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

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Damage of the Endothelial Glycocalyx in Dialysis Patients

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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 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.
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.1-4 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.5 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.5-9 Perturbation of glycocalyx occurs after provocation with inflammatory or atherogenic stimuli (such as ischemia reperfusion,10 infusion of oxidized LDL,9,11 administration of TNF-α12 or endotoxin,13 and during hyperglycemia14) and after stimulation with thrombin,15 atrial natriuretic peptide,16 or abnormal blood shear stress.17,18 Consequences of glycocalyx perturbation include a wide range of vascular abnormalities in experimental models, including increased vascular permeability followed by generation of tissue edema,19 increased rolling and adhesion of leucocytes,6 and increased platelet adhesion.9 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 noninvasively 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.14 Subsequently, a significant reduction in glycocalyx volume was found in patients with type 1 diabetes.20 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>35%</td>
<td></td>
<td></td>
</tr>
</tbody>
</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

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**Table 2. Serum levels of glycocalyx constituents, hyaluronidase activity, and sE-selectin in the study groups**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Controls</th>
<th>Dialysis Patients</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronan (ng/ml)</td>
<td>16.8 (6.4-29.5)</td>
<td>35.9 (14.5-70.0)</td>
<td>0.02</td>
</tr>
<tr>
<td>Hyaluronidase activity (U/ml)</td>
<td>24.1 (20.9-26.9)</td>
<td>28.2 (23.9-36.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Syndecan-1 (ng/ml)</td>
<td>27.5 (18.9-33.7)</td>
<td>111.0 (79.8-154.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>sE-selectin (ng/ml)</td>
<td>20.9 (14.3-30.6)</td>
<td>47.6 (27.1-67.6)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Results are presented as median (interquartile range).

**Figure 3. Relationship between serum levels of HA and total time on RRT in dialysis patients.** A positive correlation was present between serum levels of HA and time on RRT ($r^2=0.32$, $p=0.0002$).
Endothelial Glycocalyx in ESRD

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>Imaging parameters</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>Biochemical parameters</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.

[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 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>28.3 (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>45.6 (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 in vitro by inflammatory factors and activated in vivo 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),\(^{54-56}\) 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 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.
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
test depending on the distribution of the data. Only correlations with a $p$ value < 0.01 were considered significant.

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**Disclosures**

None.
References


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.