in humans are present in the umbilical cord and synovial fluid, but data in rabbits suggest that about 8% is present in the kidneys, especially the medulla and papillae.2

Most of the HA in the vasculature is incorporated into the EG and the extracellular matrix of the underlying tissue.3 Although HA accounts only for 5%-20% of the total glycosaminoglycan content of the endothelial surface layer,5 it is an important determinant of the layer’s size, mesh like structure, and function. Hyaluronan is essential for several vasculoprotective functions of the EG: mechanotransduction of fluid shear stress, NO release, permeability barrier, and adhesion barrier.3,5,6 The essential role of HA within the EG therefore turns it into an attractive potential marker for the state of the EG.

The tissue half-life of HA can range between 0.5 and 3 days and depends mainly on in situ metabolic degradation. The mechanisms for the degradation process include a breaking into smaller fragments by Hyal and also by free oxygen radicals in pathologic situations.2,3 Tissue HMWHA and the LMWHA fragments are taken up into the circulation by the lymphatic system. Lymph nodes extract up to 90% of the quantity transported to the blood stream. About 85-90% of the amount reaching the circulation is eliminated in the liver, and 10%, in the kidneys.2
Six Hyal genes have been identified in mammals. HYAL1 and HYAL2 are the most important ones in humans. The highest levels of HYAL1 messenger RNA are found in the liver, kidney, spleen, and heart. The enzyme resides intracellularly in the lysosomes, has a molecular weight of 57 kDa, and functions optimally at low pH. Hyal-1 cleaves the 1-4 glycosidic bonds of HA into smaller fragments, up to tetrasaccharides. Hyal-2 is attached to the outer cell membrane where it cleaves HA polymers to intermediate size fragments of about 20 kD. Its optimal function is at a normal extracellular pH. The assumption is that these fragments are transported intracellularly for further digestion by Hyal-1.

The sources of circulating Hyal are not evident. Plasma concentrations of Hyal-1 can be measured relatively easily, because the assay is done at low pH, but how much Hyal-2 can be detected is unclear. Hyal-2 is especially important for the EG, where HA is on the outside of the cell membrane and in direct contact with circulating blood, which makes it likely that Hyal-2 is important in glycocalyx HA turnover.

**Plasma HA and Hyal in patients with impaired kidney function**
As already stated, plasma concentrations of HA are very low because of elimination in the hepatic sinusoids, in lymph nodes, and kidney. However, concentrations increase after food ingestion, and it would therefore be sensible to take blood samples in a fasting state.

As a consequence of its elimination by the liver, plasma HA has been used to monitor progression of liver disease. At least two studies showed a relationship between the degree of renal function impairment and plasma HA. The results from the first study suggest that the LMWHA fragments are removed from the circulation by glomerular filtration, but no data on tubular handling of HA are available, although the renal localization of HA occurs especially in the medulla. The finding that urine of dialysis patients with residual renal function contains only LMWHA might more favour glomerular filtration. As has been shown in a number of studies, impaired HA removal leads to higher HA plasma concentrations in patients with end stage renal disease (ESRD), especially in those treated with dialysis.

Little is known about the ratio between LMWHA and HMWHA in the plasma of patients with normal or impaired renal function. In rats, the low molecular weight fraction constituted 26% of total HA in the outer medulla of the kidney. In the plasma of hemodialysis patients, LMWHA accounted for one third of the total HA in the 3 patients investigated. Plasma of 50 stable peritoneal dialysis (PD) patients contained 15% LMWHA during a follow-up of 6 months. These findings seem to contradict measurements obtained in healthy blood donors and in patients with rheu-
Hyaluronan and Hyaluronidase in CKD

matoid arthritis or with primary biliary cirrhosis, in whom only HMWHA could be detected. The difference supports the contention that, because of impaired renal removal of LMWHA, elevated concentrations of this potentially toxic HA fragment are present in the circulation of patients with end-stage renal disease. The elevated plasma HA concentrations reported in patients with renal failure coincide with elevated levels of various circulating peptides such as adhesion molecules, cytokines, and markers of endothelial dysfunction. Very high concentrations of HA have been associated with death. It has been postulated that the correlations between peptides and HA represent damage to the EG. However the authors putting forward that hypothesis neglect the fact that kidney failure itself leads to increased plasma HA concentrations and that the raised concentrations do not necessarily mean increased biologic effect. In that regard, a relationship between C-reactive protein and plasma HA is absent. The contention that the increased HA concentration in impaired kidney function points to damage to the EG is therefore at least premature; more probably, the increased concentration is a consequence of decreased renal excretion.

Although hardly any data on the renal handling of Hyal are available, Hyal’s molecular weight of approximately 43 kDa suggests the possibility of removal by glomerular filtration. The finding that urine Hyal concentrations are about 100 times those in plasma (a urine/plasma ratio similar to that of creatinine) supports the glomerular removal hypothesis, but could also be attributable to local release from urinary tract tissue, as appears likely in patients with bladder carcinoma.

Combined plasma concentrations of HA and Hyal have been studied in stable end-stage renal disease patients, 23 of them treated with hemodialysis and 17, with PD. Clinically, the patients showed no signs of inflammation. Values of C-reactive protein were also normal in nearly all of them, and 65% were anuric. Plasma HA and Hyal were not different between the PD and hemodialysis patients and were not related to each other; neither were they influenced by plasma C-reactive protein or by the presence or absence of cardiovascular disease. Hyaluronan was evidently related to the duration of dialysis. Plasma HA levels in patients with residual renal function and in healthy controls were similar, but levels were significantly higher in the anuric patients. In contrast, Hyal concentrations were, on average, 31% higher in patients with residual renal function than in healthy controls, but similar in the healthy controls and in the anuric patients. Interestingly, the HA/Hyal ratio was 0.7 in healthy controls and 0.61 in patients with residual renal function, but 1.8 in anuric patients. Those results make it likely that an effect of renal function on plasma HA is present, but limited in the absence of inflammation and/or cardiovascular disease. Increased Hyal concentrations suggest decreased removal by the kidney, but the normal concentrations in anuria make it likely that Hyal synthesis is impaired in the absence of functioning kidney tissue.
Hyaluronan in peritoneal dialysis

In vitro studies have shown that cultured mesothelial cells and peritoneal fibroblasts synthesize HA. Peritoneal deposition of HA in PD patients has especially been described in the submesothelial zone of the parietal peritoneum. Concentrations of HA are higher in peritoneal effluent than in plasma, suggesting local production or release, and those concentrations increase during acute peritonitis. Effluent HA showed no trend over time on PD and was also no predictor for the development of peritoneal sclerosis. A switch from conventional to more biocompatible dialysis solutions is associated with a decline in effluent HA, but no relationships with peritoneal deposition of HA or with peritoneal transport are present.

Maintenance of vascular integrity is an important function of HMWHA. Hyaluronan is important in the regulation of water homeostasis, and its negative charge might reduce protein transport through vascular membranes. A study in rats, in which the interstitial mesentery was exposed to either Hyal, chondroitinase or heparinase, showed an increase in the diffusion coefficient of solutes after exposure to Hyal only, suggesting that HA could be involved in the regulation of peritoneal transport. Furthermore, submesothelial HA might affect peritoneal water transport from the circulation to the peritoneal cavity, where the water is removed from the body. In rats with normal kidney function, the addition of HA to a dialysis solution, resulted in increased ultrafiltration because of decreased uptake of intraperitoneal fluid into the circulation. Such experiments have never been conducted in patients, which makes the value of HA in clinical PD treatment doubtful.

Summary

Hyaluronan is an important component of the extracellular matrix and the endothelial glycocalyx. The plasma concentrations of HA and, to a lesser extent, of Hyal have been studied extensively in various patient groups, including those with impaired or absent kidney function. Without full determination of the HA and Hyal types, a final interpretation is impossible. However, some facts are evident:

- Plasma HA is somewhat increased in kidney failure, and high values are associated with death.
- It is likely that the increased plasma levels of HA in renal failure are more a sign of decreased excretion than of upregulated turn-over; however, a contribution of the latter cannot be excluded.
- Biocompatible PD solutions lower HA concentrations in peritoneal effluent, but the biology of this phenomenon is unknown.
References


Part II

The Endothelial Glycocalyx in Peritoneal Dialysis
Is the Systemic Microvascular Endothelial Glycocalyx in Peritoneal Dialysis Patients Related to Peritoneal Transport?

Carmen A. Vlahu, Deirisa Lopes Barreto, Dirk G. Struijk, Hans Vink, Raymond T. Krediet

Abstract

Background
The capillary wall coated by the endothelial glycocalyx, is the main transport barrier during peritoneal dialysis (PD). Here, we investigated the relationships between measurements of the systemic endothelial glycocalyx and peritoneal transport in PD patients.

Methods
We performed Sidestream Darkfield (SDF) imaging of the sublingual microvasculature in 15 patients, measured the perfused boundary region (PBR), which includes the permeable part of the glycocalyx, and calculated the estimated blood vessel density (EBVD). All patients underwent a peritoneal permeability analysis.

Results
No relationships were present between the imaging and peritoneal transport parameters, neither in the group as a whole nor in fast transporters. In patients with non-fast peritoneal transport status, PBR had a negative relationship with EBVD and small solute transport, and a positive one with net ultrafiltration (NUF). The EBVD showed a positive correlation with glucose absorption and a negative one with NUF. We found no relationships with the peritoneal transport of albumin.

Conclusions
No relationships are present between the systemic endothelial glycocalyx, which was assessed by SDF, and peritoneal transport. In non-fast transporters, a reduction in blood vessel density caused by endothelial glycocalyx alterations or a thicker permeable phase of the glycocalyx delaying the access of small solutes to the small pores, may be important.
Introduction

In peritoneal dialysis (PD), the capillary wall is the main barrier to the exchange of solutes and water between circulation and the dialysis fluid.\(^1\) The pore theory,\(^2\) which was used for the assessment of the peritoneum as dialysis membrane, does not always explain the clinical data, as it does not allow for alterations due to inflammation or high glucose concentrations. Therefore, it has been challenged by the discovery of the endothelial glycocalyx, an additional functional barrier within the vascular wall. This layer is present on the luminal side of all blood vessels and within the intercellular clefts.\(^3,4\) Together with associated plasma molecules, it forms the endothelial surface layer. This negatively charged structure is involved in vascular permeability; in experimental studies, an intact glycocalyx functions as a barrier to the transvascular exchange of water and solutes.\(^5\)\(^-\)\(^8\)

At present, no data have been published on a possible role of the endothelial glycocalyx in peritoneal transport; only speculations have been made.\(^9,10\) Recently, Sidestream Darkfield (SDF) imaging has been developed as a non-invasive method for assessment of the endothelial glycocalyx in humans.\(^11\) Hereby, the perfused boundary region (PBR), which includes the erythrocyte-permeable part of the endothelial glycocalyx, is measured in the microcirculation. We reported previously that dialysis patients have higher PBR values than healthy controls, suggesting the presence of an altered glycocalyx barrier.\(^12\) Here, we analyzed if relationships were present between peritoneal transport characteristics and PBR obtained by SDF imaging.

Subjects and Methods

Study design and patients

This analysis was performed in a subset of 15 non-diabetic, clinically stable PD patients included in a study previously reported.\(^12\) Imaging of the microcirculation and a standard peritoneal permeability analysis were performed. The study was carried out in accordance with the principles of the Declaration of Helsinki. Approval was obtained from the Committee of Medical Ethics of the Academic Medical Center, University of Amsterdam, and all participants gave written informed consent.

To investigate the relationships between the systemic endothelial glycocalyx and peritoneal transport, we used two approaches. In approach A, the group was taken as a whole, whereas in approach B, we divided patients into a group with fast peritoneal solute transport and one without this phenomenon, based on the observations that different mechanisms may regulate peritoneal transport in the former.\(^13\)
Standard peritoneal permeability analysis
Standard peritoneal permeability analysis was performed as described previously,\textsuperscript{14,15} including the assessment of free water transport (FWT).\textsuperscript{16} The mass transfer area coefficient (MTAC) of urea, creatinine and urate, glucose absorption, the peritoneal clearance of the plasma proteins $\beta_2$-microglobulin and albumin, and net ultrafiltration (NUF) were determined. Based on a previous study,\textsuperscript{15} a fast transport status was defined as the presence of 2 out of 3 of the following parameters exceeding their 95% confidence interval: MTAC creatinine $>$12.7 mL/min, MTAC urate $>$10.5 mL/min and glucose absorption $>$72%. Using the definition of fast transport status based on PET, i.e., dialysate/plasma creatinine above the mean $+$1SD, did not change the results (data not shown).

Biochemical determinations
The following solutes were measured in plasma, serum or 4-hour effluent: C-reactive protein (CRP; immunoturbidimetric assay), interleukin (IL)-6 (ELISA, R&D Systems Europe Ltd., Abingdon, UK), and cancer antigen (CA) 125 (ELISA).

Imaging of the microcirculation
The SDF records movies of the sublingual microvasculature using a MicroScan videomicroscope (MicroVision Medical Inc., Wallingford, PA, USA). An extensive description of the method was given previously.\textsuperscript{12} In short, the median red blood cell column width (RBCW) is calculated, together with the position of the outer edge of RBC perfused lumen (perfused diameter), and the perfused boundary region, defined as the distance of median RBCW to the outer edge of the perfused diameter.

Blood vessel density
Measurement lines perpendicular to the vessel direction were placed automatically every 10 µm along each microvessel. The estimated blood vessel density (EBVD), the number of measurements lines divided by the surface area, is an indirect measure of blood vessel density.

Statistics
Results are expressed as means (SD) or medians (inter-quartile range) depending on data distribution. Continuous variables with normal distribution were compared using the unpaired Student’s t-test; the Mann-Whitney U test was used for non-normally distributed data. Analysis was performed using SPSS version 16.0 (Chicago, IL, USA). Correlations were tested using either Pearson or Spearman test depending on the distribution of the data.
Table 1. Baseline characteristics of the study patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PD Patients (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>45 ± 17</td>
</tr>
<tr>
<td>Gender (females/males)</td>
<td>5/10</td>
</tr>
<tr>
<td>Duration of PD treatment (mo)</td>
<td>24 (12-47)</td>
</tr>
<tr>
<td><strong>Primary kidney disease (no of patients)</strong></td>
<td></td>
</tr>
<tr>
<td>Renovascular</td>
<td>4</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>3</td>
</tr>
<tr>
<td>Other</td>
<td>8</td>
</tr>
<tr>
<td>Residual renal function (present/absent)</td>
<td>8/7</td>
</tr>
<tr>
<td>Plasma CRP (mg/L)</td>
<td>1.4 (1.0-9.8)</td>
</tr>
<tr>
<td>Plasma IL-6 (ng/ml)</td>
<td>7.9 ± 5.4</td>
</tr>
</tbody>
</table>

Results are expressed as medians (interquartile range) or mean ± SD

Results

**Clinical characteristics**

Clinical characteristics of the study population are listed in Table 1.

**Peritoneal membrane characteristics and imaging of the microcirculation**

*Approach A.* The study parameters are listed in Table 2. No relationships were present between the imaging parameters (PBR and EBVD) and peritoneal transport parameters, nor between PBR and EBVD, in the group as a whole, as partly shown in Figure 1.

*Approach B.* The patients were divided in fast and non-fast transport status, for further analysis. The duration of PD was 3.5 (3-30) months in fast transporters and 35 (18-69) months in the other patients (p=0.03). The study parameters are shown in Table 3. According to expectations, solute transport parameters were higher and those of fluid transport were lower in fast transporters than in non-fast transporters. Effluent levels of IL-6 were not different, but those of CA125 were higher in fast transporters. PBR and EBVD were not different between the two groups, nor were the CRP levels: 3.6 (1.0-12.8) mg/L in non-fast transporters and 1.4 (1.2-15.1) mg/L in fast transporters.

In patients with a non-fast transport status, negative correlations between PBR and small solute transport were present: for MTAC urea, p=0.03, MTAC creatinine
Furthermore, PBR was related to NUF, but not to the contribution of FWT (Figure 3). No relationships were present between PBR and the clearance of $\beta_2$-microglobulin and albumin. An extensive analysis of these relationships is given as supplementary data. A negative correlation was present between PBR and the EBVD, which reached statistical significance after excluding an obvious outlier (Figure 4). Furthermore, relationships were present between EBVD and NUF (p=0.01), and glucose absorption (Figure 5).

In fast transporters no relationship was present between the study parameters.

### Table 2. Peritoneal transport parameters of the study patients according to the standard peritoneal permeability analysis and results of SDF imaging of the microcirculation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PD patients (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peritoneal transport parameters</strong></td>
<td></td>
</tr>
<tr>
<td>MTAC urea (ml/min)</td>
<td>19.0 ± 3.9</td>
</tr>
<tr>
<td>MTAC creatinine (mL/min)</td>
<td>12.3 ± 3.6</td>
</tr>
<tr>
<td>MTAC urate (ml/min)</td>
<td>10.1 ± 3.7</td>
</tr>
<tr>
<td>Glucose absorption (%)</td>
<td>66.5 ± 11.7</td>
</tr>
<tr>
<td>CI $\beta_2$-microglobulin (mL/min)</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>CI albumin (mL/min)</td>
<td>0.10 (0.08-0.13)</td>
</tr>
<tr>
<td>NUF (mL/4h)</td>
<td>438 ± 198</td>
</tr>
<tr>
<td>FWT at 60 min (%)</td>
<td>24.5 (15.9-44.7)</td>
</tr>
<tr>
<td><strong>Effluents biomarkers</strong></td>
<td></td>
</tr>
<tr>
<td>Effluent CA 125 (U/ml)</td>
<td>15.6 ± 9.3</td>
</tr>
<tr>
<td>Effluent IL-6 (pg/ml)</td>
<td>5.7 ± 4.2</td>
</tr>
<tr>
<td><strong>Imaging parameters</strong></td>
<td></td>
</tr>
<tr>
<td>P50RBC (µm)</td>
<td>10.2 ± 0.6</td>
</tr>
<tr>
<td>PBR (µm)</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>EBVD</td>
<td>609 ± 90</td>
</tr>
</tbody>
</table>

Results are presented as medians (interquartile ranges) or means ± SD. CI = clearance; NUF = net ultrafiltration CA 125 = cancer antigen 125 P50RBC = 50th percentile of RBC column width; EBVD = EBVD expressed as measurement sites per mm².

p=0.06, MTAC urate p=0.03, glucose absorption p=0.002 (partly shown in Figure 2). Furthermore, PBR was related to NUF, but not to the contribution of FWT (Figure 3). No relationships were present between PBR and the clearance of $\beta_2$-microglobulin and albumin. An extensive analysis of these relationships is given as supplementary data. A negative correlation was present between PBR and the EBVD, which reached statistical significance after excluding an obvious outlier (Figure 4). Furthermore, relationships were present between EBVD and NUF (p=0.01), and glucose absorption (Figure 5).

In fast transporters no relationship was present between the study parameters.
To the best of our knowledge, this study is the first to investigate the relationship between the state of the systemic endothelial glycocalyx and peritoneal transport in PD. No relationships were present between PBR measured in the systemic microcirculation, and peritoneal transport parameters in 15 stable PD patients. However, when peritoneal transport status was taken into account, relationships emerged in non-fast transporters: a negative correlation was present between PBR and small solute transport, and subsequently a positive correlation with ultrafiltration. Furthermore, we found that the thicker the PBR, the smaller the estimated perfused blood vessel density in the sublingual and probably systemic circulation. Relationships between the EBVD and peritoneal transport were only evident for glucose absorption and NUF.

### Table 3. Peritoneal membrane characteristics according to peritoneal transport status and results of the SDF imaging of the microcirculation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fast transporters (n=5)</th>
<th>Non-fast transporters (n=10)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTAC urea (ml/min)</td>
<td>21.8 (19.9-24.7)</td>
<td>17.4 ± 3.4</td>
<td>0.03</td>
</tr>
<tr>
<td>MTAC creatinine (mL/min)</td>
<td>16.8 (14.5-17.4)</td>
<td>10.4 ± 2.7</td>
<td>0.001</td>
</tr>
<tr>
<td>MTAC urate (mL/min)</td>
<td>14.4 (12.8-16.1)</td>
<td>7.9 ± 1.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Glucose absorption (%)</td>
<td>82 (74.5-83)</td>
<td>60.1 ± 8.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Cl β₂-microglobulin (mL/min)</td>
<td>1.8 (1.6-2.6)</td>
<td>1.0 ± 0.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Cl albumin (mL/min)</td>
<td>0.13 (0.09-0.57)</td>
<td>0.09 ± 0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>NUF (mL/4h)</td>
<td>270.9 (208.3-340.5)</td>
<td>519.4 ± 188.6</td>
<td>0.03</td>
</tr>
<tr>
<td>FWT at 60 min (%)</td>
<td>15.9 (12.1-21.2)</td>
<td>33.6 ± 12.6</td>
<td>0.02</td>
</tr>
<tr>
<td>Effluent biomarkers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effluent CA 125 (U/ml)</td>
<td>26 (19-29)</td>
<td>11 ± 7.5</td>
<td>0.008</td>
</tr>
<tr>
<td>Effluent IL-6 (pg/ml)</td>
<td>6.8 (4.9-8.9)</td>
<td>5.0 ± 4.8</td>
<td>0.44</td>
</tr>
<tr>
<td>Imaging parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P50RBC (µm)</td>
<td>10.0 (9.6-10.7)</td>
<td>10.3 (9.8-10.7)</td>
<td>0.44</td>
</tr>
<tr>
<td>PBR (µm)</td>
<td>3.8 (3.2-3.9)</td>
<td>3.5 (3.4-3.7)</td>
<td>0.51</td>
</tr>
<tr>
<td>EBVD</td>
<td>569 (494-705)</td>
<td>605 (536-693)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

Results are expressed as medians (interquartile ranges) or means ± SD. MTAC = mass transfer area coefficient; NUF = net ultrafiltration; FWT = free water transport; CA 125 = cancer antigen 125; P50RBC = 50th percentile of RBC column width; PBR = perfused boundary region; EBVD = EBVD expressed as measurement sites per mm².
Figure 1. Lack of correlation between PBR and parameters of peritoneal transport (MTAC creatinine, glucose absorption and clearance of albumin) and EBVD (p >0.05 for all correlations).

Figure 2. Relationship between PBR and small solute transport in patients with non-fast peritoneal transport status: negative correlations between perfused boundary region and MTAC creatinine (r=-0.6, p=0.06) and glucose absorption (r=-0.8, p=0.002).
The Endothelial Glycocalyx and Peritoneal Transport

Figure 3. Relationship between PBR and fluid transport in patients with non-fast transport status (NUF: $r=0.8$, $p=0.01$, FWT at 60 min: $r=0.3$, $p=0.35$).

Figure 4. Relationship between EBVD and PBR in patients with non-fast transport status ($r=-0.8$, $p=0.02$). The asterisk represents the outlier.

Figure 5. Relationship between EBVD and glucose absorption ($r=0.7$, $p=0.05$) in patients with non-fast transport status. The asterisk represents the outlier.
PBR is the erythrocyte-permeable part between the RBC column and the endothelial layer, which includes the permeable part of the glycocalyx. This explains the difference between anatomic glycocalyx measurements, which average 0.5 µm,8 and the PBR thickness of 3.5 µm.12 To date, a role of the endothelial glycocalyx in peritoneal transport has only been speculative.

The absence of any relationship between peritoneal transport parameters and the sublingual microvascular endothelial glycocalyx was not surprising, because the peritoneal vascular bed is the only part of the systemic microcirculation that is continuously exposed to unphysiologic dialysis solutions. This may lead to functional and anatomic alterations. One of these is the presence of an early fast transport status,17 which is often reversible and probably due to epithelial-to-mesenchymal transition of mesothelial cells.18 It goes along with high effluent concentrations of CA12515 and production of biomarkers that are partly vasoactive.20 Inflammation may21 or may not be present.22 Therefore, the regulation of the peritoneal circulation in fast transporters is different from that of the systemic circulation. By chance, the number of patients with an early fast transport status was relatively high in the present study. They had a higher effluent CA125 but no signs of inflammation as evidenced by their normal serum CRP and Il-6. These considerations are supportive for a distinction between the patients with a fast peritoneal transport status and those without this phenomenon.

Most of our patients had a non-fast transport status and relationships were present between PBR and small solute transport. This may be explained by relationships between the endothelial glycocalyx and vascular density. Experimental studies have shown that alterations of the endothelial glycocalyx result in perturbation of the microcirculation, leading to a reduction in functional microvascular density.23,24 In line with these, recent unpublished clinical studies have shown a negative correlation between PBR and EBVD. Our findings are in line with these data, because in non-fast transporters a thicker PBR was associated with a lower EBVD. A lower systemic EBVD may be consistent with a reduced peritoneal vascular surface area, which leads to a decreased peritoneal transport of small solutes. This reasoning can explain the lower transport rates of small solutes from circulation to dialysate, and probably also in the opposite direction for glucose absorption. The absence of any relationships for the transport of plasma proteins may be due to either the low number of patients or to the large pore system through which their transport occurs. A similar explanation can be hypothesized for FWT, which takes place through aquaporin-1. Alternatively an increased PBR may be due to a thicker stagnant fluid layer, which delays the access of small solutes to the small inter-endothelial pores. The involvement of stagnant fluid layers in peritoneal solute diffusion has already been suggested in 1978, but without proof.25
A question that needs to be addressed is whether the sublingual measurements reflect the peritoneal microcirculation. However, in humans, the quality of peritoneal SDF imaging does not allow the calculations (unpublished data). Studies should also investigate the effects of RBC alterations on PBR. Furthermore, our study group is small and the findings should be reproduced in larger studies. The strength of the study includes the combination of a well-documented phenotype with a standard peritoneal permeability analysis, SDF imaging and assessment of effluent biomarkers, all non-invasive investigations providing a complete assessment of peritoneal membrane.

In conclusion, no relationships were present between the thickness of the erythrocyte-permeable region bordering the endothelial layer in the systemic microcirculation, and peritoneal transport parameters in PD patients. However, relationships with small solute transport were present in non-fast transporters. In fast transporters, these effects may be overruled by the presence of vasoactive substances, leading to better perfused peritoneal microvessels and a higher number of functional small pores. Our findings provide support for a role of the endothelial glycocalyx in peritoneal transport during PD, but should be confirmed in a larger group of patients.

Acknowledgements
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Disclosures
No conflict of interest to declare.
References


Chapter 6

The Endothelial Glycocalyx in the Peritoneal Microcirculation of Rats with Chronic Kidney Failure Exposed to Dialysis Solutions

Carmen A. Vlahu, Jan Aten, Marijke de Graaff, Dirk G. Struijk, Raymond T. Krediet

Manuscript in preparation
Abstract

Introduction
The peritoneal endothelial glycocalyx may affect the transport of solutes and water across the vascular wall during peritoneal dialysis (PD). The aim of the present study was to analyze the endothelial surface layer (ESL) in a rat model with chronic kidney failure (CKD) and long-term exposure to dialysis solutions (PDF), and relate the results to peritoneal transport parameters and vascular expression of glycocalyx constituents.

Methods
Forty-four rats were grouped in: NKF (normal kidney function, n=8), CKD (induced by 70% nephrectomy, n=12), CKD+PDF (CKD, daily peritoneal infusions with either Physioneal® 3.86% or Dianeal® 3.86%, n=24). After 16 weeks a standard peritoneal permeability analysis and Sidestream Darkfield imaging of the peritoneal microvasculature were performed in vivo to assess peritoneal transport capacity and to estimate peritoneal ESL as well as micro vascular density. Mesenteric tissue specimens were collected to analyse the expression of syndecan-1 heparan sulfate proteoglycan in the interendothelial junctions and the distribution pattern of heparan sulfate 10E4 epitope by immunofluorescence. Plasma levels of syndecan-1 were measured by ELISA.

Results
The thickness of the perfused boundary region (PBR) in the peritoneal microcirculation was similar in the three groups. Rats exposed to PDF had higher microvascular densities compared to non-exposed rats. Dialysate to plasma ratio of creatinine was associated with both PBR and microvascular density. Plasma levels of syndecan-1 were increased in rats with CKD and also in PDF exposed rats. Syndecan-1 expression in the interendothelial junctions was decreased in rats with CKD but similar to values in normal kidney function in rats exposed to PDF. No relationships with peritoneal solute transport were present. Heparan sulfate 10E4 had predominantly a basolateral distribution. No differences were present after exposure to Dianeal® or Physioneal®.

Conclusion
Rats with CKD and rats with CKD exposed to PDF have normal thickness of the erythrocyte permeable part of the ESL in the peritoneal microcirculation. PBR, but not the expression of syndecan-1, is associated with small solute transport, suggesting that the endothelial surface layer is important in peritoneal transport during PD. The localization of syndecan-1 and heparan sulfate 10E4 epitope precludes an essential role for these molecules in the establishment of the peritoneal endothelial surface layer.
**Introduction**

The exchange of solutes and water between blood and the peritoneal cavity during peritoneal dialysis (PD) is mainly restricted by the endothelial layer. The permeability of the endothelium is partly dependent on the inner vascular surface layer, known as the endothelial glycocalyx (EG), which coats the luminal side of all blood vessels and extends into the intercellular clefts. It is a negatively charged mesh of membranous glycoproteins, proteoglycans and glycosaminoglycans (GAGs), of which heparan sulfate is the most abundant. Various plasma molecules attach to this layer and together they form the endothelial surface layer (ESL).

A role of the endothelial glycocalyx in vascular wall permeability to water and solutes has been suggested by several experimental studies, in which enzymatic degradation of the glycocalyx was associated with increased transport of plasma proteins and solutes. In addition, plasma proteins interact with the glycocalyx and are necessary to maintain the capillary barrier. The ESL could therefore influence peritoneal transport of solutes and water during PD. To date, a possible role of the peritoneal endothelial glycocalyx in peritoneal transport has been suggested based on theoretical considerations, but not supported by experimental data.

Assessment of the systemic ESL in vivo in humans is possible by using sidestream dark field imaging (SDF) of the sublingual microvasculature. Hereby the width of the red blood cell (RBC) column is assessed and the perfused boundary region (PBR) is calculated. This reflects the RBC permeable part of the ESL. Our group reported previously that PD patients have a thicker sublingual PBR compared with age-matched controls with normal kidney function, suggestive for altered ESL barrier properties in kidney failure. Interestingly, we also found an inverse relationship between the systemic PBR and small solute transport in PD patients with a non-fast peritoneal transport status. In addition, measurements of plasma levels of several glycocalyx constituents (e.g., hyaluronic acid, syndecan-1) have been used for the assessment of the endothelial glycocalyx. Syndecan-1 is a heparan sulfate proteoglycan which via its side chain glycosaminoglycans binds various growth factors and cytokines, modulating their function. Subsequently, syndecan-1 is involved in various processes, some of which, like angiogenesis and fibrosis, occur in the peritoneum after long term treatment with PD. Therefore syndecan-1 may be important in the remodelling of the peritoneal membrane.

Noteworthy, the particular environment present in the peritoneal cavity during treatment with PD, makes it likely that specific changes may occur in the peritoneal microcirculation that are dissimilar to those in the systemic microcirculation. As the SDF imaging of the peritoneal microcirculation in patients is technically challenging (Vlahu et al., unpublished data), the aim of the present study was to investigate the state of
the peritoneal endothelial glycocalyx in a rat model with chronic kidney failure with or without long-term exposure to either a conventional or ‘biocompatible’ dialysis solution (PDF), and relate the results to peritoneal transport parameters. For this, we performed SDF imaging of the peritoneal microvasculature and investigated the distribution of the glycocalyx constituents syndecan-1 and heparan sulfate in the peritoneal blood vessels. In addition, plasma concentrations of syndecan-1 were measured in all study groups.

Materials and methods

Study design
Forty four male Wistar rats (Harlan, Zeist, the Netherlands), with a body weight (BW) of 260-280 grams, were randomly assigned to four experimental groups: 8 with normal kidney function (NKF, no nephrectomy, no peritoneal exposure), 12 with chronic kidney failure (CKD, 70% nephrectomy, no peritoneal exposure), 24 with chronic kidney failure and exposure to either a conventional or a biocompatible dialysis solution for 16 weeks (CKD+PDF, catheter implantation, 70% nephrectomy, daily peritoneal exposure to either Dianeal®, 3.86 % glucose, CKDD, n=12, or Physioneal®, 3.86 % glucose, CKDP, n=12). An extensive description of this model and of the surgical procedures (catheter implantation and nephrectomy), has been published previously. All dialysis solutions were provided by Baxter (Utrecht, The Netherlands). The protocol was approved by the Committee for Animal Experiments of the Academic Medical Center, University of Amsterdam.

Assessment of the peritoneal microcirculation
Rats were anesthetized with a mixture of ketamine, diazepam and atropine (10:10:1), 0.25ml/kg BW intramuscularly, divided over the hind legs. A blood sample was taken prior to the test. The rats were placed on a heating pad (37°C) to maintain a constant body temperature throughout the experiment. A midline incision was performed and the mesentery was exteriorized. Pre-heated (37°C) phosphate buffered saline solution (Lonza, Verviers, Belgium) was used to moisten the tissue. The probe of the camera was placed on the ileum, at 6 cm distance from the cecum and movies were recorded (Figure 1). At the end of the investigation blood was sampled by heart puncture. After sacrifice, mesenteric tissue was collected for further investigation.

Perfused Boundary Region
Sidestream Dark field (SDF) intravital microscopy (MicroVision Medical Inc., Wallingford, PA) of the peritoneal microcirculation was performed to detect the dynamics of the red blood cell column near the vessel wall, i.e., to measure the penetration of RBCs into the glycocalyx, expressed as PBR. Images were obtained from peritoneal tissue covering the ileum. Movies for the analyses consist of 40 consecutives frames of 950x700 µm
(0.665 mm²) tissue surface area. Blood vessels with a diameter between 5 and 25 µm are automatically identified and measurements lines perpendicular to the vessel direction are placed every 10 µm along each visible microvessel, defining vascular segments. The recording stops automatically when a minimum of 3000 vascular segments has been obtained. The image acquisition was mediated through the Glycocheck software (Glycocheck BV, Maastricht, the Netherlands). An extensive description of the analysis of the video recordings has been published previously. In short, the median red blood cell column width (RBCW) is calculated at each measurement site, together with the position of the outer edge of RBC perfused lumen (perfused diameter), and the perfused boundary region, which is defined as the distance of the median RBCW to the outer edge of the perfused diameter.

**Valid microvascular density**

Only the vascular segments that are filled with RBCs for more than 50% (valid vascular segments) were selected, thereby minimizing the influence of hematocrit on the PBR measurement. We calculated the valid microvascular density (VMD) by dividing the number of valid vascular segments by the surface area of visualized tissue (0.665 mm²). This parameter is an indirect measure of perfused blood vessel density.

**Red Blood Cell filling percentage**

The percentage of vascular segments with RBC present during all 40 frames of the recording is automatically calculated by the software. Combined with the VMD this yields an estimate for the quantity of microvascular perfusion.
Standard Peritoneal permeability analysis adapted for rats

At least two rats per group underwent a peritoneal transport function test before the imaging of the peritoneal blood vessels (SPARa). This assay is based on the human standard peritoneal permeability analysis (SPA) described before\textsuperscript{19} and adapted for rats. An extensive description of the SPARa has been given previously.\textsuperscript{20}

Peritoneal transport parameters

Peritoneal small solute transport was expressed as dialysate to plasma creatinine concentration after 240 minutes and as peritoneal glucose absorption, which was calculated from the difference between the amount of glucose in the dialysate at the start of the SPARa and the amount present at the end of the SPARa, relative to the instilled quantity of glucose. Albumin and IgG concentrations in plasma and dialysate were measured by enzyme-linked immunosorbent assays (ELISA plates, Maxisorp immunoplate; NUNC, Roskilde, Denmark, coated with goat anti-rat albumin, Nordic Immunology, Tilburg, The Netherlands, or goat anti-rat IgG, Nordic Immunology, Sigma-Aldrich, Steinheim, Germany) to calculate their peritoneal clearances. The lower detection limit for albumin was 0.4 mg/L and for IgG it was 0.4 µg/L.

Kidney function

24-hour urine was collected from rats in metabolic cages before the end of the study, and together with a blood sample, used for the calculation of the glomerular filtration rate (GFR), expressed as mean of endogenous creatinine and urea clearances.

Measurement of glycocalyx constituents

Plasma levels of Syndecan-1 were determined by the means of an enzyme-linked immunosassay (USCN Life Science Wuhan, PRC).

Tissue collection and immunohistochemistry

Mesenteric tissue was collected from each rat and fixed in 4% buffered formaldehyde immediately after sampling. Paraffin embedded tissue was serially sectioned at 4 µm thickness.

Immunostaining of syndecan-1

Sections were deparaffinised in xylene and alcohol (100%, 95%, 70%), followed by heat induced epitope retrieval in 10 mM Tris 1mM EDTA pH=9. The staining sequence was: 5% normal goat serum in PBS for 30 minutes; primary antibodies: rabbit anti-syndecan-1 (Invitrogen Corp, CA) mixed with mouse IgG1 anti-rat aminopeptidase P (endothelial marker) (ReliaTech GmbH, Wolfenbuttel, Germany), both diluted at 1:100 and left overnight at 4° C; the secondary antibodies were goat anti-mouse IgG1 Alexa Fluor 594 (Molecular Probes, Inc., Eugene, OR, USA) with 1% normal rat serum
Peritoneal Endothelial Glycocalyx in CKD and PD

and donkey anti-rabbit Alexa Fluor 488 (Jackson ImmunoResearch Laboratories, Inc. PA, USA), dilution 1:100 for both, were left 30 minutes at room temperature. After washing, sections were mounted using Prolong® Gold antifade reagent with DAPI (Life Technologies, USA). Negative controls consisted of sections treated in a similar way but with substitution of each of the primary antibodies with antibody diluent only (ImmunoLogic, The Netherlands), followed by staining with the corresponding secondary antibody; as control, also separate staining of each of the primary antibodies together with the corresponding secondary antibody was applied. The localization of syndecan-1 in interendothelial junctions was investigated by double staining with mouse IgG1 anti-beta-catenin (BD, Biosciences, USA).

**Immunostaining of heparan sulfate**
The distribution pattern of heparan sulfate was investigated by indirect immunostaining using monoclonal antibody 10E4 which binds an epitope that requires presence of N-sulfated glucosamine residues (mouse IgM 10E4; AMS Biotechnology, UK). Double staining with antibodies directed to Von Willebrand factor (DAKO, Glostrup, Denmark) was applied to visualize endothelial cells.

**Image analysis**
Subcellular localization of syndecan-1 was examined by confocal laser scanning microscopy using a Leica TCS SP8 X mounted on a Leica DMI6000 inverted microscope with a HCX PL APO 63x/1.40-0.60 oil-immersion objective (Leica Microsystems, Rijswijk, The Netherlands). Nuclear DAPI stain was excited with UV diode (405 nm), antibody-conjugated AlexaFluor 488 and AlexaFluor 594 were each excited sequentially using a 80 MHz pulsed White Light Laser (470-760 nm). Separate PMT and HyD detectors were used. Z-stacks of scans were recorded and analysed by deconvolution software (Huygens Remote Manager, Scientific Volume Imaging, Hilversum, The Netherlands). Epifluorescence imaging was performed using a Leica DM5000B microscope with a HCX PL APO 63x/1.40-0.60 oil-immersion objective. Filterblocks applied for DAPI, Alexa488 and Alexa Fluor 594 fluorescence were A4, L5 and TX2, respectively. Images were recorded using a Leica DFC500 camera and LAS software (Leica Microsystems). At least 8 images were recorded of each rat: examination of the slide was started in the upper left corner, to the right, till the first blood vessel with a diameter up to 25 microns was visible, thereafter every fifth field of view was examined and pictures were taken when blood vessels were present. The mean and maximum fluorescence intensities of the syndecan-1 staining in the interendothelial junctions were analyzed using ImageJ 1.44 (National Institutes of Health, USA).
Statistics
Data are presented as mean ± standard deviation or median (interquartile range). To test differences between the groups we used the ANOVA test with Games-Howell post-hoc analysis or the Kruskal-Wallis test, depending on the distribution of the data. The two groups exposed to dialysis solutions were analysed together as CKD+PDF group. When these showed significant differences, further comparisons were made. These included NKF versus all other groups, CKD versus all rats exposed to dialysis solutions (CKD+PDF), and CKDD versus CKDP. Student’s t-test or Mann Whitney U test were used for continuous variables. The associations between variables were tested using linear regression analysis. For the analysis we used the IBM SPSS statistics software, version 21 (Chicago, IL).

Results
Drop-out
Five rats were excluded from the study due to complications related to the nephrectomy or the presence of inflammatory infiltrate in the peritoneal tissue sections at the end of the study, suggestive for peritonitis (3 rats from CKDP group, 2 rats from CKDD group). One rat from the CKDD group was excluded from the analysis of the imaging of the peritoneal microcirculation due to compromised peritoneal perfusion at the end of the SPARa.

Induction of chronic kidney failure
GFR was 2.9 (2.9-3.5) mL/min in rats with normal kidney function, 1.5 (1.2-1.6) mL/min in CKD, 1.7 (1.1-1.8) mL/min in CKD+PDF (p<0.01). Kidney function was similar in the two nephrectomised groups and also in CKDD and CKDP.

Assessment of the peritoneal microcirculation
SDF imaging of the peritoneal microcirculation showed no differences in the PBR between the three groups, as shown in Figure 2A: 2.2 ± 0.2 μm in NKF, 2.1 ± 0.3 μm in CKD, 2.1 ± 0.2 μm in CKD+PDF. In contrast, the valid microvascular density was higher in the groups exposed to dialysis solutions compared to the non-exposed groups (Figure 2B), while no significant difference was present between the NKF and CKD group: 4148 (3678-4283) μm/mm² versus 3543 (3188-4181) μm/mm². In rats exposed to PDF, VMD was 4468 (3878-5177) μm/mm² (p=0.02 between CKD and CKD+PDF). RBC filling percentage was not different between groups: 71.1 ± 3.4% in NKF, 70.7 ± 4.2% in CKD, 70.9 ± 4.6% in CKD+PDF (Figure 2C). When all groups were analysed together, PBR was inversely related with RBC filling percentage (regression coefficient \( \beta = -0.515; 95\% \text{ CI} [-0.045, -0.012]; R^2 = 0.265, p=0.001 \), but not with VMD (p=0.13) as shown in Figure 3. Similar results were obtained when the rats
exposed to PDF were analysed separately: PBR was inversely associated with RBC filling % (regression coefficient $\beta=-0.685$; 95% CI [-0.041, -0.011]; $R^2=0.469$, $p=0.002$) but not with VMD ($p=0.71$).

Peritoneal transport parameters and relationships with SDF imaging parameters
Peritoneal transport parameters are shown in Table 1. Exposure to dialysis fluids tended to increase solute transport parameters, but this was only significant for peritoneal IgG clearance. In rats exposed to dialysis fluids, D/P creatinine was significantly associated
Table 1. Peritoneal transport parameters as measured by the standard peritoneal permeability analysis adapted for rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NKF (n=2)</th>
<th>CKD (n=3)</th>
<th>CKD+exposure (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/P creat (µl/min)</td>
<td>0.38 ± 0.06</td>
<td>0.45 ± 0.03</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td>Glucose absorption (%)</td>
<td>58.2 (54.0-62.3)</td>
<td>58.1 (57.9-59.8)</td>
<td>68.4 (61.9-71.1)</td>
</tr>
<tr>
<td>Albumin Clearance (µl/min)</td>
<td>1.3 (1.1-1.5)</td>
<td>1.6 (1.3-3.1)</td>
<td>2.7 (2.4-3.8)</td>
</tr>
<tr>
<td>IgG Clearance (µl/min)</td>
<td>0.4 (0.3-0.5)</td>
<td>0.6 (0.5-0.6)</td>
<td>0.8 (0.7-0.9)*</td>
</tr>
<tr>
<td>Net ultrafiltration rate (µl/min)</td>
<td>72.6 (63.6-81.6)</td>
<td>60.5 (58.4-103.0)</td>
<td>61.5 (29.2-77.5)</td>
</tr>
</tbody>
</table>

Results are presented as median (range) or mean ± SD. D/P creatinine: dialysate-to-plasma ratio creatinine; NKF: normal kidney function; CKD: chronic kidney failure; CKD+exposure: CKD exposed to either Dianeal or Physioneal. Mann-Whitney test: *p<0.05, CKD versus CKD+exposure.

Figure 4. D/P creatinine is associated with both PBR ($R^2=0.571$, $p=0.03$), (panel A), and VMD ($R^2=0.590$, $p=0.044$), (panel B) in rats exposed to dialysis solutions.

with both PBR (regression coefficient $\beta=0.756$; 95% CI [0.043, 0.598]; $R^2=0.571$, $p=0.03$) and VMD (regression coefficient $\beta=0.768$; 95% CI [0.001, 0.090]; $R^2=0.590$, $p=0.044$) as shown in Figure 4. In contrast, no relationships were present between peritoneal glucose absorption and the imaging parameters. Relationships were also absent for albumin and Ig G clearances and the imaging parameters (data not shown).

Circulating glycocalyx constituents
Plasma levels of syndecan-1 were 7.2 (4.1-14.3) ng/ml in NKF, and increased to 16.2 (11.8-29.5) ng/ml in CKD and 12.6 (9.9-16.8) ng/ml in rats exposed to dialysis fluids ($p=0.04$), as shown in Figure 5A. These levels tended to be lower in CKD+PDF
compared to CKD, without reaching statistical significance (p=0.13). Plasma concentrations of syndecan-1 were inversely related to GFR when all groups were analysed together, as shown in Figure 5B (regression coefficient $\beta=0.346$; 95% CI [-0.549, -0.024]; $R^2=0.12$, p=0.03).

**Expression of syndecan-1 in the mesenteric microvasculature**

The staining pattern for syndecan-1 in endothelial cells was predominantly localized in association with the lateral cell membranes, in the cytoplasmic compartment, and in the nucleus (Figure 6 and 7). Syndecan-1 at the lateral membrane appeared to colocalize with $\beta$-catenin, suggesting association with interendothelial adherens junctions (Figure 8). The expression of syndecan-1 in the interendothelial junctions was lower in rats with CKD compared to NKF. However, in rats with CKD exposed to PDF junctional syndecan-1 was more extensive and similar to that in rats with normal kidney function (Figure 9). The nuclear syndecan-1 expression followed the same pattern (data not shown). No relationships of amount of junctional syndecan-1 immunostaining were found with kidney function, peritoneal transport parameters, or plasma concentrations of syndecan-1 (data not shown).

**Expression of heparan sulfate in the mesenteric microvasculature**

Heparan sulfate (10E4 epitope) immunostaining of mesenteric blood vessels showed positive staining predominantly in the basolateral compartment of the endothelial cells with reduced or absent staining at their luminal side. No staining was present in the interendothelial junctions. In addition, HS 10E4 was abundant in the extracellular matrix surrounding the smooth muscle cells (Figure 10). The HS expression was increased in rats with CKD and rats exposed to the dialysis solutions.
Immunostaining of the mesenteric tissue for syndecan-1. Syndecan-1 (SDC1 – green), aminopeptidase P (APP – red), nuclei (DAPI – blue). Syndecan-1 is present predominantly in association with interendothelial junctions and in the nucleus, but also in the cytoplasmic compartment. For both nuclear and junctional syndecan-1, a reduced expression was present in CKD but this was similar to NKF in rats exposed to both dialysis solutions. For each overlay, fluorescence recorded in the corresponding split channels is displayed in small panels (grey). NKF: normal kidney function; CKD: chronic kidney failure; CKDD: chronic kidney failure + exposure to Dianeal and CKDP: chronic kidney failure + exposure to Physioneal. Scale bar 50 µm.
Figure 7. Syndecan-1 is absent at the apical membrane of vascular endothelium. Confocal laser scanning microscopy of a peritoneal venule immunostained for syndecan-1 (SDC1 – green), aminopeptidase P (APP – red), and nuclei (DAPI – blue). Transluminal line-plots (a, b, c) indicate the cellular SDC1 localization in relation to the APP-positive luminal surface. Scale bar 5 µm.

Figure 8. Colocalization of syndecan-1 and β-catenin in the peritoneal microvasculature. Syndecan-1 (SDC1 – green), β-catenin (βCAT – red), nuclei (DAPI – blue). For each overlay, fluorescence recorded in the corresponding split channels is displayed in small panels (grey). Scale bar 10 µm.
The above summarized results of SDF imaging are clearly different from those obtained in the sublingual vasculature of CKD and chronic dialysis patients, in whom an increased thickness of the PBR was found. Regrettably, sublingual assessment of the systemic glycocalyx was impossible in rats because of their anatomical characteristics. The kidney failure-induced increased systemic PBR thickness, is obviously absent in the peritoneal microcirculation of rats. Similarly, the thickness of erythrocyte permeable part of the ESL in the peritoneal microcirculation was not significantly altered in rats with CKD exposed to 3.86% glucose dialysis solution. Experimental studies have shown that the ESL has a layered structure: a more compact zone is adjacent to the endothelial cell and does not allow the access of erythrocytes, whereas the most luminal part is more permeable to solutes and RBCs. Different components modulate the penetration of solutes and RBCs into this layer. Changes in the endothelial glycocalyx composition may occur, and are not necessarily accompanied by increased access of the RBC into the glycocalyx as shown after perfusion of the hamster cremaster microcirculation with hyaluronidase. A normal PBR does not exclude changes in the structure.
Figure 10. Immunostaining of the peritoneal microvasculature for the 10E4 heparan sulfate epitope. Heparan sulfate (10E4 – green), von Willebrand factor (vWF – red) and nuclei (DAPI – blue). The heparan sulfate, as stained for the 10E4 epitope, is situated predominantly on the abluminal side of the endothelium and in the ECM surrounding the smooth muscle cells. Increased expression of HS10E4 is detected in CKD and CKD rats exposed to dialysis solutions. For each overlay, fluorescence recorded in the corresponding split channels is displayed in small panels (grey). NKF: normal kidney function; CKD: chronic kidney failure; CKDD: chronic kidney failure + exposure to Dianea and CKDP: chronic kidney failure + exposure to Physioneal.
of the endothelial glyocalyx, that could be induced by both the circulating uremic toxins but also the local conditions present in the peritoneal cavity during PD, i.e., high glucose concentrations and new blood vessel formation. The changes in the endothelial surface layer reflect the combination of direct alterations of glyocalyx structure induced in a given condition and the endothelial adaptive response. The latter may, in certain conditions, translate into increased synthesis of some of the glyocalyx constituents, like in the case of hyaluronan during inflammation. Pro-inflammatory mediators induce overexpression of HA synthase 2, resulting in increased hyaluronan synthesis, which is accompanied also by increased expression of hyaluronan receptor CD44. This is likely to occur in uremia, which is associated with increased pro-inflammatory molecules and circulating adhesion molecules. Furthermore, the synthesis of hyaluronan increases following increased availability of its precursor, as occurs in hyperglycemic conditions. It is therefore likely that changes in peritoneal glyocalyx structure do occur during treatment with PD and are different from the changes in the systemic glyocalyx.

Despite the absence of differences in PBR between the three groups, a thinner PBR was associated with an increased RBC filling. This is similar to data obtained in the sublingual microcirculation in humans, where it was suggested that a thinner PBR reflects efficient vascular perfusion. Studies on the sublingual glyocalyx showed that a thicker PBR is associated with a decreased VMD. Such relation was absent for the peritoneal glyocalyx in the exposed rats, again pointing to the presence of specific glucose-induced alterations of the peritoneal glyocalyx in the setting of kidney insufficiency. In addition, neoangiogenesis occurs during long-term PD, and newly formed vessels usually have a thinner glyocalyx.

Peritoneal dialysis as a way to remove uremic waste products is largely dependent on the state of the peritoneal microcirculation. These vessels are exposed to extremely high dialysate concentrations of glucose, that diffuse from the peritoneal cavity to the abluminal part of the vascular wall, so in the opposite direction from the situation that occurs in hyperglycemia. The concept of rigid endothelial gaps as pathway of solute transport has been challenged over the last years, by the discovery of an additional functional barrier present at the luminal side of all blood vessels and in the inter-endothelial clefts, consisting of the endothelial glyocalyx. To date there is robust evidence pointing to the importance of this structure in the permeability of many vascular beds, as reviewed by Curry et al. who proposed the ‘glyocalyx-junction-break’ model, in which the glyocalyx forms the principal molecular sieve at the capillary wall. Furthermore, the addition of glyocalyx as an extra layer on the luminal side of the capillary, resulted in a revised Starling’s law. This delicate layer is very sensitive to inflammatory mediators and hyperglycaemia, its alteration providing a mechanism for permeability changes that cannot be explained by the classical mathematical models. The relationship between peritoneal PBR thickness and peritoneal D/P creatinine in the exposed animals is in
contrast with the previously reported inverse correlation between the systemic PBR and peritoneal transport of small solutes in PD patients. Currently no obvious explanation is available. It may be that the severity of kidney failure (CKD stage 5 in humans, stage 3 to 4 in rats) is of importance, or that the RBC impermeable part of the peritoneal glycocalyx is involved. This is mainly formed by glycosaminoglycans and proteoglycans, which are likely influenced by the extremely high glucose concentrations in dialysis solutions. The contribution of the main GAGs (HS, CS, HA) to the apparent thickness of the glycocalyx and its permeability to small solutes has been investigated in post-capillary venules of rats intestinal mesentery. The study found that the GAGs are distributed unevenly within the endothelial glycocalyx and they each contribute to its permeability in a different way. Whereas treatment with chondroitinase and hyaluronidase induced increased permeability of the EG to solutes, the treatment with heparinase and fMLP decreased the diffusion coefficients of solutes and may decrease permeability. Therefore, it is of major importance to identify the specific changes induced by a particular condition in order to identify the effect on permeability.

Assessment of the endothelial glycocalyx components
The perfused boundary region reflects the luminal part of the ESL which is permeable to erythrocytes, and is calculated based on the dynamic of RBC column near the vessel wall. Although not translated into a detectable effect on the PBR, alterations in the composition of the endothelial glycocalyx may be present in our study groups. In order to test this hypothesis, we attempted gain more insight into the composition of the endothelial glycocalyx and performed immunohistochemistry of the peritoneal tissue for heparan sulfate glycosaminoglycan and syndecan-1 proteoglycan.

Heparan sulfate is the most abundant GAG within the endothelial glycocalyx. It accounts for more than 50% of the total GAG content, and is characterized by structural heterogeneity. Moreover, various cellular environment are associated with structural differences in HS and several antibodies are currently available, directed against various epitopes. In our study, the immunostaining of the 10E4 epitope showed mostly a basolateral pattern of distribution, with scarce positive staining at the luminal side of the endothelium. A similar distribution of HS 10E4 epitope was reported in mice, in which a high HS content in the basolateral compartment was associated with inflammation. This may also be one of the factors responsible for the increased HS 10E4 content observed in rats with CKD and CKD exposed to dialysis solutions. Additional experiments, targeting different HS epitopes and different endothelial markers, should be performed in order to assess the composition of the endothelial glycocalyx.

Syndecan-1 is a transmembrane proteoglycan which contains both heparan sulfate and chondroitin sulfate. It serves as co-receptor for growth factors and various ligands via its
GAGs side chains, and modulates processes like matrix remodeling, inflammation and angiogenesis. It is abundantly expressed by epithelial cells and plasma cells but can also be present on endothelial cells. In our study syndecan-1 was present predominantly in association with the interendothelial junctions, in the cytoplasmic compartment and in the nucleus, and was scarce or absent at the luminal side on the endothelium. This suggests that syndecan-1 might not be a crucial component of the glycocalyx at the luminal side of the endothelium. Our findings are in line with a recent study which showed that syndecan-1 deficient mice are able to synthesize and maintain a hydrodynamically relevant glycocalyx. Also, perfusion with fluorescent antibodies directed against syndecan-1 showed subendothelial and not luminal localization in peritoneal venules of mice with normal kidney function and without exposure to dialysis solutions.

Here plasma syndecan-1 concentrations were increased in nephrectomised rats and appeared to be mainly related to the magnitude of GFR. However, a role of the renal accumulation of syndecan-1 has been excluded previously. In view of our observation that syndecan-1 is scarce or absent on the luminal surface of the endothelium, the assumption that plasma levels of syndecan-1 reflect shedding from the endothelial glycocalyx, becomes questionable. We cannot exclude the endothelial cell as source for plasma syndecan-1, but other (cellular) sources, like plasma cells, need to be considered as well.

In our study on rats with nephrectomy-induced chronic kidney disease, the unaffected thickness of the PBR, was accompanied by a reduced syndecan-1 expression in the interendothelial junctions and endothelial nuclei in rats with CKD, but normal expression in rats exposed to dialysis fluids. This suggests a reactive increase in syndecan-1 expression in this group as a result of long term-exposure to PDF. Next we questioned whether syndecan-1 could be important for peritoneal transport of solutes. The changes in syndecan-1 expression were associated with neither small nor large solute transport parameters. Interestingly, on activated endothelial cells syndecan-1 may modulate the interaction between insulin-like growth factor 1 receptor and \( \alpha V \beta 3 \) integrin and so affect angiogenesis. New studies are needed to determine whether the PDF induced increase in junctional syndecan-1 expression is involved in the regulation of neoangiogenesis induced by treatment with PD.

In conclusion, both rats with CKD and CKD exposed to glucose-based dialysis solutions have an unaltered erythrocyte permeable region of the ESL in the peritoneal microcirculation. Remarkably, relationships are present between parameters of small solute transport and the PBR, suggesting that the ESL may be important in peritoneal transport. In addition, changes in the expression of junctional syndecan-1 occur, but are not associated with peritoneal transport parameters.
References


Part III

Peritoneal Dialysis
Effects on the Peritoneum
Chapter 7

New Insights in Effects of Chronic Kidney Failure and Dialysate Exposure on the Peritoneum

Carmen A. Vlahu, Jan Aten, Marijke de Graaff, Henk van Veen, Vincent Everts, Dirk R. de Waart, Dirk G. Struijk, Raymond T. Krediet

Perit Dial Int 2016, in press
Abstract

Introduction
Chronic uremia and the exposure to dialysis solutions during peritoneal dialysis induce peritoneal alterations. Using a long-term peritoneal exposure model, we compared the effects of chronic kidney failure (CKD) itself and exposure to either a ‘conventional’ or a ‘biocompatible’ dialysis solution on peritoneal morphology and function.

Methods
Wistar rats were grouped in: normal kidney function (NKF), CKD induced by 70% nephrectomy, CKD receiving daily peritoneal infusions with 3.86% glucose Dianeal® (CKDD), or Physioneal (CKDP). At 16 weeks a peritoneal function test was performed, and histology, ultrastructure and hydroxyproline content of peritoneal tissue were assessed.

Results
Comparing CKD with NKF, peritoneal transport rates were higher, mesothelial cells displayed increased number of microvilli, blood and lymph vasculature expanded, vascular basal lamina appeared thicker, with limited areas of duplication, and fibrosis had developed. All alterations, except lymphangiogenesis, were enhanced by exposure to both dialysis fluids. Distinct mesothelial cell alterations were observed in CKDD and CKDP, the latter displaying prominent basolateral protrusions. In addition, CKDP was associated with a trend towards less fibrosis compared to CKDD.

Conclusions
Chronic kidney failure itself induced peritoneal alterations, which were in part augmented by exposure to glucose-based dialysis solutions. Overall, the conventional and biocompatible solutions had similar long-term effects on the peritoneum. Importantly, the latter may attenuate the development of fibrosis.
Introduction

Advanced knowledge to optimize peritoneal dialysis (PD) for long-term renal replacement therapy is required, especially in view of the continuing shortage of kidneys for transplantation. Long-term PD can be associated with alterations of peritoneal morphology and function. Angiogenesis, lymphangiogenesis, hyalinising vasculopathy and fibrosis can develop and lead to changes in peritoneal solute transport and decreased ultrafiltration. Various mechanisms have been hypothesized, of which the combination of glucose with a high dialysate lactate concentration, the formation of glucose degradation products (GDPs) and the associated advanced glycation end-products (AGEs) formation are likely the main culprits.

New peritoneal dialysis solutions (PDF) have been developed to prevent PD related peritoneal damage and thereby to allow a longer duration for treatment with this dialysis modality. These ‘biocompatible’ solutions have low content of GDPs, neutral or normal pH, and are either lactate, bicarbonate or bicarbonate/lactate buffered. Since peritoneal biopsies are not readily available from clinical studies, the effects of biocompatible PDF on peritoneal morphology have been studied in animal models. All of these showed a better morphologic status, when biocompatible solutions were compared with conventional ones. However, these studies were either done in rats with normal kidney function (NKF), or peritoneal function measurements were absent or limited, or the duration of exposure was relatively short. Kidney failure itself induces morphological and functional alterations of the peritoneum, and therefore, it should be included in peritoneal exposure models. Moreover, most studies did not extensively assess the morphological and ultrastructural alterations induced by various types of dialysis solutions, especially in long-term exposure models. Presently specific markers and new methodologies are available and provide valuable tools for an improved accuracy.

The goal of the present study was to perform an extensive comparison between the functional, morphologic and ultrastructural changes in the peritoneum induced by long-term peritoneal exposure to a conventional and a biocompatible dialysis solution in a rat model with CKD.

Materials and methods

Study design

Fourty-four male Wistar rats (Harlan, Zeist, the Netherlands), with a body weight (BW) of 260-280 grams, were randomly assigned to four groups: normal kidney function (NKF, n=8), chronic kidney failure (CKD, n=12, 70% nephrectomy, no peritoneal exposure), CKD and daily peritoneal exposure to either a conventional lactate-buffered dialysis solution, 3.86% glucose, Dianeal® (CKDD, n=12), or a bicarbonate/
lactate- buffered dialysis solution, 3.86% glucose Physioneal® (CKDP). The solutions were provided by Baxter (Utrecht, The Netherlands). The exposure groups were infused daily for 16 weeks with the appropriate solution via a catheter. Before instillation the dialysis fluids were preheated to 37°C and heparinised (5 IU/ml). The rats received 6 ml/100g BW dialysis fluid intraperitoneally and the infused volume was set at 20 ml/day in case their BW exceeded 420 g. The fluid was not removed from the peritoneal cavity, but allowed to be absorbed. After 16 weeks, a standard peritoneal permeability analysis adapted for the rat (SPARa) and morphologic assessment of the peritoneum, both by light- and electron microscopy were performed. The presence of peritonitis was excluded in all rats at the end of the experiments (results not shown). The protocol was approved by the Committee for Animal Experiments of the Academic Medical Center, University of Amsterdam.

**Housing conditions, catheter implantation, nephrectomy and peritoneal function test**

Housing conditions, catheter implantation, 70% subtotal nephrectomy, blood collection and Standard Peritoneal permeability analysis adjusted for rats (SPARa), were all performed as described previously, with the following exceptions: the 70% subtotal nephrectomy was performed under anesthesia with isoflurane (4% induction and 2% maintenance) and analgesics were administered both pre- and postoperatively. In short, a vascular access port (Rat-o-Port, Access Technologies, Norfolk Medical, Skokie, IL, USA) was implanted subcutaneously in the neck and the attached catheter was tunnelled subcutaneously and inserted into the peritoneal cavity. After one week allowed for recovery, three groups underwent 70% subtotal nephrectomy (the right kidney and the upper and lower pole of the left kidney were resected) during a single procedure. Two weeks were allowed for recovery with daily infusions of 1 ml heparinised buffer (5 UI/ml).

**Peritoneal transport parameters**

The SPARa was performed in at least two animals in each group, as previously described. Dextran 70 (5 g/L Hyskon®, Medisan Pharmaceuticals AB, Uppsala, Sweden) was added to the solution as a volume marker for calculation of fluid kinetics. Peritoneal small solute transport was expressed as mass transfer area coefficients (MTAC) of urea and creatinine. Glucose absorption, peritoneal fluid kinetics (transcapillary ultrafiltration rate, net ultrafiltration rate), and the peritoneal clearances of albumin and IgG, were calculated as described before. Because of the small number of animals undergoing a SPARa in the NKF group, we did not include this group in the statistical analysis.

**Renal function**

24-hour urine was collected in metabolic cages and used for the calculation of renal function expressed as the mean of creatinine and urea clearances.
**Immunohistochemistry**
Omentum was collected, fixed in 4% buffered formaldehyde, and embedded in paraffin. Sections were deparaffinised and rehydrated followed by heat-induced epitope retrieval in sodium citrate, pH= 6. To detect blood vessel endothelial cells, monoclonal mouse IgG1 anti-rat aminopeptidase P 1:200 (ReliaTech GmbH, Wolfenbuttel, Germany) was applied followed by alkaline phosphatase (AP)-conjugated goat anti mouse IgG1 1:50 (Southern Biotech, Birmingham, AL, USA). Bound antibodies were visualized using the Vector Blue Substrate kit (Vector Laboratories, Inc, Burlingame, CA, USA). For double staining to distinguish lymphatic endothelial cells, sections were washed, heated in citrate pH=6, and incubated with monoclonal mouse anti-rat Podoplanin 1:10000 (ReliaTech GmbH) as first antibody, followed by AP-conjugated goat anti-mouse and the Vector Red Substrate kit (Vector Laboratories) for visualisation.

**Image acquisition**
Slides were scanned and TIFF images acquired (1 pixel = 648 nm x 648 nm) using a BX61VS microscope with 10x/0.40 UPlanSApo objective and an Olympus dotSlide imaging system (Olympus, Zoeterwoude, The Netherlands). TIFFs were exported as 10 megabytes files for analysis of blood vessels (BV) and lymphatic (LV) profile densities.

**Analysis of blood and lymph vessel profile density**
The stereology tool STEPanizer version 1 (Bern, Switzerland) was used to quantify the number of vascular profiles and the surface area. We analysed 8 images in each rat, selected after applying the systematic uniform random sampling (SURS) of the images. The vascular profiles were counted in a counting frame with an area of 924x924 pixels if they were located inside the counting frame, but not touching the exclusion line or its extensions. The profile density was expressed as ratio of number of vessel profiles per tissue surface area.

**Hydroxyproline (OH-proline) content in tissue specimens**
Omental tissue was collected in Tissue-Tek and frozen at -80 °C. OH-proline content was determined as described in detail elsewhere, with some modifications. In short, two samples in each rat were processed and left to hydrolyze overnight at 85 °C, followed by quantitation by high performance liquid chromatography (HPLC) on an Ultimate 3000 LC system (Dionex, Sunnyvale, CA) and fluorescence detection (FP920, Jasco Corporation, Tokyo, Japan). In three rats frozen omental tissue was not available.

**Analysis of fibrosis**
Omental sections were cut and stained with picro-sirius red, PSR (Gurr, BDH, United Kingdom). In addition omental tissue from the groups exposed to PDF, was stained for type I collagen, using rabbit polyclonal IgG anti-collagen type I α1 at 1:400
(GTX41286, GeneTex Inc., Irvine, CA) as primary antibody, followed by poly alkaline phosphase-conjugated goat IgG anti-rabbit IgG (BrightVision, ImmunoLogic, Duiven, The Netherlands) as secondary antibody. Bound antibody was detected using Vector Blue Substrate Kit.

**Semi-quantitative analysis**
Fibrosis was assessed blindly in three different areas of omentum (submesothelial, perivascular and intersegmental), using a 4-graded scoring system as described previously. Maximum over-all fibrosis score is 9.

**Image analysis**
PSR-stained sections were scanned as described above, and images were exported as 20 megabytes files. The area of PSR-positive staining was measured (ImagePro premier 9.1, Media Cybernetics, Inc., USA), and expressed as percentage of the total surface area. The latter was calculated using ImageJ 1.44 (National Institutes of Health, USA). Collagen I immunostained sections were scanned as described above and images were exported as 40 megabytes files. After SURS of the files was applied, five images were selected in each rat for analysis.

**Electron microscopy**
Mesenteric tissue collected in three rats from each group was fixed overnight in 1% glutaraldehyde and 4% paraformaldehyde in 0.1M sodium cacodylate buffer (pH=7.4) and further processed for transmission electron microscopy (Supplemental_data). Sections were examined with a FEI Tecnai-12 G2 Spirit Biotwin electron microscope and micrographs were taken with a Veleta camera (Olympus, SIS).

**Mesothelial cell layer**
A TEM operator took micrographs of the MC layer, which were blindly analysed using Leica Qwin software. We analyzed the number of all visible microvilli attached to the membrane, and expressed it per length of the membrane surface. No statistical analysis was attempted because of the limited number of rats in which MC layer was assessed.

**Vascular basal membrane**
At least twenty-five BV cross-sections were analyzed in each group. Micrographs were taken at a 23000x magnification and the analysis was done blindly. The alterations of the VBM were defined based on an adaptation of a previously published method, as: (1) *normal*: monolayered, <200nm thickness; (2) *thick*: monolayered, >200nm thickness; (3) *mild alteration*: 2-3 layers, duplication present on at least 30% of the visible blood vessel circumference; (4) *moderate alteration*: 4 layers or more, duplication present on at least 30% of the visible BV circumference. VBM thickness was measured as follows:
measurement sites were placed every 1 µm along the vessel circumference. Only measurement sites where the endothelial cell membrane was clearly delineated as a thin sharp black line were taken into account for the analysis to correct for oblique sections. To avoid error, no measurements were done in areas with pericytes. For analysis we used Leica Qwin software. Non-homogeneity, defined as variability in electron density throughout the lamina basalis, was assessed as present or absent. No statistical analysis was attempted because of the limited number of rats in which the vascular basal lamina was assessed.

Statistics
The data are presented as median and interquartile range. Differences between the groups were assessed with an ANOVA test with Games-Howell post-hoc analysis or Mann-Whitney U test for continuous variables, and the Pearson chi-square test for categorical variables. When these showed significant differences, further comparisons were made. These included NKF versus all other groups, CKD versus all rats exposed to dialysis solutions (CKD+exposure), and CKDD versus CKDP. For the analysis we used the IBM SPSS statistics software, version 21.

Results
Development of chronic kidney disease
Glomerular filtration rate was uniformly decreased at 16 weeks after 70% nephrectomy (Table 1). Five rats (2 CKDD, 3 CKDP) were excluded from further analysis, because of complications due to excessive bleeding post-nephrectomy (n=2), catheter obstruction (n=1), and peritonitis (n=2).

Peritoneal transport
The rats with kidney failure had higher small solute transport rates than the NKF animals did (Table 2). Exposure to PDF augmented this effect, showing it also for

Table 1. Renal function at 16 weeks after 70% nephrectomy. Comparison of renal function of the groups at the end of the experimental period. No difference was present between CKDP and CKDD.

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>CKD (n=12)</th>
<th>CKDD (n=10)</th>
<th>CKDP (n=8)</th>
</tr>
</thead>
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<tr>
<td>Removed kidney mass (%)</td>
<td>0</td>
<td>69.6 (68.3-70.4)*</td>
<td>69.5 (68.4-70.2)*</td>
<td>68.8 (68.7-69.6)*</td>
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<tr>
<td>GFR (ml/min)</td>
<td>2.9 (2.7-3.5)</td>
<td>1.5 (1.2-1.6)*</td>
<td>1.7 (1.4-1.9)*</td>
<td>1.4 (1.0-1.7)*</td>
</tr>
</tbody>
</table>

Results are expressed as median and interquartile range. NKF: normal kidney function; CKD: chronic kidney failure; CKDD: CKD and exposure to Dianeoal; CKDP: CKD and exposure to Physioneal. ANOVA with Games-Howell post-hoc analysis.* NKF vs CKD, CKDD, CKDP all p<0.001
Table 2. Peritoneal transport. Peritoneal transport parameters as measured by the standard peritoneal permeability analysis adapted for rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NKF (n=2)</th>
<th>CKD (n=3)</th>
<th>CKD+exposure (n=8)</th>
<th>CKDD (n=4)</th>
<th>CKDP (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTAC urea 240 (µl/min)</td>
<td>105.4(104-107)</td>
<td>160.1(129-196)</td>
<td>188.4(145-496)</td>
<td>185.4(148-206)</td>
<td>190.6(145-496)</td>
</tr>
<tr>
<td>MTAC creat 240 (µl/min)</td>
<td>41.4(40-43)</td>
<td>72.9(49-82)</td>
<td>115.7(89-150)²</td>
<td>96.0(89-150)</td>
<td>134.2(89-137)</td>
</tr>
<tr>
<td>Glucose absorption (%)</td>
<td>58.2(54.0-62.3)</td>
<td>58.1(57.9-59.8)</td>
<td>68.4(61.9-71.1)³</td>
<td>68.5(67.8-71.1)</td>
<td>67.0(62-69)</td>
</tr>
<tr>
<td>Albumin Clearance (µl/min)</td>
<td>1.3(1.1-1.5)</td>
<td>1.6(1.3-3.1)</td>
<td>2.7(2.4-3.8)</td>
<td>2.7(2.4-2.9)</td>
<td>2.7(2.6-3.8)</td>
</tr>
<tr>
<td>IgG Clearance (µl/min)</td>
<td>0.4(0.3-0.5)</td>
<td>0.6(0.5-0.6)</td>
<td>0.8(0.7-0.9)³</td>
<td>0.7(0.7-0.9)</td>
<td>0.77(0.76-0.78)</td>
</tr>
<tr>
<td>TCUFR (µl/min)</td>
<td>74.2(73.6-74.9)</td>
<td>78.2(75.4-79.6)</td>
<td>87.1(63.2-167.2)²</td>
<td>89.8(68.4-124)</td>
<td>65.2(63.2-167.2)</td>
</tr>
<tr>
<td>NUFIR (µl/min)</td>
<td>72.6(63.6-81.6)</td>
<td>60.5(58.4-103.0)</td>
<td>61.5(29.2-77.5)</td>
<td>62.4(60.2-64.4)</td>
<td>57.9(29.2-77.5)</td>
</tr>
</tbody>
</table>

Results are expressed as median (range). MTAC 240: mass transfer area coefficient at 240min; TCUFR: transcapillary ultrafiltration rate; NUFIR: net ultrafiltration rate. NKF: normal kidney function; CKD: chronic kidney failure; CKDD: CKD and exposure to Dianeal; CKDP: CKD and exposure to Physioneal; CKD+exposure: CKDD and CKDP analysed together. Mann-Whitney test: *p<0.05, CKD versus CKD+exposure
Figure 1. Mesothelial cell ultrastructure is differently modified in CKD and after exposure to dialysis solutions. Compared to MC in NKF, MC in CKD rats are enlarged, with more microvilli (black arrowhead), and higher number of cytoplasmic vesicles. MC in both exposed groups are further enlarged and occasionally contain lamellar bodies (white arrowhead). In CKDD both microvilli and intracytoplasmic vesicles (inset) are more numerous. In CKDP, cytoplasmic processes are bridging widened inter-cellular gaps (open arrow). At the basal side of the plasma membrane, invadopodia-like structures are present. Occasionally these penetrate through the mesothelial basal lamina into the sub-mesothelial stroma (inset). NKF: normal kidney function; CKD: chronic kidney failure; CKDD: chronic kidney failure and exposure to Dianeal; CKDP: chronic kidney failure and exposure to Physioneal. Scale bars represent 2 μm, in insets 0.4 μm.
Table 3. Morphological alterations of the peritoneal membrane.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NKF (n=8)</th>
<th>CKD (n=12)</th>
<th>CKD+exposure (n=19)</th>
<th>CKDD (n=10)</th>
<th>CKDP (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel densities/profiles/mm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood vessels</td>
<td>2.9 (2.2-3.8)</td>
<td>5.5 (4.4-6.9) *</td>
<td>7.0 (5.7-8.5) *†</td>
<td>7.3 (5.6-9.0)</td>
<td>7.0 (5.7-8.3)</td>
</tr>
<tr>
<td>Lymph vessels</td>
<td>0.3 (0.1-0.5)</td>
<td>0.6 (0.5-1.2) *</td>
<td>0.9 (0.6-1.2) *</td>
<td>0.7 (0.5-1.7)</td>
<td>0.9 (0.6-1.2)</td>
</tr>
<tr>
<td>Analysis of fibrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrosis score</td>
<td>0 (0-1)</td>
<td>3 (1-3) *</td>
<td>5 (4-6) *†</td>
<td>6 (5-7)</td>
<td>4 (4-5) ‡</td>
</tr>
<tr>
<td>PSR staining (%)</td>
<td>1.3 (0.9-2.2)</td>
<td>2.8 (2.0-3.5) *</td>
<td>7.9 (4.4-11.8) *†</td>
<td>7.1 (5.3-16.6)</td>
<td>8.7 (4.1-11.2)</td>
</tr>
<tr>
<td>OH-proline (µg/mg tissue)</td>
<td>0.3 (0.2-0.5)</td>
<td>0.5 (0.4-1.0)</td>
<td>0.8 (0.4-1.3)</td>
<td>1.0 (0.4-1.3)</td>
<td>0.6 (0.2-1.1)</td>
</tr>
<tr>
<td>Collagen I (%)</td>
<td>-</td>
<td>-</td>
<td>14.2 (9.7-18.8)</td>
<td>14.8 (13.1-25.8)</td>
<td>9.8 (5.9-18.5)</td>
</tr>
</tbody>
</table>

We assessed blood and lymph vessel profile densities, fibrosis by a semi-quantitative method (fibrosis score), percentage of the picrosirius red positive area from the total tissue section (PSR staining), OH-proline content in omentum, and fraction of collagen I positive area from the total tissue section. Results are expressed as median and interquartile range. NKF: normal kidney function; CKD: chronic kidney failure; CKDD: CKD and exposure to Dianea; CKDP: CKD and exposure to Physioneal; CKD+exposure: CKDD and CKDP analysed together. PSR: picro-sirius red.

* p<0.05 CKD, CKDD, CKDP versus NKF; † p<0.05 CKD+exposure versus CKD; ‡ p<0.05 and ‡ p=0.09 CKDP versus CKDD
Peritoneal Alterations in CKD and PD

Glucose absorption. Also, peritoneal clearance of IgG was higher in the exposed rats. No difference was present between CKDD and CKDP. No difference was found for fluid transport parameters.

**Mesothelial cell alterations**

Mesothelial cells (MC) in NKF rats appeared as a flattened monolayer (Figure 1). MC in CKD showed a reactive state, being enlarged with a significant increase in the number of microvilli (NKF: 0.26 microvilli/µm, CKD: 0.38 microvilli/µm), and many intracytoplasmic vesicles. Areas of detachment from the basal lamina were present. The exposure to both dialysis fluids led to further enlargement of MC and alterations of their plasma membrane resulting in increased numbers of microvilli (CKDD: 0.44 microvilli/µm, CKDP: 0.51 microvilli/µm). MC in CKDD contained numerous intracytoplasmic vesicles, but maintained extensive cell-to-cell lateral contact. In contrast, MC in CKDP displayed prominent cytoplasmic protrusions forming bridged intercellular spaces. In addition, similar processes were present at the basal membrane, occasionally traversing the lamina basalis and protruding into the submesothelial compartment (Figure 1). Occasional necrotic MC were found in CKDD, in association with areas of denudation.

**Blood and lymph vessel profile densities**

All groups with kidney failure had higher blood and lymph vessel profile densities compared to NKF, as shown in Table 3. Angiogenesis, but not lymphangiogenesis, was more extensive in the exposed groups compared to CKD (Figure 2, upper panel, and Supplemental_Figure_1 for a higher magnification). LV and BV profile densities were similar in CKDD and CKDP (see also Supplemental_Figure_2).

**Vascular basement membrane (VBM) alterations**

CKD rats tended to have a thicker VBM compared to NKF, with areas of duplication (Figures 3 and Supplemental_Figure_3). The severity of these alterations was further increased by exposure to PDF, and no difference was evident between CKDD and CKDP. In the majority of vessels however, the duplication was mostly focally distributed. In addition, variability in electron density of VBM was observed in CKD compared to NKF, and this alteration was more evident in the exposed groups.

**Peritoneal fibrosis**

Both the semi-quantitative analysis and whole slide image analysis of picro-sirius red (PSR) staining (Figure 2, lower panel) showed more fibrosis in CKD compared to NKF (Table 3, Supplemental_Figure_2). The exposure to PDF further enhanced the amount of fibrosis. Although statistical significance was not apparent, the hydroxyproline content of peritoneal tissue from CKD was higher than in NKF and further increased in rats...
Figure 2. Upper panel. Both angiogenesis and lymphangiogenesis are induced in CKD and only the former is further enhanced after exposure to both dialysis solutions. These alterations occurred to a similar extent in CKDD and CKDP. Double immunostaining was performed to detect endothelial cells in blood vessels using anti-aminopeptidase P and in lymph vessels using anti-podoplanin. Mesothelial cells also stain for podoplanin. NKF: normal kidney function; CKD: chronic kidney failure; CKDD: chronic kidney failure and exposure to Dianeal; CKDP: chronic kidney failure and exposure to Physioneal.

Lower panel. Fibrosis is increased in CKD and further enhanced in CKDD and CKDP. Omental tissue was stained with Picro Sirius Red to detect collagen. NKF: normal kidney function; CKD: chronic kidney failure; CKDD: chronic kidney failure and exposure to Dianeal; CKDP: chronic kidney failure and exposure to Physioneal. Scale bar represents 200 µm.
Figure 3. Lamina basalis of blood vessels is thickened in CKD and duplicated after exposure to both dialysis solutions. Lamina basalis is indicated by arrow heads. NKF: normal kidney function; CKD: chronic kidney failure; CKDD: chronic kidney failure and exposure to Dianeeal; CKDP: chronic kidney failure and exposure to Physioneal. Scale bars represent 1 µm.
exposed to dialysis solutions. The semi-quantitative analysis showed less fibrosis in the CKDP rats compared to the CKDD ones. In agreement, the whole slide image analysis, the OH-proline content of homogenized peritoneal tissues, and collagen I immunohistochemistry (Supplemental_Figure_4) showed a trend towards less fibrosis in the CKDP rats (Table 3, Supplemental_Figure_2).

**Discussion**

This comprehensive analysis of the effects of long-term exposure to PDF on the peritoneum in a rat model with CKD, similar to stage 3 to 4 in humans, shows that CKD itself induced mesothelial cell alterations, new blood and lymph vessel formation, duplication of the vascular basal lamina, peritoneal fibrosis, and high peritoneal transport rates. Long-term exposure to PDF further increased these transport rates and enhanced the ultrastructural alterations, angiogenesis and the extent of fibrosis, but had no effect on new lymph vessel formation. The biocompatible PDF was associated with increased MC membrane surface area and a trend towards less fibrosis, compared to the conventional solution.

Mesothelial cells are at the interface between peritoneum and PDF during PD. They are metabolically active, constitute a source of growth factors, extracellular matrix molecules, and various immunomodulatory mediators, and contribute to the structural and functional integrity of the peritoneum. CKD induced MC alterations which is in line with data in patients. An additional effect of exposure to PDF was present. The increased number of microvilli found in our study seems at odds with some studies in which exposure to PDF induced a decrease in the number of microvilli. It is likely that mesothelial degeneration prevailed in those studies, while in others and here, activation and regeneration were more prominent. Importantly, an influence of the kind of dialysis solution administered was present. Whereas in CKDD the increased number of vesicles was predominant, in CKDP cytoplasmic protrusions were present at the basal side causing an additional increase in the MC plasma membrane surface area. The occasional extension of these structures into the submesothelial stroma is consistent with the migratory phenotype of MC during epithelial-to-mesenchymal transition as described previously by Yanez-Mo et al. Noteworthy, in patient studies the switch from a conventional to a biocompatible solution is associated with an increase in effluent cancer antigen 125 (CA125), a mucinous glycoprotein mainly located at the cell membrane of MC. The higher levels of this biomarker are thought to reflect the increased MC mass and turnover in this condition, consistent with less damage to the peritoneum. Little is known about the mechanisms which lead to increased CA125 in peritoneal effluent. Both increased production by mesothelial cells and increased shedding from the cell
membrane may be involved. Mesothelial cells with enlarged cell membrane area, as we describe in CKDP rats, may be the source of higher CA125 effluent levels, as found in PD patients after the switch to a biocompatible PDF. Unfortunately, due to the unavailability of a suitable antibody we could not determine effluent CA125 levels in our rats.

CKD was associated with higher peritoneal blood and lymph profile densities compared to NKF. Previous experimental studies investigating angiogenesis in PD, used endothelial markers such as CD31, von Willebrand factor or eNOS, which are expressed by both blood and lymphatic endothelium. We used specific staining to distinguish BV from LV and applied unbiased stereological methods for the analysis. Aminopeptidase P, a glycosyl-phosphatidylinositol membrane-linked aminoacylproline aminopeptidase, is expressed specifically by endothelial cells of BV only. By using this methodology we confirmed that exposure to PDF causes a further increase in new BV formation. CKDD and CKDP were not different in this respect. This contrasts with the reduction in vessel density reported previously using a non-specific endothelial staining, after exposure to a biocompatible PDF for various periods of time. The peritoneal alterations are time dependent. It may be that the difference between the two solutions is important at the start of the treatment and becomes less important in time. Limited data is available in patients, but a recent study reported an increased BV density in patients treated with a neutral dialysis solution compared to those treated with acidic ones. Importantly, the similar BV densities in the CKDD and CKDP rats are in line with the lack of a difference in peritoneal solute transport in both rats and patients. CKD was associated with some increase in small solute transport, confirming previous reports. This is in line with the observation that patients with normal renal function had lower small solutes transport rates during PD than uremic patients. Exposure to PDF augmented these effects in the present study without any difference in peritoneal transport between the two solutions. This is in accordance with the situation in patients, where most studies have shown no effect of biocompatible PDF on peritoneal solute transport after short to median time exposure. A recent randomized controlled trial showed a small difference in the time-course of peritoneal transport with lower D/P creatinine values in patients treated with a biocompatible solution. The difference between these clinical studies may either have been caused by the use of different solutions or by the duration of follow-up.

At the ultrastructural level, uremia itself causes some increase in thickness, reduced homogeneity and duplication of the VBM. Although hypertension may have been the cause, vascular abnormalities are present as well in patients with renal failure without hypertension. The exposure to PDF augmented these ‘diabetiform’ alterations without any evidence for a difference between the two glucose-based solutions. The fact that
these solutions differ only in pH and GDPs content, strongly suggests that glucose itself, possibly enhanced by hypertension, is by far the most important factor in the genesis of the vascular alterations associated with long-term PD.

Limited data is available on new LV formation induced by PD. Lymphangiogenesis develops after short-term exposure to PDF in both non-uremic and uremic rats, but data is lacking in long-term exposure models. In the present study CKD induced an increase in LV density. Noteworthy, lymphangiogenesis was not further enhanced by exposure to PDF, which is in line with the clinical data. In patients, no difference was found between those exposed to either a neutral or an acidic dialysis solution, and predialysis patients. Our findings are also in agreement with the functional data which showed that no time trend is present for the effective peritoneal lymphatic absorption rate in stable PD patients.

CKD itself induced peritoneal fibrosis which was further enhanced in the groups exposed to PDF. The presence of a catheter in the peritoneal cavity of the exposed animals may itself have caused inflammation and induced structural and functional alterations of the peritoneum. However, these reaction patterns tend to occur predominantly during the initial phase of PD and decrease significantly after 8 weeks of exposure. Also in the present study, an initial effect of the catheter may have been present, but an exact contribution to peritoneal alterations cannot be established. Importantly, the long duration of exposure to PDF in the present study allowed us to focus on the long term effects of the dialysis solutions on the peritoneum. The semi-quantitative analysis of the PSR staining indicated that the biocompatible solution induced less fibrosis compared to the conventional one. In agreement, the whole slide analysis, OH-proline determinations, and collagen I staining analysis also showed a trend towards less fibrosis for the biocompatible PDF. This is in line with findings in non-uraemic rats and in patients, showing a thinner submesothelial compact zone after exposure to a biocompatible solution compared to the conventional one. It is speculative whether this can be explained by a lower content of GDPs, causing reduced formation and accumulation of AGEs. In order to gain more insight into the organization of the collagen fibers, we considered using polarization microscopy. Although extensively used in literature for the characterization of collagen fibers, recent data show its limited value.

The particular strength of the present study is the application of long term daily peritoneal exposure to dialysis solutions in the setting of advanced chronic kidney disease, thereby truly modelling clinical peritoneal dialysis. Using this model, this study is the first to compare exposure of the peritoneal cavity to either conventional or biocompatible dialysis solutions combining functional peritoneal transport characteristics with detailed morphological and structural analyses. Systematic uniform random sampling,
followed by computer assisted stereology for the quantification of vascular profile densities, ensured accurate estimates for these parameters. Our model may further deepen insight into the pathogenesis of peritoneal fibrosis and offer reliable exploration of potential therapeutic interventions.

In summary, chronic renal failure itself induces peritoneal alterations, which are overall comparably augmented by long-term exposure to both conventional and biocompatible dialysis solutions. Interestingly however, in contrast to angiogenesis, lymphangiogenesis was not further enhanced by exposure to either glucose-based PDFs. Distinct mesothelial cell phenotypes were induced by each of the dialysis solutions. Importantly, the use of a biocompatible solution tends to be beneficial with respect to the development of peritoneal fibrosis.

Acknowledgements
This work was supported by grant GHOL5988 from Baxter Healthcare. The authors kindly acknowledge O.J. de Boer and N. Claessen for the excellent technical assistance.

Disclosure
The authors declare no financial conflict of interest.
References


Supplemental Data

Electron microscopy

After fixation, the mesentery was washed in 0.1 M phosphate buffer followed by a washing step in distilled water, osmicated for 75 minutes in 1% OsO4 in water and washed again in distilled water. For contrast enhancement, biopsies were block stained overnight in 1.5% aqueous uranyl acetate and then dehydrated through a series of ethanol and embedded in Epon 812 (EMS). The resin blocks were polymerized for 48 hours at a temperature of 60 °C. Ultrathin sections of 80 nm were cut on a Reichert EM UC6 with a diamond knife, collected on formvar coated grids and stained with uranyl acetate and lead citrate.

Supplemental Figure 1. Both angiogenesis and lymphangiogenesis are induced in CKD and only the former is further enhanced after exposure to both dialysis solutions. These alterations occurred to a similar extent in CKDD and CKDP. Double immunostaining of omental tissue was performed to detect endothelial cells in blood vessels using anti-aminopeptidase P (blue) and in lymph vessels using anti-podoplanin (red). NKF: normal kidney function; CKD: chronic kidney failure; CKDD: chronic kidney failure and exposure to Dianec; CKDP: chronic kidney failure and exposure to Physioneal. Scale bar represents 100 µm.
Supplemental Figure 2. Quantitative analysis of angiogenesis, lymphangiogenesis and fibrosis in the study groups. Symbols represent values for each individual rat. The mean group value is shown as well.

A. Analysis of blood vessel profile density. Results are expressed as BV profiles/mm².
B. Analysis of lymph vessel profile density. Results are expressed as LV profiles/mm².
C. Semi-quantitative analysis of PSR staining. Results are expressed as fibrosis score.
D. Whole slide image analysis of PSR staining. Results are expressed as percentage of PSR positive surface area.

E. Determination of hydroxyproline content in peritoneal tissue.
F. Image analysis of collagen I immunostaining in the groups exposed to dialysis solutions. Results are expressed as percentage collagen I positive tissue surface area.

NKF: normal kidney function; CKD: chronic kidney failure; CKD+exposure: rats exposed to dialysis solutions analysed together; CKDD: chronic kidney failure and exposure to Dianeal; CKDP: chronic kidney failure and exposure to Physioneal.

Supplemental Figure 3. Vascular basal lamina is thickened and occasionally duplicated in CKD and these alterations are further enhanced by exposure to dialysis fluids. The class of alterations is indicated in the figure. Each column represents one individual rat. NKF: normal kidney function; CKD: chronic kidney failure; CKDD: chronic kidney failure and exposure to Dianeal; CKDP: chronic kidney failure and exposure to Physioneal.
Supplemental Figure 4. Collagen I immunostaining is increased to a higher extent in CKDD compared to CKDP rats. CKDD: chronic kidney failure and exposure to Dianea; CKDP: chronic kidney failure and exposure to Physionea. Scale bar represents 200µm.
Peritoneal Alterations in CKD and PD
Chapter 8

Lymphangiogenesis and Lymphatic Absorption Are Related and Increased in Chronic Kidney Failure, Independent of Exposure to Dialysis Solutions

Carmen A. Vlahu, Marijke de Graaff, Jan Aten, Dirk G. Struijk, Raymond T. Krediet

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Abstract

Background
Increased lymphatic absorption might contribute to ultrafiltration failure in peritoneal dialysis (PD). Lymphangiogenesis develops during PD, but little is known about the relationship between its morphologic and functional parameters. The relationships between lymph vessel density, the effective lymphatic absorption rate (ELAR), and fibrosis were investigated in a rat model of chronic kidney failure (CKD) with exposure to dialysis solutions.

Methods
Wistar rats \( (n = 44) \) were allocated to these groups: NKF (normal kidney function), CKD (70% nephrectomy), CKDD [CKD, with daily intraperitoneal (i.p.) Dianeal 3.86% (Baxter Healthcare BV, Utrecht, Netherlands)], CKDP [CKD, with daily i.p. Physioneal 3.86% (Baxter Healthcare BV)]. After 16 weeks, a peritoneal function test was performed, and the ELAR was calculated from the disappearance rate of i.p. dextran 70. The lymph vessel profile density (LVPD) was assessed using STEPanizer image analysis (Java application from Tschanz SA, Bern, Germany) of omental sections after anti-podoplanin immunostaining.

Results
Fibrosis was quantified by picro-sirius red staining. The LVPD was significantly increased in CKD rats compared with NKF rats, and no additional effect of dialysis solutions was present. The ELAR was increased in uremic rats compared with NKF rats. For all rats together, the LVPD correlated positively with the ELAR and with the amount of fibrosis.

Conclusions
Chronic kidney disease itself induces lymphangiogenesis and fibrosis and increases the ELAR, independent of exposure to dialysis fluids. The ELAR is related to the LVPD in peritoneal tissue.
Lymphangiogenesis and ELAR in PD

Introduction

Ultrafiltration failure is an important reason for discontinuation of treatment with peritoneal dialysis (PD).1-3 High lymphatic reabsorption can contribute to impaired ultrafiltration. Some PD patients have a high lymphatic absorption rate, but no time trend is evident.4 Lymphangiogenesis is part of normal development during embryogenesis and occurs in several pathogenic conditions,5-9 including peritoneal exposure to dialysis solutions, during which its development is associated with peritoneal fibrosis by the transforming growth factor β–vascular endothelial growth factor C pathway.10,11 Furthermore, compared with uremic patients before the start of PD, PD patients with ultrafiltration failure have been described to show an increase in the number of lymph vessels in peritoneal tissue.11

To date, little is known about the relationship between the morphologic and functional data of lymphangiogenesis. In the present study, we used a rat model of chronic kidney failure, with exposure to either conventional or biocompatible dialysis solution, to investigate whether the density of lymph vessels in peritoneal tissue and the effective lymphatic absorption rate (ELAR) are related. The ELAR was calculated based on the rate of dextran 70 disappearance from the peritoneal cavity during a standard peritoneal permeability analysis adapted for rats (SPARa).12 In addition, we tested whether lymphangiogenesis and the extent of fibrosis of peritoneal tissue were related in this model.

Methods

Study design

Male Wistar rats (n = 44; Harlan, Zeist, Netherlands) with a body weight of 260 – 280 g were randomly assigned to four experimental groups: normal kidney function (NKF), n = 8, no peritoneal exposure; chronic kidney failure (CKD), n = 12, 70% nephrectomy, no peritoneal exposure; CKD exposed to Dianeal (CKDD), n = 12, 70% nephrectomy, with daily peritoneal exposure to Dianeal (Baxter Healthcare BV, Utrecht, Netherlands), a conventional lactate-buffered dialysis solution, 3.86% glucose; CKD exposed to Physioneal (CKDP), n = 12, 70% nephrectomy, daily peritoneal exposure to Physioneal (Baxter Healthcare BV), a bicarbonate/lactate–buffered dialysis solution, 3.86% glucose.

All dialysis solutions were provided by Baxter Healthcare BV. The rats received 6 mL dialysis fluid per 100 g body weight intraperitoneally, and the infused volume was set at 20 mL daily when the body weight of the animal exceeded 420 g. After 16 weeks, at least 2 rats in each group underwent a SPARa, and morphology assays of the peritoneum were performed in all rats. The protocol was approved by the Committee for Animal Experiments of the Academic Medical Center, University of Amsterdam.
Procedures
Housing conditions, catheter implantation, nephrectomy, and SPARa were all performed as previously described.\textsuperscript{13}

Peritoneal transport parameters
Peritoneal small-solute transport was expressed as dialysate-to-plasma creatinine (D/P Cr), determined by an enzymatic method. The ELAR was calculated based on the rate of dextran 70 disappearance from the peritoneal cavity—that is, the difference between the instilled and recovered dextran mass divided by the geometric mean of the dialysate dextran concentrations. Intraperitoneal dextran 70 was measured by gel permeation chromatography.\textsuperscript{14}

Renal function
Just before the end of the study, 24-hour urine collected from rats in metabolic cages was used for the calculation of endogenous creatinine clearance.

Immunohistochemistry
Formalin-fixed, paraffin-embedded omental tissue was used for immunohistochemistry. Sections of 4 µm thickness were deparaffinized, and heat-induced epitope retrieval in sodium citrate (pH 6) was then performed. The staining sequence was 5% normal goat serum in phosphate-buffered saline for 30 minutes; the primary antibody [mouse anti-rat podoplanin, 1:10000 (ReliaTech GmbH, Wolfenbuttel, Germany)] was then left for 2 hours at room temperature. The secondary antibody [alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G1, 1:50 (Southern Biotech, Birmingham, AL, U.S.A.)] was followed by washing in Tris-buffered saline with Tween (Sigma–Aldrich, St. Louis, MO, U.S.A.) and visualization using a Vector Red Substrate kit (Vector Laboratories, Burlingame, CA, U.S.A.). Fibrosis was analyzed on sections of formalin-fixed, paraffin-embedded omental tissue, stained with picro-sirius red (Gurr: BDH Chemicals, Poole, U.K.).

Image acquisition
A BX61VS microscope with 10x/0.40 UPlanSApo objective and an Olympus dotSlide imaging system (Olympus, Zoeterwoude, Netherlands) was used to scan slides for the acquisition of TIFF (tagged image file format) images (1 pixel = 648 nm x 648 nm). The TIFF images were exported as 10 MB files for analysis of lymph vessel profile density and as 20 MB files for quantification of fibrosis. In the latter case, the area of picro-sirius red–positive staining was measured (ImagePro premier 9.1: Media Cybernetics, Rockville, MD, U.S.A.) and expressed as a percentage of the total surface area.
Analysis of lymph vessel profile density (LVPD)
The stereology tool STEPanizer (version 1: Java application from Tschanz SA, Bern, Germany) was used to quantify the number of vascular profiles and the surface area.\textsuperscript{15} We analyzed 8 images from each rat, selected after application of a systematic uniform random sampling of the images. Using a counting frame (area: 924x924 pixels), the vascular profiles were counted if they were located inside the counting frame, but not if they were touching the exclusion line or its extensions. The profile density is reported as a ratio: the number of lymph vessel profiles per tissue surface area.

Statistics
Data are presented as means, with standard deviations. Possible differences between the study groups were assessed using analysis of variance and Games–Howell \textit{post hoc} analysis, or the Mann-Whitney U-test for comparisons between specific groups. Correlations between continuous variables were tested using the Pearson correlation test or the Spearman rho.

Results
Because of complications related to nephrectomy, to catheter obstruction, or to suspicion of peritonitis, 5 rats were excluded from the study (2 from CKDD, 3 from CKDP).

Renal function
At the end of the study period, endogenous creatinine clearance was highest in the NKF animals (4.3 ± 0.7 mL/min) and decreased in nephrectomized rats (CKD: 1.8 ± 0.5 mL/min; CKDD: 2.3 ± 0.6 mL/min; CKDP: 1.9 ± 0.5 mL/min; \( p < 0.001 \) for all groups vs. NKF).

Lymph vessel profile density
The LVPD was 0.3 ± 0.2 profiles/mm\(^2\) in NKF rats and increased significantly in rats with kidney failure (CKD: 0.8 ± 0.4 profiles/mm\(^2\); CKDD: 1.0 ± 0.6 profiles/mm\(^2\); CKDP: 0.9 ± 0.4 profiles/mm\(^2\); \( p = 0.009 \) for the differences between groups). No significant differences were evident between the CKDP and CKDD groups (\( p = 0.9 \)), nor between the CKD group and the groups exposed to dialysis fluids (\( p = 0.8 \) for CKDD, \( p = 0.7 \) for CKDP). When CKDD and CKDP were analyzed together, the difference between the CKD group and the combined groups remained nonsignificant (\( p = 0.4 \)).

Peritoneal transport parameters
The ELAR was 12 ± 3 µL/min in NKF rats and was increased in rats with kidney failure (CKD: 19 ± 2 µL/min; CKDD: 29 ± 22 µL/min; CKDP: 23.2 ± 9 µL/min; \( p = 0.09 \).
or all the rats with CKD vs. NKF rats). Although the ELAR was somewhat higher in the groups exposed to dialysis solution than in the CKD group, the difference was not significant ($p = 0.4$). The D/P Cr was $0.38 \pm 0.06$ in NKF rats and somewhat higher in CKD rats ($0.45 \pm 0.03$). Exposure to dialysis fluids induced an additional significant increase (CKDD: $0.59 \pm 0.09$; CKDP: $0.63 \pm 0.05$; $p = 0.002$ for both groups vs. the CKD group). No difference between the CKDD and CKDP groups was evident ($p = 0.9$ for both parameters). The ELAR was related to the D/P Cr ($p = 0.04$, $r = 0.6$).

**Fibrosis of peritoneal tissue**

The surface area positive for picro-sirius red was $1.5\% \pm 0.8\%$ in NKF rats and was increased in CKD rats ($2.9\% \pm 1.3\%, p = 0.05$). Exposure to dialysis fluids was associated with a further increase (CKDD: $9.9\% \pm 6.0\%$; CKDP: $8.0\% \pm 4.2\%; p = 0.02$ vs. the CKD group).

To investigate the relationships between the LVDP, the ELAR, and fibrosis, we analyzed the ELAR as a continuous variable. As Figure 1 shows, the ELAR and LVDP demonstrated a positive relationship ($p = 0.04$; Spearman rho: 0.63). A strong positive correlation was present between the LVDP and the amount of fibrosis expressed as percentage of positive staining by picro-sirius red ($p < 0.001$; Spearman rho: 0.7).

**Discussion**

Analysis of the LVDP in peritoneal tissue shows that lymphangiogenesis develops in CKD and that it is associated with fibrosis. Long-term exposure to dialysis fluids had no significant additional effect. Importantly, the ELAR was also higher in CKD rats than in NKF rats, and it was positively correlated with the LVDP and with small-solute transport. The relationship between the ELAR (which is a functional parameter) and

![Figure 1. Relationship between the effective lymphatic absorption rate (ELAR) and the lymph vessel profile density (LVDP) in omental tissue ($p = 0.04$; Spearman rho: 0.63).](image-url)
the LVPD has not previously been reported and supports our contention that the ELAR indeed represents lymphatic absorption\textsuperscript{10} - a finding hitherto not recognized by some authors.\textsuperscript{17}

The presence of uremia-induced lymphangiogenesis in rats has previously been reported, and exposure to dialysis fluids for 4 weeks has been claimed to lead to a further increase in the number of lymph vessels.\textsuperscript{10,18} Also, simple exposure to dialysis fluids induced an increase in the number of lymph vessels in rats with normal kidney function.\textsuperscript{19} In our study, although the absolute values of the LVPD in the groups exposed to dialysis fluids were higher than the values in the CKD group, we found no additional significant effect of the exposure to dialysis fluids. The methods used for the quantification of lymphangiogenesis in other studies and in ours are different. In addition, we cannot exclude the possibility that a lymphangiogenic effect of exposure to dialysis fluids might be found only early, at initiation of peritoneal dialysis, and that after a longer follow-up or in long-term uremia, the effect could become less important.

The ELAR was calculated as peritoneal dextran clearance, which includes both the subdiaphragmatic and interstitial pathways of uptake into the lymphatic system. As previously reported in patients,\textsuperscript{20} the ELAR and the transport rates of small solutes (determined as D/P Cr) were positively correlated, suggesting that a large lymphatic surface area develops in concert with a large surface area of blood vasculature.

The combination of functional and morphologic data, together with the use of computer-assisted stereology for the quantification of LVPD, are among the strengths of the present study; the small number of rats undergoing the SPARa could be considered a weakness.

**Conclusions**

The present study shows that lymphangiogenesis is present and is associated with fibrosis in the peritoneal tissue of rats with CKD. An additional significant effect of dialysis solutions could not be established. Importantly, the ELAR, a functional parameter calculated as peritoneal dextran clearance, was related to the LVPD in peritoneal tissue.

**Disclosures**

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Chapter 9

Summary and conclusions
General discussion
Nederlandse samenvatting
The endothelial surface layer: a new target of research in kidney failure and peritoneal dialysis

This thesis reports investigations aimed at defining the state of the endothelial glycocalyx in chronic kidney failure. More specifically, in part I we investigated the state of the endothelial surface layer in patients with end-stage renal disease, and after successful kidney transplantation. In part II we focused on the role of the endothelial surface layer in peritoneal dialysis. For this, we assessed the peritoneal microvascular endothelial glycocalyx, and investigated the relationships with peritoneal transport. In part III we described the peritoneal alterations induced by long-term exposure to high glucose concentration dialysis solutions in an experimental model of chronic kidney disease.

Chapter 1 gives a summary of the endothelial glycocalyx structure and functions, followed by a description of the assessment methods which are currently available in humans. Then, we give an overview of chronic kidney failure and peritoneal dialysis as method of renal replacement therapy. Patients with chronic kidney failure form a high risk patient group because of the high prevalence of cardiovascular disease which is associated with their condition. The uremic milieu is associated with increased oxidative stress, a chronic inflammatory state, decreased availability of antioxidant systems and hypervolemia, which all contribute to the vascular endothelial dysfunction and may impact the endothelial glycocalyx. Through its numerous vasculoprotective functions, the endothelial glycocalyx is essential in maintaining vascular integrity, and its alteration will increase endothelial vulnerability. The gap in the current knowledge on the state of the endothelial surface layer in kidney disease has led to the investigations performed in chapter 2. In patients with ESRD treated with hemodialysis or peritoneal dialysis, we used Sidestream Darkfield imaging of the sublingual microcirculation, to measure the perfused boundary region (PBR), which reflects the erythrocyte-permeable part of the endothelial surface layer. The results were compared with those from healthy individuals. Both HD and PD patients had a thicker PBR and larger perfused diameters in the sublingual microcirculation, indicating a change in the RBC-excluding properties of the endothelial surface layer. In addition, dialysis patients had higher plasma concentrations of glycocalyx constituents hyaluronan, syndecan-1, and increased hyaluronidase activity compared with healthy individuals. Higher plasma concentrations of C-reactive protein in dialysis patients were associated with a thicker erythrocyte-permeable part of the endothelial surface layer. Anuric patients had higher plasma hyaluronan concentrations and reduced hyaluronidase activity compared with patients with residual renal function. Interestingly, the presence of cardiovascular disease was not associated with additional changes in any of the study parameters. In conclusion, by analyzing the dynamic variations of erythrocyte column width in the sublingual microcirculation, we showed that dialysis patients, treated with either HD or PD, have changes in glycocalyx barrier properties.
These patients have high concentrations of hyaluronan and syndecan-1 in blood, but the extent to which these are caused by shedding from the endothelial surface layer, has not been determined. The role of kidney function in their removal from circulation should be considered as well, and should be carefully investigated.

In chapter 3, we used similar methods to assess the endothelial surface layer in renal transplant recipients with stable graft function. Successful kidney transplantation is associated with a survival benefit compared to patients treated with dialysis. Transplantation diminishes inflammation, oxidative stress and uremia-related complications, and therefore might impact the endothelial glycocalyx. In renal transplant recipients, the erythrocyte-permeable region of the endothelial surface layer was thicker compared with healthy controls, and the magnitude of this alteration was dependent on graft function. In addition, plasma hyaluronan concentrations were similar to those in controls, and only modest increases in plasma syndecan-1 and hyaluronidase activity were found in patients. In conclusion, successful kidney transplantation may be beneficial and induce normalization of the turnover within the endothelial glycocalyx. The restoration of the endothelial surface layer barrier properties is dependent on graft function.

Measurements of plasma concentrations of glycocalyx constituents provide an indirect tool for the assessment of the endothelial glycocalyx. Because of its presumed vasculo-protective effects within this layer, hyaluronan, together with the regulating enzyme hyaluronidase, have been described in literature, but almost never from a clinical point of view and without much focus on renal function. In chapter 4, we addressed the utility of plasma hyaluronan levels and hyaluronidase activity as potential markers for endothelial glycocalyx damage in patients with chronic kidney failure. Hyaluronic acid is an important constituent of the extracellular matrix and the endothelial glycocalyx. It is a negatively charged, unsulfated GAG, synthesized at the cytosolic side of the cell membrane by hyaluronan synthases. The major part of hyaluronan has a molecular weight exceeding 1000 kD, but small amounts of low molecular weight HA with pro-inflammatory and pro-angiogenic effects, can also be found. Hyaluronidase-2 activity may be important for the composition of the endothelial glycocalyx due to its localization at the outer cell membrane. In patients with kidney failure, plasma levels of hyaluronan are increased. However, the precise mechanism is unknown, and also, mutual relationships with hyaluronidase have not been investigated. Consequently, correct interpretation of a high plasma hyaluronan concentration without other determinations is impossible.

The second part of the thesis focuses on the importance of endothelial glycocalyx in peritoneal dialysis. During PD various solutes and water are transported from the circulation into the peritoneal cavity through the peritoneum. The peritoneal membrane
Summary and conclusions

consists of three main anatomical layers: the capillary wall, the intersitium and the mesothelium, the former creating the main resistance to transport. Endothelial permeability is partly dependent on the negatively charged glycocalyx that extends into the interendothelial clefts. In chapter 5 we investigated the relation between the state of the endothelial surface layer assessed in the sublingual microcirculation, and parameters of peritoneal transport in stable PD patients. A fast solute transport status was defined based on glucose absorption and the mass transfer coefficient of creatinine and urate. This condition is associated with rapid disappearance of the osmotic gradient, thereby impairing efficient dialysis. We found no relation between the imaging parameters and peritoneal transport parameters neither in the group as a whole, nor in patients with a fast transport status. However, in patients with non-fast transport status, peritoneal transport parameters were related to the perfused boundary region: the thicker the erythrocyte-permeable part of the endothelial surface layer in the sublingual vasculature, the lower the transport rates of small solutes and the higher the net ultrafiltration through the peritoneal membrane. The interpretation of these findings is challenging. Besides the relationships between perfused boundary region, blood vessel density and solute transport, which can explain the results, an increased diffusion distance is one of the possibilities. Unlike the systemic endothelial glycocalyx, peritoneal endothelial cells are exposed to high glucose concentrations, which may result in specific alterations of the peritoneal endothelial glycocalyx. Therefore, the measurements performed in the sublingual microvasculature may not reflect the peritoneal microvasculature, and subsequently, the relationships between peritoneal transport and the peritoneal endothelial glycocalyx may be different. SDF imaging of the peritoneal microcirculation in PD patients is currently impossible.

In chapter 6 we therefore attempted to address this question in a rat model of chronic kidney failure and exposure to dialysis solutions. In contrast to the data in patients, the PBR thickness in the peritoneal microcirculation was similar in rats with chronic kidney disease (CKD), rats with CKD exposed to dialysis solutions, and rats with normal kidney function. The exposure to dialysis fluids was associated with higher peritoneal microvascular densities. Relationships were present between small solute transport and peritoneal microvascular density, and PBR. A thicker erythrocyte-permeable region of the endothelial surface layer was associated with higher transport rates for small solutes. Taken together, our findings confirm our hypothesis that specific changes occur in the peritoneal microcirculation during PD, that are not captured by the assessment of the sublingual vasculature. Plasma concentrations of syndecan-1 were increased in rats with CKD and also in PDF exposed rats, and were associated with renal function. In order to gain insight into the biochemical alterations that occur within the endothelial glycocalyx, we performed immunostaining of heparan sulfate (HS) 10E4 epitope and syndecan-1, a proteoglycan to which both HS and chondroitin sulfate attach. HS 10E4 had predomi-
nantly a basolateral distribution and syndecan-1 was expressed in the interendothelial
junction, nucleus, and the cytoplasmic compartment. Syndecan-1 expression in inter-
endothelial junctions was decreased in rats with CKD, whereas in rats exposed to peri-
toneal dialysis fluids, the expression was similar to that in rats with normal kidney func-
tion. No relationships with peritoneal solute transport were present. Taken together,
our findings suggest that the endothelial surface layer may be important in peritoneal
transport during PD. The localization of syndecan-1 and HS 10E4 epitope precludes
an essential role for these molecules in the establishment of the peritoneal endothelial
glycocalyx at the luminal side.

In the third part of this thesis, we made an extensive analysis of the peritoneal membrane
alterations induced by long term exposure to either a conventional or a biocompatible
dialysis solution, both with the same high glucose concentration. For this, we used the
above mentioned model of rats with chronic failure exposed to dialysis solutions for 16
weeks. In contrast to the current literature on this topic, we used specific endothelial
markers and applied new methodologies, such as unbiased stereology, for the analysis.
This is currently considered to be the best-practice method for quantitative histology
as it enables high accuracy of the results. In chapter 7, we report that chronic kidney
failure itself induces mesothelial alterations, fibrosis, angiogenesis, lymphangiogenesis
and these are partially augmented by exposure to dialysis solutions. Interestingly, the
biocompatible dialysis solution seemed to induce less fibrosis than the conventional
one, and different mesothelial cell phenotypes were associated with the two solutions.
However, contrary to published data from various experimental models of peritoneal
exposure, we could not show a beneficial effect of the biocompatible solutions with regard
to neither morphologic parameters such as neoangiogenesis, nor peritoneal transport.
Our findings are consistent with the results of clinical studies, which showed no definite
advantage of biocompatible dialysis solutions on transport parameters. Lymphangiogen-
esis developed during CKD and in rats with CKD exposed to dialysis solutions, and
was associated with fibrosis. In chapter 8, we showed that new lymph vessel formation
and the effective lymphatic reabsorption rate, a functional parameter measured during a
standard peritoneal permeability analysis, are both increased in CKD, and are positively
related.
Conclusions

The endothelial glycocalyx is an important regulator of vascular homeostasis, and damage to this complex structure results in increased vascular vulnerability. Because of its vasculoprotective effects, the endothelial glycocalyx is highly relevant in the context of high vascular risk conditions, such as chronic kidney failure. Patients with end-stage renal disease have endothelial dysfunction and are at high risk to develop cardiovascular disease. The changes in the erythrocyte-excluding properties of the endothelial surface layer in the sublingual microcirculation together with the high plasma concentrations of glycocalyx constituents indicate that alterations of the endothelial glycocalyx occur in dialysis patients, regardless of the type of dialysis treatment they receive. Successful renal transplantation may have a beneficial effect on the endothelial glycocalyx and the restitution of the endothelial surface layer RBC-excluding properties is dependent upon graft function. Future studies should also address the effects of various immunosuppressive therapies on the ESL in this condition.

A critical appraisal of changes in plasma concentrations of glycocalyx constituents should be done to establish their value as markers for the endothelial glycocalyx, taking the role of the kidneys in their clearance from circulation into account.

The exposure of the peritoneal vasculature to high glucose concentration during peritoneal dialysis results in specific changes in the endothelial surface layer in the peritoneal microcirculation that are not reflected in the sublingual vasculature. Relationships are present between peritoneal small solute transport parameters and the thickness of the erythrocyte-permeable region of the endothelial surface layer, supporting a role for the endothelial glycocalyx in peritoneal transport. Because of their localization, the relevance of both syndecan-1 and HS10E4 for luminal endothelial glycocalyx seems to be limited. The expression of syndecan-1 in the peritoneal microvasculature changes in chronic kidney disease and after long-term exposure to glucose-based dialysis solutions and its role in peritoneal neoangiogenesis and fibrosis should be addressed in future studies. The expression of different glycocalyx constituents, like glypicans and other glycocalyx constituents, such as other glycosaminoglycans or additional heparan sulfate epitopes, may offer more insight into the biochemical composition of the peritoneal endothelial glycocalyx during peritoneal dialysis.

Chronic kidney disease itself induces peritoneal alterations, and these are partly augmented by exposure to dialysis solutions with high concentrations of glucose. The use of a biocompatible dialysis solution is likely beneficial with regard to the development of fibrosis, despite identical glucose concentrations compared to the conventional one. Specific endothelial markers and new methodologies, such as stereological methods that allow for unbiased and accurate morphological data, are currently available and should be used for quantitative histology.
General discussion

The endothelial glycocalyx in chronic kidney failure

The aim of this thesis was to gain insight in the alterations of the endothelial glycocalyx that occur in chronic kidney failure. Damage to the endothelial glycocalyx results in increased vascular vulnerability\(^1\) and therefore, this layer is of major importance in the setting of high vascular risk conditions such as chronic kidney failure. Non-invasive assessment of the endothelial surface layer in humans is currently possible by using Side-stream darkfield imaging of the sublingual microcirculation. In normal conditions, the presence of the glycocalyx limits the access of erythrocytes towards the endothelial cells.\(^2\) Based on experimental studies, the concept was formulated that alterations of the glycocalyx are associated with perturbation of its erythrocyte-excluding properties\(^3\), reflected by the variation in the RBC column width, from which the perfused boundary region (PBR) is calculated. This parameter reflects the part of the endothelial surface layer that is permeable to red blood cells and therefore, it is not a measure of the anatomic thickness of this structure. The ability to restrict red blood cells depends on the complex organization and interactions between glycocalyx components. Importantly a normal thickness of the perfused boundary region does not exclude changes in the endothelial glycocalyx structure or permeability to various solutes. This was demonstrated by Henry \textit{et al} who showed that treatment of hamster cremaster muscle with hyaluronidase resulted in increased permeability for various macromolecules but did not affect the access of the red blood cells into the glycocalyx.\(^4\) Both dialysis patients and renal transplant recipients have a thicker perfused boundary region in the sublingual microcirculation, suggesting that changes in the erythrocyte excluding properties of the glycocalyx occur in these patients. In a given condition, the changes in the endothelial surface layer reflect the combination of damage to the endothelial glycocalyx and the adaptive response of the endothelium. In the setting of chronic kidney failure, the oxidative stress, proinflammatory mediators and overhydration may all induce damage to the glycocalyx. The endothelial response may translate into increased expression of certain glycocalyx constituents. This is probably the case for hyaluronan (HA) in chronic kidney failure. Chronic kidney disease stage 4 and 5 is associated with increased plasma concentrations of hyaluronan and adhesion molecules, and relationships are present between these parameters.\(^5\) It is unknown if an increased plasma HA reflects a higher hyaluronan content of the microvascular glycocalyx or shedding from this structure into circulation. The endothelial synthesis of HA is triggered by pro-inflammatory cytokines which also increase expression of various adhesion molecules. High molecular weight hyaluronan is synthesized and will interact with CD44 present on both endothelium and immune cells, contributing to inflammation. The precursor availability is also of importance for HA synthesis, especially in hyperglycemic conditions.\(^6\) Renal failure is also associated with increased plasma concentrations of proinflammatory mediators and adhesion molecules, which makes it likely that in this condition hyaluronan synthesis is upregulated.
Hyaluronan is very hydrophilic and attracts water.\textsuperscript{7,8} Because of its length of several microns and its ability to bind water, hyaluronan is thought to have an essential contribution to the glycocalyx volume.\textsuperscript{9} Goa \textit{et al} investigated the composition of the endothelial glycocalyx and its relation to its thickness and diffusion of small solutes, in rat mesentery.\textsuperscript{10} The authors used techniques of macromolecule exclusion to measure the glycocalyx thickness, and the diffusion of the fluorescent dye FITC to assess the permeability of the glycocalyx to small solutes. It appeared that various GAGs are unevenly distributed throughout the endothelial glycocalyx. Hyaluronan and chondroitin sulfate (CS) are distributed towards the endothelial cell membrane whereas heparan sulfate is mainly present in the most luminal part of the glycocalyx. The measurements of diffusion coefficients of FITC indicated the presence of a more compact, denser sublayer adjacent to the endothelial cell membrane, in which the diffusion of small solutes is hindered compared to the more luminal part of the glycocalyx where loss of distal GAGs into the circulation occur. HA seems to contribute to the formation of this denser sublayer near the endothelial cell surface. Treatment with hyaluronidase decreased the thickness of the barrier to dextran 70 and increased the diffusion coefficient of small solutes in both the luminal part of the glycocalyx and in the more compact part adjacent to the cell membrane. The contribution of each GAG to the permeability of the endothelial glycocalyx varies. Heparan sulfate seems to provide the structural support of the upper part of the glycocalyx, whereas CS and HA contribute significantly to glycocalyx permeability to solutes.\textsuperscript{10}

It follows from the above that renal failure probably leads to an increase of the hyaluronan content of the microvascular glycocalyx. This would contribute to the formation of the denser sublayer on the microvascular luminal surface, which hinders the diffusion of small solutes. This is of interest especially in the setting of peritoneal dialysis where small solutes diffuse from the circulation into the interstitial space and peritoneal cavity.

\textbf{The endothelial surface layer in the peritoneal microcirculation}

The endothelial glycocalyx in peritoneal dialysis may be of importance for peritoneal transport, but also for the development of peritoneal membrane alterations, such as fibrosis and angiogenesis. The current knowledge on the state of the peritoneal endothelial glycocalyx is limited and needs to be expanded. In contrast to the situation in the sublingual vasculature, the endothelial glycocalyx in the peritoneal microvasculature is exposed not only to the uremic milieu present at the luminal side, but also to the high glucose concentration present in the peritoneal cavity. Whereas physiologic glucose concentrations may provide a substrate for glycocalyx synthesis, extremely high glucose concentrations are associated with generation of reactive oxygen species, which would add to the uremia-associated harmful stimuli and may result in additional glycocalyx damage.\textsuperscript{11} It is possible that the increased HA content in the endothelial glycocalyx
induced by chronic kidney failure is overruled by the harmful effect of high glucose concentrations to which the peritoneal vasculature is exposed. In contrast to the thicker PBR measured in the sublingual vasculature of PD patients, the PBR in the peritoneal microcirculation of rats with kidney failure exposed to dialysis solutions was not different from the control group. Our results suggest that specific changes occur in the peritoneal endothelial glycocalyx. However, the peritoneal measurements were done in rats and therefore the difference in species as cause of this dissimilarity, cannot be ruled out. Importantly, as mentioned previously, the unaltered thickness of the erythrocyte-permeable region of the endothelial surface layer does not exclude the presence of glycocalyx alterations in the peritoneal microcirculation. The experiments that we report here were aimed to gain insight into the biochemical composition of the glycocalyx, and revealed that syndecan-1 and HS 10E4 are probably not major constituents of the luminal endothelial glycocalyx. Syndecan-1 may be important with regard to the development of peritoneal alterations such as fibrosis or neangiogenesis during PD, and future studies should address these aspects. Heparan sulfate is the main glycosaminoglycan within the endothelial glycocalyx. Its structure is complex and highly variable, and antibodies directed various epitopes are currently available. The HS 10E4 epitope occurs in native heparan sulfate chains, is nitric oxide-sensitive and is partly inaccessible in the HS chains attached to glypican-1 proteoglycan. N-acetyl groups are essential for 10E4 binding. In our study HS 10E4 had predominantly basolateral distribution. Antibodies against different HS epitopes should be used in order to assess the HS alterations in PD.

Peritoneal alterations associated with peritoneal dialysis
Another aim of this thesis was to do an extensive analysis of the peritoneal alterations induced by chronic kidney failure and long term-exposure to dialysis solutions with high glucose-concentrations. In addition, we specifically addressed the changes induced by a conventional and a biocompatible dialysis solution. Some of our findings deserve special attention and raise additional questions. An important limitation to long-term treatment with PD is the development of structural and functional membrane alterations, which may lead to technique failure. The 'so-called' biocompatible solutions were developed in order to reduce the peritoneal alterations induced by treatment with peritoneal dialysis. Numerous experimental studies have shown better morphological parameters associated with the biocompatible solution. Contrary to expectations, the biocompatible solutions were associated with no obvious advantage when the peritoneal transport characteristics were investigated in patients, making the benefit of biocompatible solutions on outcomes uncertain. Here, we combined the functional, morphologic and ultrastructural analysis of the peritoneal alterations to address this question.
In our study, both the conventional and the biocompatible solutions induced in general similar peritoneal alterations: neoangiogenesis, lymphangiogenesis, mesothelial cell alterations, vascular alterations. However, the extensive analysis of peritoneal fibrosis showed that biocompatible solutions may have a beneficial effect with regard to the development of fibrosis. Our findings may be of interest also with regard to the development of encapsulating peritoneal sclerosis (EPS). EPS is a rare but very severe complication of treatment with peritoneal dialysis, and is characterised by loss of mesothelial cells and progressive fibrotic thickening of the peritoneum. It has been argued that the use of poorly biocompatible acidic glucose-based dialysis solutions may be of importance for the development of this complication. Studies evaluating the incidence of EPS and factors related to EPS occurrence are currently being performed in PD patients using dialysis solutions with a low content of glucose degradation products. This is in line with observations from our center and other centers in the Netherlands that experienced a decrease in EPS incidence in the last years, as orally reported by the EPS registry. This coincided with the increased availability and use of biocompatible solutions.

We also showed that the biocompatible and the conventional solutions were associated with a different mesothelial cell phenotype. To our knowledge this is the first report to address this point. The mesothelium represents the cellular source of effluent cancer antigen 125 (CA125). CA125 is a large glycoprotein mainly located at the cell membrane and, regardless of the glucose concentration used, it follows a linear course in the effluent during a PD exchange. Several studies have suggested that CA125 may be a good marker for mesothelial cell mass, but this hypothesis has been questioned by others. To date, a combination of biochemical determinations of CA125 with morphometric analysis has not been done. Therefore it has not been established with certainty whether the effluent CA125 is a marker for mesothelial cell viability or damage. Effluent CA125 increases in patients after switch to a biocompatible solution, suggesting a different effect of the relatively new solutions on the mesothelium. Our findings are consistent with these data in patients. Rats treated with a biocompatible solution have an enlarged cell membrane area compared to the conventional group. Further research should investigate whether this could be the source for higher effluent levels of CA125, as found in patients. Unfortunately, we have not been able to analyze the level of CA125 in our rats, since the antibody against CA125 is generated in mice and has cross-reactivity with rats. Studies on mesothelium as source for CA125 in combination with morphometric analysis are required to solve the issue of mesothelial cell turn-over or mesothelial cell mass.

Overall, it can be concluded that chronic renal failure leads to alterations in the systemic glycocalyx, that are partially adaptive. Peritoneal dialysis leads to damage of the peritoneal endothelial glycocalyx, which may be caused by the high glucose content of the dialysis solutions. Biocompatible solutions are useful to limit the formation of peritoneal fibrosis.
References


Nederlandse samenvatting

De binnenkant van alle bloedvaten is bekleed met een laag van suikerstructuren, de endotheliale glycocalyx genaamd. Deze laag bestaat uit proteoglycanen en glycosaminoglycanen, en deze beschermt de vaatwand tegen de schadelijke invloeden vanuit de bloedstroom. De glycocalyx kan onder verschillende omstandigheden worden beschadigd, waardoor de kwetsbaarheid van het endotheel toeneemt. In dit proefschrift wordt ons onderzoek naar de endotheliale glycocalyx bij chronische nierinsufficiëntie beschreven. Hierbij ligt de focus op peritoneale dialyse en het belang van de endotheliale glycocalyx in de peritoneale bloedvaten.

Hoofdstuk 1 geeft een inleiding over de structuur en functies van de endotheliale glycocalyx en hoe deze bij patiënten gemeten kan worden. Daarna volgt een overzicht van chronische nierinsufficiëntie en peritoneale dialyse. Tevens beschrijven wij de functie van het peritoneum wat betreft het transport van water en deeltjes en de veranderingen van het peritoneale membraan tijdens lang-durige PD.

Deel I is gericht op het meten van de endotheliale glycocalyx in dialyse patiënten, niertransplantatie patiënten en gezonde vrijwilligers. Patiënten met chronische nierinsufficiëntie hebben een verhoogd risico op het ontwikkelen van hart- en vaatziekten. De niertransplantatie vermindert de uremische complicaties en verbetert de overleving van deze groep patiënten. In hoofdstukken 2 en 3 gebruiken wij een niet-invasieve beeldvormingstechniek, Sidestream Darkfield imaging, om de perfused boundary region (PBR) te meten in de bloedvaten onder de tong. De PBR is de dikte van het luminale deel van de glycocalyx dat permeabel is voor erytrocyten. Bij dialyse patiënten hebben wij een dikkere PBR gevonden (er treedt een verandering op in de erytrocyten-excluderende eigenschap van de glycocalyx), samen met hogere plasma concentraties van glycocalyx producten hyaluronzuur, syndecan-1 en toegenomen hyaluronidase activiteit. Dit suggereert dat bij dialyse patiënten veranderingen in de endotheliale glycocalyx optreden. Bij getransplanteerde patiënten was alleen de PBR dikker, en gerelateerd aan de nierfunctie. In hoofdstuk 4 bespreken wij het nut van plasma concentraties van hyaluronan en hyaluronidase als markers voor de kwaliteit van de endotheliale glycocalyx bij nierinsufficiëntie. Hyaluronzuur is een belangrijk bestanddeel van de extracellulaire matrix en de endotheliale glycocalyx. Deze is heel belangrijk voor de beschermende rol van deze laag in het vaatstelsel, en tevens een aantrekkelijk molecuul als marker voor de endotheliale glycocalyx. Het merendeel van het hyaluronzuur in het bloed bestaat uit een hoog moleculair gewicht hyaluronan (boven 1000 kDa), maar een laag moleculair gewicht hyaluronan is ook te vinden en heeft pro-inflammatoire en pro-angiogene eigenschappen. Naast de lever, is ook de nier belangrijk voor de verwijdering van hyaluronzuur vanuit de bloedstroom. Hyaluronidase-2 kan aanwezig zijn op de apicale celmembraan en kan daarom belangrijk zijn voor de endotheliale glycocalyx.
Patiënten met nierinsufficiëntie hebben hogere hyaluronzuur concentraties in het bloed maar zonder aanvullende bepalingen moet dit voorzichtig geïnterpreteerd worden.

**Deel II** van dit proefschrift is gericht op de endotheliale glycocalyx tijdens peritoneale dialyse. In hoofdstuk 5 beschrijf ik de relaties tussen de sublinguale glycocalyx metingen en het transport van deeltjes en water tijdens peritoneale dialyse in patiënten. In het algemeen was er geen relatie aanwezig tussen de parameters. Niettemin, bij patiënten met niet-snel peritoneaal transport bleek dat een dikkere PBR was geassocieerd met een trager transport van kleine deeltjes. In hoofdstuk 6 tonen wij aan dat er een tegenovergestelde relatie is tussen de peritoneale endotheliale glycocalyx metingen en peritoneaal transport. Om de glycocalyx in de peritoneale bloedvaten te bestuderen, gebruiken wij een rattenmodel met chronische nierinsufficiëntie en langdurige blootstelling aan dialyse vloeistoffen met hoge glucose concentraties. In tegenstelling tot het sublinguale vaatstelsel, wordt het peritoneum tijdens peritoneale dialyse blootgesteld aan heel hoge glucose concentraties en hierdoor kunnen verschillende veranderingen in de peritoneale endotheliale glycocalyx optreden. De dikte van dit voor rode bloedcellen toegankelijke deel van de glycocalyx was hetzelfde in ratten met chronische nierinsufficiëntie, ratten met chronische nierinsufficiëntie blootgesteld aan dialyse vloeistoffen en in ratten met normale nierfunctie. Om inzicht te krijgen in de veranderingen van de peritoneale endotheliale glycocalyx hebben we kleuringen voor syndecan-1 proteoglycan en het 10E4 epitoot van heparansulfat glycosaminoglycaan toegepast. Syndecan-1 was aanwezig met name in de interendotheliale verbindingen en niet op de apicale membraan van het endotheel. De expressie van syndecan-1 neemt af bij chronische nierinsufficiëntie maar wordt na langdurige blootstelling aan dialyse vloeistoffen gelijk aan die bij ratten met normale nierfunctie. Ook, het heparansulfat 10E4 epitoot bevond zich in die condities vooral aan de abluminale zijde van het endotheel.

In hetzelfde rattenmodel de veranderingen beschreven die optreden in het peritoneale membraan bij chronische nierinsufficiëntie en na langdurige blootstelling aan dialyse vloeistoffen. Tevens hebben wij de effecten van een conventionele met die van een biocompatible (minder afwijkend van de samenstelling van plasma) dialyse vloeistof vergeleken. Chronische nierinsufficiëntie induceert veranderingen in het mesotheel, fibrose, het verdikken en duplicatie van de basale lamina in de peritoneale bloedvaten, lymphangiogenese en neoangiogenese. Deze veranderingen nemen deels toe door de blootstelling aan dialyse vloeistoffen met hoge glucose concentratie. In het algemeen hadden de twee dialyse vloeistoffen dezelfde schadelijke effecten op het peritoneum. Bovendien waren de transport karakteristieken vergelijkbaar. Niettemin lijkt de biocompatible vloeistof geassocieerd te zijn met minder fibrose dan de conventionele vloeistof. Tevens was in de twee groepen een verschillend mesotheelcel fenotype te zien.
Conclusies

De endotheliale glycocalyx is heel belangrijk voor de bescherming van de vaatwand tegen schadelijke prikkels vanuit de bloedstroom. Daardoor is de glycocalyx van belang in condities met endotheliale dysfunctie en een verhoogd risico op het ontwikkelen van cardiovasculaire aandoeningen, zoals chronische nierinsufficiëntie. Bij dialyse patiënten treden veranderingen in de systemische endotheliale glycocalyx op, onafhankelijk van het soort dialyse. Een geslaagde niertransplantatie lijkt een positief effect te hebben op de endotheliale glycocalyx. Echter, dit effect is afhankelijk van de functie van de getransplanteerde nier. Nader onderzoek moet worden gedaan naar de effecten van de immunsuppressiva op de endotheliale glycocalyx.

Een kritische evaluatie moet plaatsvinden van de veranderingen in de plasma concentraties van glycocalyx bestanddelen om hun waarde als markers voor de endotheliale glycocalyx te bepalen, rekening houdend met de rol van de nier bij hun verwijdering vanuit het bloed.

De blootstelling van de peritoneale bloedvaten aan dialyse vloeistoffen met hoge glucose concentratie leidt tot specifieke veranderingen die niet in de sublinguale bloedvaten te zien zijn. Er zijn relaties aanwezig tussen het transport van kleine deeltjes en de dikte van het deel van de endotheliale glycocalyx dat toegankelijk is voor erytrocyten. Dit suggereert dat de endotheliale glycocalyx belangrijk kan zijn voor peritoneaal transport. Nader onderzoek moet worden gedaan naar de veranderingen die in de endotheliale glycocalyx optreden in relatie tot de veranderingen in de peritoneale membraan. Syndecan-1 en het 10E4 epitoot van heparan sulfaat lijken niet essentieel te zijn voor de samenstelling van de glycocalyx aan de luminale kant van het endotheel. Om inzicht te krijgen in de biochemische structuur van de endotheliale glycocalyx, moeten andere bestanddelen zoals glypicanen, andere glycosaminoglycanen en heparan sulfaat epitopen bestudeerd worden.

Chronische nierinsufficiëntie leidt tot veranderingen in de peritoneale membraan. Deze nemen deels toe na de blootstelling aan dialyse vloeistoffen met hoge glucose concentraties. De biocompatible vloeistof lijkt een positief effect te hebben op de ontwikkeling van peritoneale fibrose.

Specifieke endotheliale markers en nieuwe methodologie zoals stereologische methoden waar mee onbevooroordeelde en accurate morfologische data verkregen kunnen worden, zijn momenteel beschikbaar en moeten gebruikt worden voor kwantitatieve histologie.
Appendices

List of abbreviations
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List of abbreviations

AGE Advanced glycation end-products
ALAT Alanine aminotransferase
APP Aminopeptidase P
BMI Body mass index
BP Blood pressure
BV Blood vessel
BVPD Blood vessel profile density
CA125 Cancer antigen 125
CRP C-reactive protein
CS Chondroitin sulfate
CKD Chronic kidney disease
CKDD Chronic kidney disease exposed to Dianeal
CKDP Chronic kidney disease exposed to Physioneal
CVD Cardiovascular disease
DAPI 4’,6-diamidino-2-phenylindole
diabetes mellitus
D/Perf Perfused diameter
D/P ratio Cr Dialysate-to-Plasma ratio of creatinine
EBVD Estimated blood vessel density
EDRD End-stage renal disease
EG Endothelial glycocalyx
eGFR Estimated glomerular filtration rate
ELAR Estimated lymphatic absorption rate
ELISA Enzyme-linked immunosorbent assay
eNOS Endothelial nitric oxide synthase
ESL Endothelial surface layer
FWT Free water transport
GAG Glycosaminoglycans
HA Hyaluronic acid
HbA1c Glycated hemoglobin
HD Hemodialysis
HDL High-density lipoprotein
HI Healthy individuals
Hyal Hyaluronidase
HMWHHA High molecular weight hyaluronan
HS Heparan sulfate
HSPGs Heparan sulfate proteoglycan
IL-6 Interleukin-6
LDL Low-density lipoprotein
LMWHHA Low molecular weight HA
LVPD Lymph vessel profile density
MC Mesothelial cells
MDA Malondialdehyde
MTAC Mass transfer area coefficient
MW Molecular weight
NKF Normal kidney function
NUF Net ultrafiltration
NUFR Net ultrafiltration rate
PBR Perfused boundary region
### Appendices

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<tr>
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<td>Peritoneal dialysis</td>
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<td>Proteoglycans</td>
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<tr>
<td>PSR</td>
<td>Picro-sirius red</td>
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<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>RBCW</td>
<td>Red blood cell column width</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SDC-1</td>
<td>Syndecan-1</td>
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<td>Sidestream Darkfield</td>
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<td>Standard peritoneal permeability analysis</td>
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<tr>
<td>SPARa</td>
<td>Standard peritoneal permeability analysis adapted for rats</td>
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<tr>
<td>SURS</td>
<td>Systematic uniform random sampling</td>
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<td>TCUFR</td>
<td>Transcapillary ultrafiltration rate</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor alfa</td>
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<td>Vascular basement membrane</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>Valid microvascular density</td>
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<td>UFF</td>
<td>Ultrafiltration failure</td>
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**Name**  
Carmen Anca Vlahu

**Start PhD Program**  
January 2010

**Title Thesis**  
The endothelial surface layer: a new target of research in kidney failure and peritoneal dialysis

**Promotor**  
Prof. Dr. R.T. Krediet

**Co-promotor**  
Dr. D.G. Struijk

**Defence of the thesis**  
May 17th 2016

### General courses

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- *Damage of endothelial glycocalyx in dialysis patients.* 31st Annual Dialysis Conference 2011 0.5
- *The endothelial glycocalyx in end-stage renal disease.* PLAN dag 2010 0.5
- *A damaged glycocalyx in peritoneal dialysis patients.* 30th Annual Dialysis Conference 2010 0.5

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- Nephrology Dialysis Transplantation
- Atherosclerosis
- Peritoneal Dialysis International
- International Urology and Nephrology
### Awards and Prizes

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<td>The endothelial glycocalyx and Na(^+) and fluid overload in hemodialysis patients. 50th ERA-EDTA Congress</td>
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Appendices
List of publications

Vlahu CA, Aten J, de Graaff M, van Veen H, Everts V, de Waart DR, Struijk DG, Krediet RT.

Vlahu CA, Krediet RT.
Can plasma hyaluronan and hyaluronidase be used as markers of the endothelial glyco-

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Appendices