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The peritoneal membrane as a window for microvascular pathophysiology in chronic kidney disease.

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Not everything that can be counted, counts, and not everything that counts can be counted.

Albert Einstein
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<td>α-SMA</td>
<td>alpha-Smooth Muscle Actin</td>
</tr>
<tr>
<td>AII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-Converting Enzyme</td>
</tr>
<tr>
<td>ACEI</td>
<td>Angiotensin-Converting Enzyme Inhibitors</td>
</tr>
<tr>
<td>ADMA</td>
<td>Asymmetric Dimethylarginine</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced Glycation End products</td>
</tr>
<tr>
<td>AOPP</td>
<td>Advanced Oxidation Protein Products</td>
</tr>
<tr>
<td>APD</td>
<td>Automated Peritoneal Dialysis</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin II Receptor Blockers</td>
</tr>
<tr>
<td>CAPD</td>
<td>Continuous Ambulatory Peritoneal Dialysis</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
</tr>
<tr>
<td>CV</td>
<td>Cardiovascular</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>DDAH</td>
<td>Dimethylarginine Dimethylhydrolase</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle’s Balanced Salt Solution</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>eGFR</td>
<td>estimated Glomerular Filtration Rate</td>
</tr>
<tr>
<td>EMPs</td>
<td>Endothelial Microparticles</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-Mesenchymal Transition</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ESRD</td>
<td>End Stage Renal Disease</td>
</tr>
<tr>
<td>EUTox</td>
<td>European Uraemic Toxin work group</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-Methionine-Leucine-Phenylalanine</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GDPs</td>
<td>Glucose Degradation Products</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
</tr>
<tr>
<td>HD</td>
<td>Haemodialysis</td>
</tr>
<tr>
<td>HPMCs</td>
<td>Human Peritoneal Mesothelial Cells</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole Acetic Acid</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IS</td>
<td>Indoxylsulfate</td>
</tr>
<tr>
<td>KDIGO</td>
<td>Kidney Disease: Improving Global Outcomes</td>
</tr>
<tr>
<td>KDOQI</td>
<td>Kidney Disease Outcomes Quality Initiative</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus Amebocyte Lysate</td>
</tr>
<tr>
<td>LMWPs</td>
<td>Low-Molecular Weight Proteins</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1</td>
</tr>
<tr>
<td>MDRD</td>
<td>Modification of Diet in Renal Disease</td>
</tr>
<tr>
<td>MM</td>
<td>Middle Molecules</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetyl-Cysteine</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor - κB</td>
</tr>
<tr>
<td>NKF</td>
<td>National Kidney Foundation</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>pCS</td>
<td>p-Cresylsulfate</td>
</tr>
<tr>
<td>pCG</td>
<td>p-Cresylglucuronide</td>
</tr>
<tr>
<td>PD</td>
<td>Peritoneal Dialysis</td>
</tr>
<tr>
<td>PDF</td>
<td>Peritoneal Dialysate Fluid</td>
</tr>
<tr>
<td>PET</td>
<td>Peritoneal Equilibration Test</td>
</tr>
<tr>
<td>PM</td>
<td>Peritoneal Membrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PMNL</td>
<td>Polymorphonuclear Leukocytes</td>
</tr>
<tr>
<td>PP</td>
<td>Parietal Peritoneum</td>
</tr>
<tr>
<td>PRMT</td>
<td>Protein Arginine Methyltransferase</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-Angiotensin-Aldosterone System</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal Replacement Therapy</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDMA</td>
<td>Symmetric Dimethylarginine</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>sTF</td>
<td>soluble Tissue Factor</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TonEBP</td>
<td>Tonicity-responsive Enhancer Binding Protein</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>UF</td>
<td>Ultrafiltration</td>
</tr>
<tr>
<td>UFF</td>
<td>Ultrafiltration Failure</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VP</td>
<td>Visceral Peritoneum</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1. **General Aspects**
1.1. Background

The kidneys are located in the retroperitoneal space, usually one on each side of the spinal column. They normally are bean shaped, and have the size of a fist.

The kidneys have several important functions in the homeostasis of the body. Most importantly, they regulate the water balance. The kidneys also purify the blood from water soluble metabolites and waste products, by eliminating them in the urine. They also have a hormonal function. The secretion of erythropoietin stimulates the bone marrow to make red blood cells preventing anaemia, and renin regulates blood pressure via the renin angiotensin aldosterone axis. The production of 1.25 dihydroxyvitamin D, the active form of vitamin D, regulates the calcium and phosphorus homeostasis and the production of parathyroid hormone. Vitamin D essentially affects bone metabolism but has an impact on many other organ systems as well, e.g. immunomodulation.

A persistent decrement in kidney function is named “chronic kidney disease (CKD)”, as suggested by the Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines from the US National Kidney Foundation (NKF), to represent the entire spectrum of disease that occurs following the initiation of kidney damage. In 2002, the guidelines defined CKD as either presence of kidney damage, or an estimated glomerular filtration rate (eGFR) below 60 ml/min/1.73m² for at least 3 months\(^1\). Kidney damage is defined as the presence of pathologic abnormalities, or markers of damage, including abnormalities in blood or urine or at imaging studies. According to the KDOQI guidelines, CKD was initially classified into five stages based on the level of GFR and on proteinuria for the first two stages, and on GFR for the other 3. In 2009, the Kidney Disease: Improving Global Outcomes (KDIGO) agreed to retain the current definition of CKD based on GFR levels or presence of kidney damage, but they modified the CKD classification (table 1) by adding albuminuria stages in addition to GFR stages, by subdividing stage 3 in subgroups a and b depending on severity of renal failure, and by emphasising clinical diagnosis\(^2\). Patients with CKD 5 (eGFR < 15 ml/min/1.73m²) require renal replacement therapy (RRT) such as kidney transplantation or dialysis to survive when their eGFR further declines.

Of note, this classification also incorporates subjects whose decrease in eGFR is attributable to the natural and physiological decline of renal function with age, and as such the terminology “kidney disease” can be confusing.\(^3\)
Chapter 1: Introduction

The gold standard to determine Glomerular filtration rate (GFR) is inulin clearance. Other exogenous compounds used in a similar way to determine GFR are radiolabelled isotopes such as Iodine-125 iothalamate and Chromium-51 ethylenediamine tetraacetic acid. These determination methods are however costly, labour intensive, and cumbersome. In practice GFR is most often estimated (eGFR) by equations that take into account serum creatinine, age and gender. Body weight is additionally implemented in the Cockcroft-Gault equation and race in the Modification of Diet in Renal Disease (MDRD) study equation$^{2,4,5}$.

Table 1: Revised Chronic Kidney Disease (CKD) classification (KDIGO 2009)

<table>
<thead>
<tr>
<th>Stage</th>
<th>eGFR (ml/min/1.73 m$^2$)</th>
<th>Description</th>
<th>Albuminuria (ACR, mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt; 90</td>
<td>Kidney damage with normal or ↑ eGFR</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>60-89</td>
<td>Kidney damage with mild ↓ eGFR</td>
<td></td>
</tr>
<tr>
<td>3 a</td>
<td>45-59</td>
<td>Mild - Moderate ↓ eGFR</td>
<td>&lt; 30 (optimal and high-normal)</td>
</tr>
<tr>
<td>3 b</td>
<td>30-44</td>
<td>Moderate - Severe ↓ eGFR</td>
<td>30-299 (high)</td>
</tr>
<tr>
<td>4</td>
<td>15-29</td>
<td>Severe ↓ eGFR</td>
<td>&gt; 300 (very high)</td>
</tr>
<tr>
<td>5</td>
<td>&lt; 15, dialysis or transplantation</td>
<td>Kidney failure</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ACR: albumin-to-creatinine, eGFR: estimated glomerular filtration rate
1.1.2. Uraemic retention solutes

Uraemic retention solutes are those compounds that are retained in the body when kidney function declines. Under normal conditions, the glomerular filter clears water soluble molecules with a molecular weight (MW) up to 58 kDa. The decline in clearance of these solutes in renal failure results in a progressive accumulation of these molecules. Partial metabolic transformation and elimination by other than renal pathways (e.g. hepatic or gastrointestinal elimination) may compensate for this loss of renal clearance.

During the last few years, an immense progress has been made in the identification and quantification of uraemic solutes. Nevertheless, a substantial number of retention molecules still remains unidentified. In a number of publications by the European Uraemic Toxin work group (EUTox), the toxicity of a number of retention solutes was described with special attention for their potential for cardiovascular damage.

The uraemic molecules are preferentially classified into three major groups according to their physico-chemical characteristics which have a subsequent impact on their clearance during dialysis. The small water soluble compounds (MW<500 Da), the middle molecules (MM) (MW>500 Da) and the protein bound compounds. For all retained solutes, the concentration depends on the interplay between removal, which in most cases is decreased, and the degree of generation.

Only the solutes which are studied in this thesis will be discussed in more detail below.
1.1.2.1. Small water-soluble compounds

Prototypes for this group are urea and creatinine. In fact, creatinine belongs to the larger group of guanidines.

Guanidino compounds are structural metabolites of arginine. Increased guanidine levels have been determined in serum, urine, cerebrospinal fluid, and brains of uraemic patients. The neurotoxicity of several guanidines is extensively described. Guanidino compounds have also been shown to enhance baseline immune function and lipopolysaccharide (LPS)-stimulated intracellular TNF-α by normal monocytes. Their potential cardiovascular impact was, until recently, mainly attributed to asymmetric dimethylarginine (ADMA), which inhibits nitric oxide synthase (NOS). A holistic and standardized evaluation of ten guanidino compounds in different in vitro models showed that especially symmetric dimethylarginine (SDMA), the structural counterpart of ADMA formerly considered biologically inert, contributes to biological effects related to cardiovascular complications in uraemia. These effects may be involved in altering the prevalence of cardiovascular disease in CKD, as confirmed by clinical data pointing to an in vivo association of SDMA with IL-6 and TNF-α in CKD at different stages. Both ADMA and SDMA are only weakly correlated to eGFR.

In general, the water-soluble compounds are easily removed by any dialysis strategy. Although guanidine compounds are water-soluble, they have a different kinetic behavior from urea because of their large distribution volume which makes them more difficult to remove. A two-compartmental kinetic model demonstrated that prolonged dialysis and increased dialysis frequency are effective in significantly reducing the concentrations of the guanidino compounds compared to standard haemodialysis.

1.1.2.2. Middle Molecules

The prototype molecule for this group is β2-microglobulin. Middle molecules are presently considered as solutes in the large MW range (500-60,000 Da). This range incorporates many toxins, which were only identified after the formulation of the original middle molecule hypothesis, in which the upper MW limit was 2,000 Da. Most currently known uraemic retention solutes are low molecular weight peptides and proteins (LMWPs). Up till now, at least 40 middle molecules or groups of middle molecules have been identified. Many of these solutes have been linked with inflammation, endothelial cell damage, smooth muscle
cell proliferation and thrombocyte activation, all processes associated with enhanced cardiovascular (CV) damage. Apart from these effects associated with CV damage, middle molecules have a toxic impact on other cell and organ systems as well. However, these compounds will not be discussed further, since they are not part of this thesis.

1.1.2.3. Protein-bound compounds

Protein-bound uraemic retention solutes constitute a heterogeneous group of compounds which are retained in patients with kidney failure. Although most of these compounds are molecules with low MW, they behave like high MW substances as a result of their binding to albumin (MW 68 kDa) or another protein(s). This large group of uraemic solutes should not be considered as a homogenous entity, as retention pattern, and the removal during dialysis depend upon the degree and strength of protein binding, and the number and type of protein binding sites, which are different for all of them, so that each subgroup should be considered separately. Prototypes of this group are the phenols and indoles. As a substantial part of the generation of these products is regulated in the intestine, the option to decrease tissue concentrations by manipulating intestinal generation by dietary intervention or intestinal absorption has insufficiently been explored, although it is of major importance especially when considering phenolic and indolic compounds.

P-cresylsulfate and p-cresylglucuronide

Intestinal fermentation of the amino acid tyrosine generates 4-hydroxyphenylacetic acid which is decarboxylated to p–cresol or demethylated to phenol\(^{30}\) and very likely also phenylacetic acid as well as other phenols are generated.\(^{31;32}\) Over the last twenty years, it has generally been conceived that after its generation, p-cresol was absorbed as such by the intestine and then distributed over the body, since upon analysis the molecule was found in serum of subjects with normal and disturbed renal function.\(^{33}\) In reality, after its absorption and passage through the intestinal mucosa, p-cresol is metabolized by a cytosolic sulfotransferase to p–cresylsulfate, its main conjugate, as well as to p–cresylglucuronide by a glucuronosyltransferase. (figure 1) What might remain of p-cresol after transfer into the portal vein is further on conjugated in the liver.\(^{34;35}\) In addition, the lung mucosa also contains sulfotransferase and glucuronosyltransferase activity, conjugating the toxic phenolic compounds that might enter the body via inhalation.
The reason why p-cresol was considered for a long time as a major phenolic compound in the body is attributable to an artefact created during sample preparation. Until a few years ago, virtually all analytical methods used deproteinisation by acidification as a first step, causing hydrolysis of the p-cresol conjugates. Deproteinisation without acidification left p-cresylsulfate (and although this was not measured, probably also p-cresylglucuronide) intact, with virtually no detectable p-cresol. P-cresylsulfate concentration is increased in patients with renal failure and has a protein binding of ± 94%. In contrast, p-cresylglucuronide, the other p-cresol conjugate, shows virtually no substantial protein binding.

In contrast to the mother compound p-cresol which is a strong inhibitor of leukocyte response, p-cresylsulfate has a proinflammatory impact on monocytes and lymphocytes. P-cresylsulfate has also been linked to endothelial microparticle release, an indicator of endothelial and vascular damage. Clinical studies showed a relation between p-cresol (acting as a surrogate for p-cresylsulfate) and clinical outcomes. Recent studies confirmed that also p-cresylsulfate levels were predictive for mortality at different stages of CKD and for cardiovascular and all-cause mortality in haemodialysis patients. The relation between p-cresylsulfate and coronary artery disease in patients with no or only moderate degrees of CKD extends the association of this compound with cardiovascular outcomes beyond the scope of pronounced uraemia.

Figure 1: Organs and enzymatic pathways involved in the generation of p-cresyl conjugates
Indoxylsulfate

Indoxylsulfate is produced by the metabolism of dietary tryptophan. Briefly, tryptophan is metabolized to indole by intestinal bacteria. After intestinal absorption, indole is metabolized in the liver to indoxyl and then to indoxylsulfate.

As excretion of this compound depends mainly on proximal tubular secretion, indoxylsulfate levels, mainly as its albumin-bound form (± 90% bound), are markedly increased in patients with renal failure. Indoxylsulfate enhances drug toxicity by competition with acidic drugs at the protein binding sites. Indoxylsulfate inhibits the active tubular secretion of acidic drugs and anionic solutes. A host of in vitro and in vivo animal studies associate indoxylsulfate with inflammation, endothelial dysfunction, vascular smooth muscle cell proliferation, cardiac fibrosis and disturbances of bone metabolism. In addition, it has repeatedly been associated with loss of residual renal function, by itself a factor with strong impact on outcome.

Inhibition of intestinal absorption of indoxylsulfate by the absorbant AST-120 (Kremezin)® has been associated with a delay in the initiation of dialysis, a delay in the progression of renal failure as estimated by eGFR decline and, if applied before the start of dialysis, with better prognosis once dialysis was started. Although controlled, these studies have been performed in small populations and thus need confirmation. Recently, serum indoxylsulfate has also been associated with vascular disease and mortality in CKD patients.

Other strategies to remove protein-bound solutes are discussed more in dept in chapter 4.1.
1.1.3. **Cardiovascular disease and inflammation in CKD**

Cardiovascular disease (CVD) remains a major cause of morbidity and mortality in CKD patients, either treated by haemodialysis, peritoneal dialysis or renal transplantation, as well as in earlier stages of CKD not requiring renal replacement therapy.

Lindner *et al* were the first authors pointing out that dialysis patients are exposed to cardiovascular problems and suffer from accelerated and severe atherosclerosis\(^57\). Two decades later, Foley *et al* observed a far greater risk of CVD in haemodialysis (HD) patients compared to the general population\(^58\); similar problems were registered as well in the pre-dialytic stage (from CKD 3 on)\(^59\). Recently, it was shown that even in mild renal failure, from a GFR of 60 ml/min/1.73 m\(^2\) on or even earlier, the cardiovascular risk starts to increase\(^60,61\). This has led to the concept that renal disease is a model of accelerated vascular ageing where vascular changes can already be observed at young age.

![Figure 2: Schematic presentation of traditional and novel (or uraemia-specific) cardiovascular risk factors in chronic kidney disease. (From 62)](image)
Traditional risk factors for CVD like age, gender, smoking, diabetes, dyslipidemia and hypertension, which predict cardiovascular mortality in the general population, are present in CKD, but can only partially explain the high cardiovascular risk in this population. So-called novel risk factors for CVD, such as inflammation, endothelial dysfunction, oxidative stress, malnutrition, uraemia and vascular calcification are highly prevalent in CKD patients and seem to play a far more important role in vascular disease than in the general population\textsuperscript{62-68}. (figure 2) In particular, research has focused on factors specific to the uraemic condition leading to the identification of an increasing number of uraemic solutes with direct vasculotoxic effects.\textsuperscript{7}

Atherosclerosis is the most important underlying cause of CVD, both in the general as well as in the CKD population. It is a disease of the arterial intima characterized by the presence of plaques and occlusive lesions, in which the above mentioned risk factors contribute.\textsuperscript{69} Initially, atherosclerosis was previously considered to be a degenerative disease, but it is currently recognized as an inflammatory disorder.\textsuperscript{70} A key role in this process is played by leukocyte-endothelial interactions, causing vascular damage by products of inflammation such as free radicals. Atherogenesis, the process leading to atherosclerosis is accelerated from even minor decrements of renal function, long before renal replacement therapy is needed. Clinically, atherosclerosis causes ischaemic problems such as angina pectoris, myocardial infarction, stroke and peripheral vascular disease.

The other type of vascular lesion is arteriosclerosis, characterized by diffuse dilatation and hypertrophy of large arteries with loss of arterial elasticity.\textsuperscript{71} The main adverse effects of arterial stiffening are an increased systolic blood pressure with a normal or decreased diastolic blood pressure, left ventricular hypertrophy and diminished coronary perfusion.\textsuperscript{72} This arterial stiffening is a normal ageing process, but in CKD, arterial enlargement and increased stiffness are already observed in the early stages.\textsuperscript{73,74} It is predictive for CVD\textsuperscript{75,76}, but is also associated with inflammation and endothelial dysfunction.\textsuperscript{77,78}

In chronic renal failure, inflammation and CVD are linked to each other. CKD is characterized by a state of chronic micro-inflammation, as evidenced by increased levels of inflammatory markers, such as C-reactive protein (CRP), IL-6 and white blood cell count.\textsuperscript{79} This baseline inflammatory status is associated with oxidative stress, endothelial dysfunction, vascular calcification and wasting and thus with atherosclerosis.\textsuperscript{80} In contrast, the blunted
immune response upon activation results in an increased susceptibility for infection and a higher risk of malignancy.\textsuperscript{81,82}

This duality in immune response in CKD patients originates at different levels. Some uraemic retention solutes exert pro-inflammatory effects (e.g. guanides, advanced oxidation protein products (AOPP)), whereas others are anti-inflammatory (e.g. purines). In this way, uraemic retention solutes can contribute to both CVD and the propensity to infection of uraemic patients.\textsuperscript{21,47,83,84}
1.1.4. Renal Replacement Therapy (RRT) for CKD 5

Once CKD is diagnosed, patients need to be evaluated to determine the type of kidney disease, the stage of CKD, the complications related to the level of kidney function, the risk for further loss of kidney function and the risk to develop CVD. Based on this information, treatment can be started. Initially, drug therapy will be instituted, which will mainly focus on slowing down the progression of kidney failure and preventing and treating CVD and other complications of reduced kidney function. At more developed stages of CKD, the patient will be prepared to start renal replacement therapy, which will be considered when eGFR decreases to approximately 15 ml/min/1.73 m² (European Renal Best Practice). This renal replacement therapy can either be dialysis or renal transplantation.

1.1.4.1. Renal Transplantation

While transplantation at first sight is the preferred therapy for kidney failure, there is a shortage of donor kidneys, transplants may fail and the majority of end stage renal disease (ESRD) patients is not suitable for transplantation; as a consequence, the majority of the CKD stage 5 patients will be treated with either haemodialysis or peritoneal dialysis, while awaiting transplantation or because they are no valid candidates for a graft.

1.1.4.2. Haemodialysis

Haemodialysis (HD) is an extracorporeal technique. The blood is purified through an artificial kidney, which contains two chambers separated by a membrane. The blood flows in one compartment and dialysate in the other, in opposite directions. Waste products are cleared from the blood by diffusion induced by a concentration gradient across a semi-permeable membrane, and convective clearance and fluid removal are achieved by ultrafiltration induced by hydrostatic transmembrane pressure.

1.1.4.3. Peritoneal Dialysis

In this form of dialysis, the peritoneum, which surrounds the abdominal cavity and envelopes the intestine, serves as a semi-permeable membrane across which dialysis can occur. In peritoneal dialysis (PD), dialysis fluid is instilled into the peritoneal cavity via a permanent catheter, placed in the lower part of the abdomen. At regular intervals, the fluid is drained and replaced. (figure 3)
PD is based on the principles of diffusion and osmosis. Uraemic toxins and solutes move across the membrane, by diffusion, from the blood stream to the dialysis fluid, or vice versa, depending on the concentration gradient. Fluid removal from the body is induced by osmosis, due to the addition of an osmotic agent, usually glucose, to the dialysate. Other osmotic agents can also be used, such as icodextrin, a glucose polymer, or amino acids. The pores in the peritoneal membrane are large enough to allow the waste products to pass into the abdominal cavity, but small enough to only allow the passage of larger proteins to a limited extent.

There are two types of PD: continuous ambulatory peritoneal dialysis (CAPD) and automated peritoneal dialysis (APD). With CAPD, dialysate is instilled into and drained from the peritoneal cavity by gravity. No machine is needed. There is almost continuously dialysate fluid present in the peritoneal cavity and during the day, the fluid is changed on regular relatively short intervals, with a longer dwell time overnight. In APD, a cycler performs several exchanges overnight, whereas in the morning a dwell time starts that can last longer.
Continuous removal of waste products achieved using PD results in improved well-being of the patients, partly as a result of stabilization of the amount of waste products in the body. This in contrast with haemodialysis (HD), where waste products accumulate in-between two dialysis treatments. Patients on PD have increased flexibility compared to HD, since PD can be done by the patient himself at home. In addition, PD is less expensive for the society.

Currently, there are more than 190,000 patients on PD worldwide, representing approximately 10% of the total patient population receiving dialysis. It is expected that the number of PD patients will increase during the next decade, especially in developing countries.
1.2. **Peritoneal Membrane Biology**
In 1730, James Douglas made the first modern description of the peritoneum. He observed that it was smooth and flat, and lubricated by a fluid to preserve it from the inconveniences of continuous friction. As observed by Douglas, the main function of the peritoneum and other serous membranes was to reduce friction between moving organs.

For efficient peritoneal dialysis, the peritoneum must function as a dialyzing membrane, although this is not its primary purpose. The peritoneum is the most extensive serous membrane in the body; it covers the visceral organs (visceral peritoneum) and lines the abdominal cavity (parietal peritoneum). Due to the arrangement of peritoneal folds, its surface area is equivalent to that of the skin (2 m²). Specialized regions of the peritoneum include the omentum and the mesentery. Normally the peritoneum is very thin, 20-40 µm in the greater omentum and somewhat thicker in the parietal and visceral peritoneums.

The peritoneal membrane is composed of a monolayer of flat mesothelial cells that rest on a continuous basement membrane, and a submesothelial layer of connective tissue with variable thickness and structure, containing blood and lymphatic vessels, nerve fibres and different cell types (macrophages, fibroblasts, mast cells, leukocytes, adipocytes) immersed in an extracellular matrix. (Figure 4) Adipocytes can be seen in all peritoneal preparations, as they form fat deposits and constitute the majority of cells in the mesentery and great omentum. However, their role in the peritoneal pathophysiology remains unclear.
The mesothelium

The mesothelium consists of a continuous monolayer of mesothelial cells of mesenchymal origin, joined by various intercellular junctions. The luminal surface of mesothelial cells has numerous microvilli that markedly increase the functional mesothelial surface area, up to 40 m², for exchange between mesothelial cells and the peritoneal cavity. The number of microvilli expressed on each cell varies under different physiological and pathological conditions. Microvilli protect the delicate mesothelial surface from frictional injury by entrapping water and serous exudates, which act as lubricants for the cells. Mesothelial cells also possess cilia on their apical surface that are typically five times longer than adjacent microvilli. The quantity increases with increasing cell density, which suggests they play an essential role in mesothelial cell polarity and cell-cell adhesion. Although the mesothelium offers little resistance as a biological barrier, it contains abundant anionic sites and intercellular junctions that potentially allow it to act as a selective barrier to the passage of plasma molecules.

Furthermore, mesothelial cells provide the first line of defense in peritoneal protection during long-term or repeated exposure to unphysiologic PD solutions and against micro-organisms during infection. Mesothelial cells are active cells that can synthesize a plethora of pro-, anti-, and immunomodulatory mediators including growth factors, cytokines, hyaluronan, nitric oxide, components of the RAAS system and prostaglandins. These mediators are released in response to bacterial infection or chemical/surgical injury to initiate the reparative processes and restoring the homeostatis in the peritoneum. Repeated insult triggers chronic inflammatory responses that often result in the induction of peritoneal fibrosis. Mesothelial cells are able to secrete chemotactic peptides following activation with proinflammatory cytokines and live bacteria or active metabolites secreted by bacteria, and hence contribute to the intraperitoneal recruitment of leukocytes during peritoneal inflammation. Phagocytosis of a foreign agent by mesothelial cells induces cell activation and the release of proinflammatory cytokines and chemokines, that further amplify the inflammatory response and induce effective endothelial activation, essential for migration of leukocytes into the peritoneal cavity. It has been postulated that chronic irritation of mesothelial cells results in dedifferentiation, a process called epithelial-to-mesenchymal transition (EMT). (figure 5) During this process, mesothelial cells lose expression of several genes, such as cytokeratins and E-cadherin and gain others, such as alpha smooth muscle actin (α-SMA) and snail.
Trans-differentiated mesothelial cells are able to migrate from the superficial cell layer into the interstitial space, where they become active as myofibroblasts, which are probably responsible for the increased submesothelial thickness and fibrosis observed in long term PD patients.

**Figure 5: Epithelial-to-mesenchymal transition (EMT) (From {Aguilera, 2005 127 /id})**

Glucose, advanced glycation end products (AGEs), glucose degradation products (GDPs), low pH and inflammatory mediators (peritonitis) stimulate epithelial-mesenchymal transition (EMT) of mesothelial cells (MCs). MCs lose expression of several genes, such as E-cadherin and cytokeratins, and gain others, such as snail and smooth-muscle actin (αSMA). Trans-differentiated MCs are able to invade the peritoneal sub-mesothelium and produce a high quantity of extracellular matrix (ECM) and vascular endothelial growth factor (VEGF), a pro-angiogenic factor associated with peritoneal membrane failure. Intercellular adhesion molecule 1 (ICAM-1), interleukin 1 (IL-1), matrix metalloprotease (MMP), plasminogen activator inhibitor 1 (PAI-1), transforming growth factor β (TGF-β).
The basement membrane

The basement membrane, underlying the mesothelial cells, is a thin (±40 nm) laminar network composed of mainly type IV collagen, laminin, fibronectin and proteoglycans. It functions as a support for the mesothelium and as a selective cellular barrier, allowing macrophages and lymphocytes to pass through it, but preventing fibroblasts from the underlying connective tissue to contact the mesothelial cells.\textsuperscript{96}

The submesothelial layer

The submesothelial interstitial tissue is a complex network of collagen fibres (type I and III), elastic fibres and a gel containing macromolecules. The main component is hyaluronan, a non-sulfated glycosaminoglycan, which can bind enormous quantities of water and forms together with proteoglycans a gel-like substance that behaves like a filter. Thus, the interstitial matrix probably plays a major role in the transport of solutes and solvents. This extracellular matrix (ECM) provides mechanical strength to the tissue and acts as a framework for cell attachment and migration.\textsuperscript{91}

Under normal circumstances, fibroblasts produce the macromolecules that constitute the ECM (collagens, elastic fibres, GAGs), but when stimulated, they can, like mesothelial cells, generate chemotactic and proinflammatory factors, which contribute to the recruitment of leukocytes.\textsuperscript{97,98}

Resident macrophages, underneath the basement membrane and around the blood vessels, are activated by invading microorganisms or their secreted products and subsequently produce and/or secrete a broad variety of proinflammatory cytokines. In addition, interaction of macrophages with microorganisms also causes an increased secretion of chemoattractive agents, resulting in migration of leukocytes from the bloodstream to the site of inflammation.

Next to macrophages, mast cells further increase the peritoneal response to injury by releasing a large amount of inflammatory mediators and chemoattractive agents.\textsuperscript{99}

Other important structures are blood and lymph vessels. Peritoneal blood vessels, especially capillaries and post-capillary venules, take care of nutrient transport and waste removal from tissues and organs within the peritoneal cavity. Lymphatic vessels represent a drainage system, which helps to maintain the normal level of intraperitoneal fluid, by returning
excessive fluid and proteins to the systemic circulation. Furthermore, they can also participate in host defence by removing leukocytes carrying foreign bodies, from the peritoneal cavity.\textsuperscript{96}

\textit{The peritoneal microcirculation}

The large vessels supplying blood to the visceral peritoneum function primarily as conduits to supply blood to the visceral organs. The arterioles and capillaries are organised as a network (figure 6), with redundant capacity and bypass circuits, which can lead to a substantial recruitment of normally closed loops when needed, e.g during inflammation. This capillary recruitment, which involves already existing capillaries, should be clearly distinguished from neo-angiogenesis, where neoformation of capillaries takes place.

![Diagram of peritoneal microcirculation](image)

\textbf{Figure 6: Intravital microscopy of the rat peritoneal microcirculation. (Adapted from 100)}

Arterioles divide into a thin capillary network that forms postcapillary venules and venules.
Chapter 1: Introduction

The arterioles are the major sites of microvascular resistance and regulate flow to capillary beds. They are lined by endothelial cells resting on a basal lamina surrounded by a layer of smooth muscle cells. Terminal arterioles may participate in the exchange process as they have a discontinuous muscle layer. However, the relative contribution to overall peritoneal transport is minimal since the surface area and permeability of these vessels are much less than that of capillaries and postcapillary venules.

Capillaries are the principal sites for solute and fluid exchange. The wall of the capillary is composed of an endothelium and a basal lamina. Capillary size is approximately 5-8 µm, which is large enough to let red blood cells (average diameter 7.5 µm) through, usually one at a time and with some deformity.

Small venules that are located just distal to the capillaries are often termed postcapillary venules. They participate in fluid and solute exchange, are an important site for microvascular leukocyte adhesion, and may demonstrate dramatic changes in permeability during inflammatory conditions. Postcapillary venules are generally 10-40 µm in diameter and are composed of endothelial cells resting on a basal lamina surrounded by pericytes. Larger venules are enclosed by muscular media.

The peritoneal microcirculation plays a critical role in inflammatory responses associated with peritoneal dialysis. An important aspect of this response is the interaction of leukocytes with the vascular endothelium.

Intravital microscopic studies have demonstrated that the attachment and migration of leukocytes from the vascular space to the extravascular space (leukocyte recruitment) is localized primarily to postcapillary venules.101 (Figure 7)
Leukocyte recruitment occurs within postcapillary venules and is dependent on a cascade of events involving the selectins as the primary molecules that induce and support rolling. As the inflammatory process proceeds, the number of rolling leukocytes increases and the velocity of the rolling decreases. The adhesive interaction between the leukocytes and the endothelium is mediated by a complex, highly coordinated dynamic interplay between adhesion molecules expressed on the surface of both the leukocytes and the endothelium.

The selectin-family and their carbohydrate-containing ligands mediate leukocyte rolling. This brings the leukocyte into transient, but close contact with the endothelial cells. If the appropriate stimuli are present, the leukocyte prepares for firm adhesion and transendothelial migration by activation of a second family of adhesion molecules, the integrins, which interact with Ig-like molecules.
Molecular compounds of the peritoneal microcirculation

The capillary endothelium constitutes one of the barriers for water and small solute transport during PD. Transport rate across the peritoneal membrane depends on the intrinsic permeability to each solute and the effective peritoneal surface area (EPSA), reflecting the number of perfused capillaries within the peritoneum in contact with the peritoneal dialysis solution.\(^{102}\)

The permeability for water and solutes across the endothelium is best described by the three-pore model, which includes transcellular, ultrasmall pores (radius: 3 to 5 Å) called aquaporins (AQP), and which are exclusively permeable to water, small pores (radius: 40 to 50 Å) permeable to water and small solutes, and large pores (radius > 150 Å) permeable to macromolecules.\(^{103}\) The water channel AQP1 is the molecular counterpart of the ultrasmall pore, involved in the free water permeability explaining the sodium sieving during the first 30 min of a hypertonic dwell.\(^{102;104}\)

Histopathological studies have shown that long-term PD is associated with modifications of the peritoneal membrane (see chapter 1.3), including perivascular and submesothelial fibrosis, alterations of the mesothelium, and replication of capillary basement membrane.\(^{105;106}\) In particular, vascular proliferation and increased endothelial area within the peritoneal membrane have been documented in long-term PD patients.\(^{105;107}\) Thus, vascular proliferation and, possibly, vasodilatation of peritoneal vessels, might represent the structural basis for increased EPSA in long-term PD.

Nitric oxide (NO) plays a role in the regulation of vascular tone and permeability,\(^ {108}\) and interacts with angiogenic growth factors.\(^ {109}\) It might thus regulate EPSA and ultrafiltration (UF) during PD.\(^ {110}\)

NO is synthesized from L-arginine by three nitric oxide synthase (NOS) isoforms: the neuronal NOS (nNOS), the endothelial NOS (eNOS), and the inducible NOS (iNOS). In long-term PD, peritoneal NOS activity increased fivefold above levels observed in controls and uraemic subjects prior to the onset of PD.\(^ {107}\) Furthermore, the increased NOS activity correlated positively with PD duration and was solely attributable to the upregulation of eNOS – itself reflecting the increase in endothelial area.\(^ {107}\) Thus, the release of NO, secondary to NOS upregulation, might be a major regulator of UF in PD patients.
Vascular endothelial growth factor (VEGF) is a potent regulator of angiogenesis and vascular permeability.\textsuperscript{109} VEGF is expressed in the human peritoneal membrane and is located in the endothelium lining peritoneal capillaries. Its expression is clearly upregulated in long-term PD patients.\textsuperscript{107} VEGF has also been detected in the dialysate, where its abundance correlates with the permeability for small solutes and the loss of ultrafiltration.\textsuperscript{44} The synthesis of VEGF by cultured mesothelial and endothelial cells isolated from the peritoneum has been demonstrated.\textsuperscript{111} Thus, by analogy with other angiogenic diseases, upregulation of VEGF may trigger vascular proliferation in the peritoneal membrane in long-term PD. The putative stimuli involved in the regulation of VEGF in the peritoneal membrane include other growth factors, local ischaemia, or inflammatory cytokines, or even direct glucose mediated effects.\textsuperscript{109,112}

Figure 8: A model for the different molecular mechanisms involved in peritoneal membrane dysfunction in long term peritoneal dialysis. (Adapted from {Devuyst, 2002 416 /id})

Abbreviations: PD, peritoneal dialysis; AGEs, advanced glycation end products; GDPs, glucose degradation products; TGF-\(\beta\)\(_1\), transforming growth factor beta 1; VEGF, vascular endothelial growth factor; eNOS, endothelial nitric oxide; NO, nitric oxide; EPSA, effective peritoneal surface area; UF, ultrafiltration
Multiple interactions between NO, eNOS and VEGF occur within endothelial cells. Both NO and eNOS are required for VEGF-driven angiogenesis and vascular permeability. On the other hand, VEGF is known to upregulate eNOS and NO production. In turn, NO modulates and even suppresses the hypoxic induction of VEGF, which creates a negative feed-back between NO and VEGF induction.
1.3. **DETERIORATION OF THE PERITONEAL**
**MEMBRANE**
Long-term peritoneal dialysis (PD) is associated with functional and structural deterioration of the peritoneal membrane. Predominant causes for technique failure are ultrafiltration failure (UFF)\(^{115}\) and recurrent or severe peritonitis.\(^{116,117}\) Dysfunction of the peritoneum as a dialysis organ is thought to be the result of structural changes that develop in the peritoneal membrane with time on PD.

The most consistent changes in peritoneal tissues of PD patients are 1) an increase in the submesothelial thickness, which is associated with peritoneal fibrosis; 2) angiogenesis; and 3) epithelial-to-mesenchymal transition (loss of mesothelial cell layer) (EMT).\(^{106,117,118}\) Fibrosis and angiogenesis mostly occur together\(^ {119}\), and interventions that reduce angiogenesis mostly reduce fibrosis and vice versa.\(^ {120}\) Fibrosis and angiogenesis appear to be linked through upregulation of common initiating growth factors (TGF-β, VEGF), inflammatory cytokines, and the EMT process.\(^ {121}\) Factors that selectively inhibit these growth factors can protect the peritoneal membrane against deterioration.

Causes of these peritoneal changes are multifactorial. Nevertheless, many of these factors are modifiable, and attention should be paid to them in the clinical practice to maintain peritoneal membrane integrity.
1.3.1. PD-related factors affecting peritoneal membrane quality

1.3.1.1. Glucose & glucose degradation products

Low pH, lactate buffer, the presence of glucose in the dialysate fluid, and the generation of glucose degradation products (GDPs) and advanced glycation end products (AGEs) out of glucose as a result of heat sterilization of the PD fluid\textsuperscript{122,123}, have attracted most attention as culprits for the deterioration of the peritoneal membrane.

Peritoneal dialysis solutions using glucose as osmotic agent have been used for more than two decades as effective treatment for patients with end-stage renal disease. Although alternative osmotic agents such as amino acids and macromolecular solutions, including polypeptides and glucose polymers, are now available, glucose is still the most widely used osmotic agent in PD. It has been shown to be safe, effective, readily metabolized, and inexpensive. On the other hand, it was assumed for years that exposure of the peritoneal membrane to high glucose concentrations contributes to both structural and functional changes in the dialyzed peritoneal membrane. However, comparing heat sterilized and filtered sterilized dialysis solutions, containing the same glucose content and having the same low pH, pointed out that the latter two were of less importance than GDPs.\textsuperscript{124} Both \textit{in vitro}\textsuperscript{125-129} and \textit{in vivo}\textsuperscript{130,131} studies suggest a link between high concentrations of GDPs and AGEs in the dialysate, and induction of peritoneal fibrosis and angiogenesis through stimulation of TFG-β and VEGF production by mesothelial cells.\textsuperscript{132}

This led finally to the development of solutions low in GDPs and with a physiological pH. Although these solutions perform clearly better in terms of biocompatibility in an \textit{in vitro} setting\textsuperscript{135-139} and in animal models\textsuperscript{140-144}, their benefit in clinical conditions on preservation of peritoneal membrane integrity is not convincing so far\textsuperscript{145,146}. This might be due to substantial differences in GDP content between the different brands of “low GDP, normal pH” solutions. It has been demonstrated for example that Physioneal\textsuperscript{®} (Baxter, Illinois) contains nearly identical concentrations of 3,4-deoxyglucosone-3-ene, considered as being the most toxic GDP\textsuperscript{147}, as its classic counterpart Dianeal\textsuperscript{®}, which might explain the disappointing outcome of the study by Fan \textit{et al.}\textsuperscript{146}

The discrepancy can also be explained by the fact that, besides GDPs and low pH, many other factors impact on the properties of the peritoneal membrane. These factors are often neglected
in clinical studies, potentially leading to unnoticed differences in case-mix and blurring the interpretation of the potential impact of the novel solutions.

1.3.1.2. Peritonitis

Repetitive or protracted peritonitis episodes are associated with long-term peritoneal membrane changes, leading to increased solute transport and decreased ultrafiltration. However, if promptly and efficiently treated, peritonitis does not cause any permanent change in small solute transport. Peritonitis induces inflammation and a subsequent fibrogenic response. Inflammatory cytokines, which are induced in the peritoneal cavity during peritonitis, may further promote chronic inflammation and fibrosis. However, it is likely that the inverse is true as well: patients with an already damaged peritoneal membrane with defective mesothelial and defensive functions, are more prone to infection, and have difficulties to clear the responsible germs. Relapsing peritonitis is thus most likely a sign of bad peritoneal viability, rather than a cause of it.

1.3.1.3. Additives protecting the peritoneal membrane integrity during PD

To date, interventions designed to preserve the peritoneum by means of pharmacological manipulation (e.g. benfotiamine, pyrodoxamine, Glycosaminoglycans - GAGs) are promising, but clinical relevance is disappointing. Of all substances used as additives, GAGs have been investigated most extensively, but with inconsistent and even conflicting results.

Hyaluronan (HA) is a GAG composed of repeated dimers of N-acetylglucosamine and glucuronic acid. High-molecular weight HA added to dialysis fluid exerts anti-inflammatory and antifibrotic actions on in vitro cultured mesothelial cells. Animal studies indicated that intraperitoneal administration of HA results in improved ultrafiltration and solute clearances and can protect the peritoneal membrane during peritonitis or after repeated infusion of dialysis solutions. A clinical study by Wang et al confirmed a decreasing peritoneal fluid absorption, leading to a better ultrafiltration rate. Moberly et al reported a trend for higher solute clearance and net ultrafiltration and supported the acute safety of HA when administered intraperitoneally in the dialysis solutions of a small series of PD patients.
Heparin is a GAG with well-known anticoagulant activity\textsuperscript{161;167}, a property used in peritoneal dialysis to maintain catheter patency and to prevent the development of peritoneal adhesions.\textsuperscript{161;168} However, heparin also has a host of other biological actions beyond its role as an anticoagulant, including immunomodulating, anti-inflammatory and antiproliferative effects, and effects on the extracellular matrix, and angiogenesis.\textsuperscript{167;169;170} Low-molecular weight heparins can inhibit the effects of fibroblast growth factor (FGF) and of vascular endothelial growth factor (VEGF)\textsuperscript{171}, of which the latter has been linked to peritoneal membrane inflammation and angiogenesis during PD therapy.\textsuperscript{172-174} On the other hand, using a rat model of peritoneal perfusion, Schilte \textit{et al} failed to demonstrate a beneficial impact of heparin.\textsuperscript{156} Moreover, heparin use is accompanied by serious side effects, such as bleeding tendency, and in addition, it can only be administered intraperitoneally or subcutaneously, limiting its everyday applicability.\textsuperscript{175-177} In theory, heparin can also be administered intravenously, but in the context of peritoneal dialysis, this is not a viable or relevant option. Therefore, it might be of interest to submit other GAGs to evaluation, preferentially with oral administration.
Prevention of PD-induced ultrafiltration failure and peritoneal membrane changes has been a very important research topic over the last few years. Using angiogenesis inhibitors may lead to reduced vessel formation, resulting in sustained ultrafiltration (UF) and improved well-being of patients due to a more continuous removal of waste products. Until today, several protocols have been developed to reduce angiogenesis in PD.

Cyclooxygenase (COX) enzymes are involved in prostaglandin synthesis. COX-2 is known to be an angiogenesis stimulator by upregulating the mRNA transcription and the production of VEGF. Studies have already shown a possible role for COX-2 inhibitors in the reduction of fibrosis and angiogenesis both in vitro and in vivo. Treatment with the COX-2 inhibitor celecoxib in a rat exposure model prevented PD-induced angiogenesis in both the omentum and parietal peritoneum, but VEGF levels were unaffected. However, ultrafiltration capacity was restored. Celecoxib may therefore be of interest in the prevention of ultrafiltration failure in PD patients. Unfortunately, the use of Cox-2 inhibitors is associated with an increased incidence of acute myocardial infarction and death from cardiovascular disease.

Another interesting possibility to prevent angiogenesis is the use of tyrosine kinase inhibitors. Sunitinib, for example, is involved in the inhibition of VEGF and platelet-derived growth factor receptor signaling.

A case study with a PD patient with metastatic renal cell carcinoma treated with sunitinib demonstrated a stabilization of the abdominal metastasis as well as the thickness of the peritoneal membrane and D/P creatinine. These observations suggest that sunitinib may have prevented peritoneal membrane angiogenesis. In addition, animal studies showed prevention of new vessel formation in the omentum as well as the mesentery by sunitinib at the end of a 5 week period of PD treatment. However, sunitinib treatment did not prevent an increase of PD-induced VEGF levels. Interestingly, a loss of mast cells in the mesentery was observed in the sunitinib-treated animals. Together with previous studies, in which mast-cell-deficient rats also showed significantly reduced angiogenesis in the omentum, this suggests an important role for mast cells in new blood vessel formation upon PD.

There are also studies focusing on the prevention of the onset of angiogenesis. Peroxisome proliferator-activated receptor-γ agonists are known to control glycaemia by improved insulin sensitivity and have anti-inflammatory, and therefore probably also antifibrotic, properties. Administration of a drug of this group, rosiglitazone, in a PD mice
exposure model led to reduced AGE formation and inflammation, a preserved mesothelial cell layer, and improved ultrafiltration. Nevertheless, although promising for inhibiting the alterations due to PD, rosiglitazone treatment of patients with type II diabetes has been linked to several severe side effects.

Since epithelial-to-mesenchymal transition (EMT) also leads to angiogenesis, there has been recently a focus on the prevention of EMT in the context of PD. In a rat PD exposure model, EMT as well as angiogenesis were inhibited after treatment with recombinant bone morphogenic protein-7 (BMP-7). BMP-7 antagonises TGF-β and as a consequence can block mesenchymal-to-epithelial transition. Administration of two TGF-β blocking peptides (P17 or P144) to PD-fluid instilled mice significantly reduced fibrosis, but also angiogenesis. Administration of two TGF-β blocking peptides (P17 or P144) to PD-fluid instilled mice significantly reduced fibrosis, but also angiogenesis.

Inhibitors of angiotensin-converting enzyme (ACE) and Ang II receptor are shown to attenuate the production of VEGF in mesothelial cells in vitro. Previously, administration of these inhibitors/blockers was already shown to attenuate structural and functional alteration of the peritoneal peritoneum in a PD rat model.

Rapamycin, a commonly used immunosuppressive agent, has been demonstrated to prevent angiogenesis in the mesenteric tissue of portal hypertensive mice, at least in part through an anti-VEGF activity.

Whether the above-mentioned positive interventions used in animal studies can be translated into clinical medicine remains to be elucidated. Currently, the COX-2 inhibitor celecoxib, the only COX-2 antagonist still in use in the USA, is under study in a clinical trial. Nevertheless, we have to be careful in using blocking agents. A lot of pathways leading to, for example, EMT are also involved in pathways necessary for a good functioning of the body. Agents directly blocking TGF-β cannot be easily used in the clinical practice of PD, at least for long-term treatments, because TGF-β has important modulating functions of the immune and inflammatory responses. Blocking TGF-β could thus lead as a counterpart to chronic inflammation.
1.3.2. Non-PD related factors affecting peritoneal membrane quality

1.3.2.1. Salt intake

There is much debate about the potential benefit of a reduction in dietary salt intake in the normal population, but for patients with kidney disease, there seems to be a consensus that a too high salt intake should be avoided, although there is no agreement on the ideal daily dose of salt. In any case, it is evident that salt intake induces thirst, and will thus inevitably lead to a higher intake of fluids, which in its turn will enhance the need to increase ultrafiltration. The latter is in PD patients often pursued by using hypertonic glucose exchanges. In addition, salt intake will increase blood pressure quite rapidly, which might again lead to a need for more ultrafiltration by the introduction of hypertonic peritoneal dialysate bags. Since these hypertonic solutions are able to induce peritoneal damage, salt restriction should be advocated in PD patients, to indirectly preserve the peritoneal membrane integrity.

In addition, there is evidence that dietary salt intake by itself, even without causing hypertension or volume overload, might be deleterious, resulting in cardiac remodelling, fibrosis and left ventricular hypertrophy. Also in the kidney, it has been demonstrated that salt intake leads to enhanced glomerulosclerosis and deterioration of residual renal function. It has been postulated that upregulation of transforming growth factor beta 1 (TGF-β1) might be one of the underlying mechanisms.

1.3.2.2. Genetic factors

As for many biological processes and structures, the integrity of the peritoneal membrane can be altered by genetic polymorphisms. Some polymorphisms with functional repercussions have been described, but it is very likely that many more exist, and that they partially explain differences in the propensity to develop peritoneal dialysis related complications such as encapsulating peritoneal sclerosis.

The most important genetic polymorphism is probably the one for interleukin 6 (IL-6), of which a G and a C allele have been described. IL-6 has been associated with systemic inflammation, but has also been linked with peritoneal inflammation, and is most likely the link between the two phenomena. IL-6 was not only associated with increased small solute transport, but also with albumin leakage, and thus an increase of large pore flow, by itself a marker of inflammation.
Patients with a GC or CC disposition have much higher IL-6 levels in serum and in the drained dialysate, while an upregulation of IL-6 mRNA in the peritoneal membrane was associated with much faster transport of small solutes. In the same study, also polymorphisms of VEGF and eNOS were evaluated, but no impact on peritoneal membrane was detected. Hwang et al described an IL-6 T15A SNP polymorphism, which was associated with baseline peritoneal membrane transport rate after adjustment for diabetes, comorbidity and CRP.

Endothelial nitric oxide synthase (eNOS) is involved in the autoregulation of blood vessel tone. A gene polymorphism of the eNOS gene, with impact on the circulating NO levels, has been described. eNOS genotype "aa" or "ab", versus "bb", was an independent predictor of lower peritoneal membrane transport rate at initiation of PD, after adjustment for gender, body weight, and diabetes.

1.3.2.3. Medication

Angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARB) are used extensively in patients with renal diseases because of their beneficial effects on the cardiovascular system, and their ability to reduce proteinuria and to influence the progression of renal failure in patients with various types of glomerulopathies. The latter effect is likely due to the fact that angiotensin II (AII) has the properties of a growth factor. It has been shown that these effects are mediated through Ang II induction of transforming growth factor-β (TGF-β) synthesis and secretion. That explains an ability of AII inhibitors to attenuate development of renal fibrosis, predominantly by suppressing the activity of TGF-β.

TGF-β1 is a member of a family of cytokines and is involved in many wound healing processes. This profibrotic protein is believed to be a central mediator of fibrotic diseases in many tissues (liver, lung, kidney, central nervous system). More specifically, TGF-β1 has been proposed to be a key molecule in PD fluid-induced peritoneal membrane deterioration. Blocking TGF-β1 has recently been shown to protect the peritoneal membrane from dialysate-induced damage. ACE inhibitors have also been shown to have a positive impact on the development of peritoneal membrane morphological alterations such as fibrosis and neoangiogenesis. All inhibition prevents the increase in small solute transport and slows down the rate of decline in ultrafiltration in long-term PD. Thus, these drugs have a positive influence on PD technique survival.
1.3.3. **Uraemia-related factors affecting peritoneal membrane quality**

There is evidence that the uraemic milieu is toxic for the peritoneal membrane. Both animal and human biopsy studies suggest that uraemia can induce fibrotic changes in the peritoneum.\(^{106,220}\)

In a subtotal nephrectomy rat model, uraemia induced fast transport status. Further exploration indicated that in uraemia, there was peritoneal vascular proliferation and fibrosis, corresponding to upregulation of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).\(^{220}\) Similar observations were confirmed by Vrtovsnik et al in a uraemic rat model.\(^{221}\) In a Japanese cohort study including uraemic patients before the start of PD, the average peritoneal thickness was already increased and the lumen/vessel diameter ratio was decreased with an increase in thickness of the postcapillary venules.\(^{222}\) This is congruent with the findings of the peritoneal biopsy study group, based on a worldwide sampling of peritoneal membranes, showing that, even before initiation of dialysis, end-stage renal failure patients exhibit a peritoneum that is thickened and presents more vasculopathy as compared with peritoneum in healthy control subjects.\(^{106}\)

The mechanism by which uraemia alone induces changes in the peritoneum is not clear, but these effects may be related to the chronic inflammation, observed in patients with end-stage renal disease. As mentioned earlier in this introduction, leukocyte function is altered in uraemic patients, who are characterized by a chronically stimulated baseline inflammatory state, but also by an impaired response upon stimulation. This can explain also the increased susceptibility for recurrent peritonitis in PD patients.

Many alterations in important leukocyte functions are caused by the presence of uraemic retention solutes. The exact definition and classification of the individual retention products responsible for these effects in uraemic plasma is important, as identification of the responsible toxins would allow a tailored approach to removal by blood purification.
1.3.4. Animal models to study peritoneal membrane quality

The development of peritoneal dialysis has been paralleled by a growing interest in establishing suitable experimental models to better understand the functional and structural processes operating in the peritoneal membrane. Visceral and parietal peritoneum from mice and rats is effectively undistinguishable from that described in humans. The majority of studies are performed in rat and rabbit models. Rats are easy to handle and affordable. Rabbit models allow long periods of PD time and mimic certain aspects of the human situation (the ratio of peritoneal surface area and exchange volume in rabbits and humans is similar). However, rabbits are very sensitive animals and difficult to house. The possibility of using genetically modified mice cannot be underestimated as a tool to investigate the molecular aspects of peritoneal pathophysiology during PD and to pave the way for the development of novel, specifically targeted diagnostic and therapeutic strategies. Knockout mice have been used for example to demonstrate the importance of aquaporin-1 for fluid transport during the first hour of a PD-dwell. One of the disadvantages of mice is their small size, making manipulations difficult and more prone to deviations from the described standard approach. For example, a 25 g mouse has a plasma volume of only approximately 1.5 ml. Thus, sampling and injections should be downscaled to the microliter range. It is also not possible to cannulate more than a limited number of vessels in a mouse. In addition, mice are very sensitive to temperature changes, so a rigorous temperature control system is needed. Nevertheless, Ni et al were the first to show that a mouse model of PD is feasible for physiological experimentation.

For all these reasons and because of the previous experience in our laboratory, we have opted to use the rat as an animal model to study PD-related interventions.

However, it would be interesting to have eventually also experiments with larger animals, which have a longer life span and are probably more realistic in mimicking the situation of long-term PD in humans than are the short-term rat models we have at this moment. Animal models using larger animals have however the disadvantage that they are far more expensive, in labor time and in cost of feeding, lodging and breeding. Larger animals will also necessitate longer time frames to set up and conduct new research projects, as the longer
lifecycle of larger animals is advantageous in relevance to the human long-term PD situation; however there is also the implication that it may take months before changes in the peritoneal membrane can be observed.
1.4. OUTLINE AND AIMS
The condition of the peritoneal membrane is crucial to continue peritoneal dialysis (PD) as a renal replacement therapy. Morphological deterioration of the peritoneal membrane leads to the necessity to discontinue PD therapy. As described above, the causes of these peritoneal changes are multifactorial. The general objective of this thesis is to gain a better insight, by using chronic and acute rat models, in the mechanisms that alter peritoneal membrane function and to find strategies to prevent or attenuate these processes.

The specific aims of this thesis are:

1) To evaluate the potential protective effect of a specific additive, applied as an oral formulation, on peritoneal function and anatomy in a chronic rat model of peritoneal perfusion.

2) To investigate the direct impact of dietary intervention on peritoneal morphology and function.

3) To investigate the *in vivo* toxicity of specific uraemic retention solutes on leukocyte recruitment in the peritoneal microcirculation.

Chapter 1 describes how chronic kidney disease progresses and what the treatment options are in chronic kidney disease stage 5. An overview of the uraemic retention solutes, which were evaluated in the scope of this thesis, is given. The peritoneal membrane morphology and changes during PD are described, together with their different causes.

Chapter 2 describes the effects of oral sulodexide treatment on the development of functional and morphological changes of the peritoneal membrane using a standardized rat model of chronic peritoneal dialysate fluid (PDF) exposure. Sulodexide is a highly purified mixture of GAGs containing 80% low-molecular weight heparin and 20% dermatan sulphate. After 12 weeks of PDF exposure, a functional peritoneal equilibration test (PET) was executed and (immuno)histological analyses of the peritoneum were performed.

In chapter 3, the direct morphological impact of dietary salt loading on the peritoneal membrane of healthy rats was explored. 2% NaCl was given in the drinking water of normal Wistar rats. After 2 weeks, peritoneal samples were taken for histology and determination of gene expression.
In addition to its clinical importance for peritoneal dialysis patients, the peritoneum provides a unique and simple organ structure for \textit{in vivo} microvascular research. In \textbf{chapter 4}, the acute effects of specific uraemic retention solutes on leukocyte recruitment in the peritoneal microcirculation are evaluated, using intravital microscopy. In contrast with \textit{in vitro} models, this approach enabled us to evaluate directly this crosstalk, as intravital microscopy allows visualization of leukocyte recruitment in mesenteric postcapillary venules.

\textbf{Chapter 4.1.} gives an update of the most important protein-bound uraemic retention solutes. An overview of \textit{in vitro} and observational \textit{in vivo} data is given and removal strategies are discussed. The effects of p-cresylsulfate, p-cresylglucuronide and indoxylsulfate on leukocyte recruitment, capillary leakage and hemodynamic parameters are described in \textbf{chapter 4.2}.

The inflammatory properties of the small and water soluble guanidino compounds asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are evaluated in \textbf{chapter 4.3}. ADMA is generally accepted to be an inhibitor of nitric oxide synthase (NOS) and a marker of endothelial dysfunction. Furthermore, this molecule is linked with all cause mortality and cardiovascular events, both in the general as well as in the chronic kidney disease population. SDMA, the structural isomer of ADMA, was until recently considered biologically inert. In the present study, both ADMA and SDMA were compared for their impact on leukocyte recruitment, capillary leakage and hemodynamic parameters.

In \textbf{chapter 5}, the general conclusions resulting from this thesis are summarized and discussed. Proposals on how future research could contribute to better preservation of the peritoneal membrane, resulting in a prolonged time in PD treatment are made.
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CHAPTER 2

CHRONIC PERITONEAL EXPOSURE TO PD-FLUIDS AND ORAL SULODEXIDE
2.1. **Oral Supplementation with Sulodexide Inhibits Neo-angiogenesis in a Rat Model of Peritoneal Perfusion**

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2.1. Abstract

Peritoneal dialysis (PD) is associated with functional and morphological alterations of the peritoneal membrane (PM). It is hypothesized that vascular endothelial growth factor (VEGF) plays a role in this process. Sulodexide is a glycosaminoglycan with effects on vascular biology. Therefore, the impact of oral sulodexide on PM function and morphology in a rat model of peritoneal perfusion was evaluated.

Rats received 10 mL peritoneal dialysate fluid (PDF) twice daily via a tunneled PD catheter. The test-PD group (Sul) received 15 mg/kg/day oral sulodexide versus none in the control-PD group (Con). A third group received no PDF (Sham). After 12 weeks, a peritoneal equilibration test was performed and the PM was sampled. Neo-angiogenesis was evaluated using immunostaining with von Willebrand, and epithelial-to-mesenchymal transition (EMT) using co-localization of cytokeratin and α-smooth muscle actin. VEGF was determined in the dialysate by enzyme-linked immunosorbent assay.

PD induced loss of ultrafiltration, also in the sulodexide group. Creatinine and glucose transport were better preserved, and sodium dip was more pronounced in the sulodexide group versus control. Submesothelial thickness, neo-angiogenesis and EMT were more pronounced in the Con versus Sul versus Sham group. VEGF in the dialysate, corrected for diffusion was higher in Con and Sul versus Sham.

Oral sulodexide administration diminishes neo-vascularization, submesothelial thickening and EMT induced by exposure to PDF in a rat model. As there was no difference in VEGF at the protein level in the dialysate, we hypothesize that oral sulodexide inhibits VEGF locally by binding.

2.1.2. Introduction

Peritoneal dialysis (PD) is an accepted and established renal replacement modality [1, 2]. Whereas it is advocated as a first line technique, the most important limiting factor for its long-term use is the progressive reduction in dialytic efficacy and ultrafiltration. This decrease is due to a progressive degradation of structure and function of the peritoneal membrane (PM) [3–5]. The changes are highly comparable to those observed in diabetes and mainly consist of two distinct but concurrent processes: fibrosis with submesothelial thickening and neo-angiogenesis [6], leading to increased small solute clearance and
ultrafiltration loss, and finally to technique efficacy failure. Conceivably, these pathophysiologic similarities have been related to the presence of high concentrations of glucose and its degradation products [7, 8]. The non-physiology of dialysis solutions and the subsequent peritoneal failure have prompted the search for preventive options, one of these being the use of glycosaminoglycans (GAGs).

Heparin is a GAG with a well-known anticoagulant activity [9], a property used in animal models of PD to maintain catheter patency and to prevent the development of peritoneal adhesions [10]. Heparin has a host of biologic actions beyond its role as an anticoagulant, including immunomodulating effects, antiproliferative effects, effects on the extracellular matrix, effects on angiogenesis and anti-inflammatory effects [9, 11, 12]. Low-molecular weight heparins can inhibit fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) activity [13]. The PM is specifically subjected to inflammation, fibrosis and angiogenesis during the course of PD therapy, and some of these processes have been linked to VEGF [14–16]. All this provides ample rationale for the study of GAGs in PD.

Heparin use is accompanied by serious side effects, such as bleeding tendency and can only be administered intraperitoneally or subcutaneously (s.c.), limiting its everyday use [17–19]. Therefore, alternative GAGs should be evaluated.

Sulodexide is a highly purified mixture of GAG containing 80% low-molecular weight heparin and 20% dermatan sulphate [18–20]. The pharmacological behaviour of sulodexide differs substantially from unfractionated heparin and is mainly characterized by a prolonged half-life and a reduced effect on global coagulation and bleeding parameters. Sulodexide has been approved for human use and has been related to improvement of proteinuria and cardiovascular disease in diabetes [21–23]. Experiments using an intraperitoneal formulation in humans [24] or an s.c. formulation in rats [25] demonstrated a beneficial impact of sulodexide on the, respectively, functional and morphological properties of the PM. Sulodexide administration can thus potentially have a beneficial impact on PD patients through conservation of the integrity of the PM.

As intraperitoneal or s.c. administration are both labour intensive and prone to generating side effects, such as peritonitis or local bleeding, it is important to test whether oral supplementation with sulodexide might be effective. The low-molecular weight of both sulodexide fractions allows for better oral absorption compared to heparin [19]. After oral administration of sulodexide in rats, pharmacodynamic effects are observed within hours, and
fluorescent sulodexide was observed in tissues of kidney and liver and in the endothelium of veins and arteries [26]. Several clinical studies demonstrated the safety and good tolerance of the drug in humans [27] and in animals [28]. Fracasso et al. [29] orally administered sulodexide to patients, showing a beneficial functional effect but without morphological exploration.

For all these reasons, sulodexide appears to be a good candidate for an oral drug to protect the PM during PD. Therefore, this study analyses the effect of oral sulodexide on peritoneal function and anatomy in a rat model of peritoneal perfusion.

### 2.1.3. Materials and methods

#### Laboratory animals

Forty-nine female Wistar rats (Iffa Credo, Brussels, Belgium) with a mean body weight of $237 \pm 11$ g at the beginning of the experiment were investigated. They were kept under constant temperature and humidity in a 12 h controlled dark/light cycle and were allowed 1 week of acclimatization before the start of the experiment. During the experiment, rats were housed in groups of four per cage. The ethical committee of experimental animals at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocol.

#### Rat model

A subcutaneous mini access port (PMINA-CBAS-C30 Soloport; Instech Solomon, Almere, The Netherlands) was implanted in a sterile manner in the neck under isoflurane anaesthesia (Forene®, Abott, Louvain-la-Neuve, Belgium). The attached polyurethane heparin-coated [10, 30] catheter (Instech Solomon) was tunnelled into the abdominal cavity through the left flank. Antibiotics were provided to prevent peritonitis, as described earlier (oxacillin; 2.5 mg/day and gentamycin; 0.04 mg/day, Bristol-Myers Squibb, Brussels, Belgium) [10, 31]. In the first week after implantation, the catheter was flushed daily with 1 mL Earle’s Balanced Salt Solution at 37 °C (EBSS; Invitrogen, Merelbeke, Belgium) to allow wound healing. Then, the ports were punctured twice a day under sterile conditions to allow infusion of 10 mL 3.86% glucose peritoneal dialysate fluid (PDF) at 37 °C (Dianeal; Baxter SA, Lessines, Belgium). The area of the port was disinfected with ethanol 97% twenty seconds before puncture.
Chapter 2: Peritoneal exposure to PD-fluids

**Experimental design**

Three groups of experimental animals were studied. The animals of group Sul ($n=23$) received peritoneal perfusion as described above. In addition, they received oral sulodexide (Biofer SpA, Medolla, Italy) mixed in powder form with their standard chew (rat and mice maintenance chow; Carfil, Oud Turnhout, Belgium) in a dose of 15 mg/kg/day. This dosage was based on those used in the clinical setting [29, 32] and animal studies [25, 33]. Before start of this study, food intake was monitored for 1 week. The lowest food intake weight was used to mix the sulodexide in. Rats in group Con ($n=20$) received peritoneal perfusion, but no oral sulodexide and served as a positive control. The rats in group Sham ($n=6$) were not surgically treated (no catheter and no peritoneal perfusion) and served as a negative control group.

The weight of the rats was recorded weekly. Catheter patency and the integrity of the skin of the abdomen and around the port were evaluated twice a day. In the case of catheter obstruction, an attempt was made to infuse fluids under isoflurane anaesthesia. Rats in the Sham group were left uninstrumented during the 12 weeks.

**Peritoneal transport studies**

After 12 weeks of PDF exposure, a modified peritoneal equilibration test (PET) was used in all three groups to investigate peritoneal transport parameters. Briefly, rats were placed on a thermopad at 37 °C after anaesthesia with thiobutabarbital (Inactin®; 100 mg/kg s.c.; Sigma, St Louis, MO). The trachea was intubated, the right jugular vein was cannulated for continuous saline infusion (0.9% NaCl, 3 mL/h), and the right carotid artery was cannulated for tension and blood sampling. A silicone catheter (Venflon, 18GA 1.77 IN; Becton-Dickinson, Erembodegem, Belgium) was inserted into the peritoneal cavity 30 min after anaesthesia and 15 mL of 4% glucose PDF at 37 °C (BicaVera, Fresenius) was instilled (= time zero).

For the PET test, plasma (P) and dialysate (D) samples were collected at time zero and after 30, 60 and 240 min of dwell time for determination of creatinine (high-performance liquid chromatography), urea (urease), glucose (hexokinase), sodium (ion-selective electrodes calibrated for use in PDF), total protein (Biuret method) and VEGF [enzyme-linked immunosorbent assay (ELISA)] levels.

At the end of all the PETs, PDF was recovered from the peritoneal cavity through the silicone catheter, after which the abdomen was opened by a midline incision to collect the remaining
dialysate and for tissue sampling. Net ultrafiltration was calculated and transport of low-
molecular weight solutes was evaluated by calculating the dialysate-to-plasma (D/P) ratios for 
creatinine, sodium and the D/D₀ ratio for glucose at 0, 30, 60 and 240 min of dwell time. 
VEGF levels in the dialysate were quantified by ELISA (Quantikine, MMV00; R&D, 
Minneapolis, MN) according to the manufacturer’s instructions and corrected for diffusive 
transport according to the method provided by Zweers et al. [34] to obtain an estimate of 
local production.

Adequate attention was paid to maintain a hygienic environment and to prevent peritonitis. 
Furthermore, the tunnelled polyurethane catheter was removed in a sterile way and the tip 
was cultured. PDF samples were obtained for culture and white blood cell (WBC) counting in 
a Bürker chamber. Infection was arbitrarily defined as a positive dialysate culture with a 
dialysate WBC count >1000/mm³ [35]. Rats positive for peritoneal infection were excluded 
from further analysis.

Study of peritoneal morphology

Parietal peritoneum (PP) and visceral peritoneum (VP) was carefully dissected in a 
standardized fashion in order to obtain the same anatomical region and to avoid mechanical 
trauma to mesothelial cells. The PP was collected at the contralateral side to the tip of the 
implanted catheter. Samples were immediately fixed in 4% neutral buffered formalin for 2 h 
at 4 °C, rinsed with phosphate-buffered saline and embedded in paraffin. Sections (5 µm) of 
the peritoneum were cut for histology with a Leica RM 2145 sliding microtome (Leica 
Microsystems, Nussloch, Germany).

To determine the thickness of the PP, sections were sliced perpendicularly to the peritoneal 
surface and a classic Masson’s Trichome staining was performed. Double 
immunofluorescence stainings for alpha smooth muscle actin (α-SMA) and cytokeratin on VP 
sections were performed. These two stainings were executed as described previously [36]. 
For visualization of blood vessels, adjacent VP sections were stained with monoclonal 
antibodies [anti-von Willebrand Factor (anti-vWF)] reactive with endothelial cells. Sections 
were deparaffinized, rehydrated and pretreated with Proteinase K (S3020-2; Dako, Heverlee, 
Belgium) for 7 min. Thereafter, sections were blocked for endogenous peroxidase for 30 min 
with 3% H₂O₂. After rinsing under running water, aqua dest (AD) and buffer [Tris-buffered 
solution (TBS) + Tween 0.05%], sections were incubated with UltraSense Block
(Immunologic kit, Klinipath, Olen, Belgium) for 10 min to avoid cross reactions. Subsequently, they were incubated with the primary antibody anti-FVIII (1/250, A0082, Dako) for 1 h, followed by incubation with the secondary antibody (Immunologic kit) for 10 min and streptavidin peroxidase (Immunologic kit) for 10 min. 3,3’-diaminobenzidine (Dako) was used as the chromogenic substrate to visualize immunolabeling resulting in a brown precipitate. Finally, counterstaining was performed with Haematoxyline Mayer (Mallinckrodt Baker, Deventer, The Netherlands). Between every step, slides were rinsed in buffer solution.

**Morphometric analysis**

All slides were coded and blindly analysed by the same operator with an Olympus BX41 microscope (Olympus, Aartselaar, Belgium). From each experimental animal, three peritoneal samples were analysed. For each sample of peritoneum, three sections were digitalized and quantified with a computerized image analysis system (CellID software; Olympus).

The submesothelial thickness (micrometers) was measured using the Masson’s Trichome staining. Microvascular density of the vWF immunostainings was determined by counting the number of blood vessels (number per square millimeters) and by calculating the percentage of the stained area per section. The double a-SMA/cytokeratin staining was viewed with a fluorescence microscope (Axioscoop, Zeiss, Germany) and pictures were taken using CellF Software (Olympus Soft Imaging Solutions, Münster, Germany). A semiquantitative assessment was performed independently and blindly by two operators. Each section was screened to estimate the extent and distribution of colocalization of a-SMA and cytokeratin. Staining results were classified using a visual score: 0 = no co-localization at all, 1 = suggestive for co-localization, 2 = evidence of co-localization and 3 = clear colocalization and migration of mesothelial cells into the interstitium. The results were calculated as the mean of the individual scores of the two operators for each animal.

**Statistical analysis**

Data analysis was performed with SPPS version 15.0 (SPSS Inc,Chicago, IL). For all analyses, one-way analysis of variance for the three groups and post-hoc testing with Least Significant Difference was used. The results are expressed as mean ± SD. The significance level was set at $P \leq 0.05$. 
2.1. Sulodexide and Peritoneal Dialysis

2.1.4. Results

Technique survival

During the experiment, the well-being of the animals was monitored daily and no apparent abnormalities were observed. Body weight was similar in the different experimental groups at all time points (data not shown). Technique survival was 100% in all groups. Two animals of group Sul were excluded because of positive dialysate culture (one streptococcus viridans, one with Bacteroides species), positive tip culture (Paecilomyces sp.) and increased dialysate leukocyte count. In the Con group, one dialysate culture yielded Corynebacterium species but without increased dialysate leukocyte count.

Peritoneal transport studies

Exposure to PDF (Sul and Con) reduced net ultrafiltration significantly after 240 min of dwell time as compared to Sham, but sulodexide did not have a protective effect (Figure 1A).

In addition to the decrease of net ultrafiltration capacity, PDF in the Con group induced a significantly enhanced creatinine transport (Figure 1B) compared to Sham at every time point and compared to Sul group after 60 and 240 min. Addition of sulodexide partially prevented this enhancement of diffusive creatinine transport, because no significant differences in D/Pcreatine were found between the Sul and Sham group.

Glucose absorption was significantly increased in the Con compared to Sham group after 30 and 60 min (Figure 1C).

Sodium dip was less pronounced and earlier in the Con group versus the Sul and the Sham group (Figure 1D).

Protein loss was significantly higher in the Con group versus the Sham and Sul group after 240 min (Figure 1E).
Figure 1. Peritoneal Transport Parameters
†P < 0.05 versus Sham, *P < 0.05 Con versus Sham, **P < 0.01 Con versus Sham, °°P < 0.01 Con versus Sul, °P < 0.05 Con versus Sul. Bars and line graphs represent mean ± SD.
Peritoneal morphology

The Masson’s Trichrome staining showed a significant thickening of the submesothelial layer of the PP in the groups on peritoneal perfusion (Table 1 and Figure 2), with significant lower values in the Sul group compared to the Con group. Neo-angiogenesis was more expressed in the Con group as compared to the Sul group and the Sham group, without a difference between the latter two (Table 1 and Figure 3) and this both for the number of vessels per square millimeters as for the percentage surface stained with vWF. There was a higher score for epithelial-to-mesenchymal transition (EMT) in the Con versus Sul versus Sham group (Table 1 and Figure 4).

![Figure 2. Submesothelial thickness of parietal peritoneum](image)

(A) Significant differences were found between groups on peritoneal perfusion (Con: 19.9 ± 7.26 & Sul: 15.5 ± 4.86) and control group (Sham: 10.0 ± 1.57). *p<0.01 vs Sham,**p<0.05 vs Sham,°°p<0.05 vs Sul. Bars represent mean±SD.

(B) Histological images of the Masson’s Trichome staining. (magnification, 400x) The submesothelial thickness of the parietal peritoneum was significant higher in the groups on peritoneal perfusion (Con & Sul), but with significant lower values for the Sul-group, compared to the Con-group.
Figure 3. Vascularisation of the visceral peritoneum.

Significantly less blood vessels (23.6±9.3 vs 31.8±12.6 vs 48.1±16.2) and % surface positive for vWF staining (0.28±0.17 vs 0.48±0.26 vs 1.05±0.64) were observed in the Sham vs Sul vs Con group. *p<0.01 vs Sham; °p<0.01 vs Sul; **p<0.01 vs Sham. Bars represent mean±SD.

Pictures show representative Von Willebrand immunostainings of visceral peritoneum (magnification 200x).
2.1. Sulodexide and Peritoneal Dialysis

Figure 4. α-SMA, cytokeratin and a double staining for α-SMA and cytokeratin (+ DAPI nuclear staining) of the visceral peritoneum.

Representative pictures are shown (magnification, 400x). Serial sections of the visceral peritoneum from Sham-rats (A-C), Sul-rats (D-F) and Con-rats (G-L) were stained for cytokeratin (A,D,G,J), α-SMA (B, E, H, K) and double stained for α-SMA and cytokeratin (C, F, I, L). In the Sham-animals, only mesothelial cells stain for cytokeratin (A), only vascular smooth muscle cells stain for α-SMA (B) and virtually no α-SMA/cytokeratin co-localization (C) occurs. In the peritoneal membrane of the Sul- and the Con-animals, α-SMA staining is found not only in the vascular smooth muscle layer of blood vessels, but also in submesothelial tissue (E, F & H, I resp.). In the Con-rats, cytokeratin staining is additionally found in the interstitial tissue (J,L). Co-localization of α-SMA and cytokeratin is pronounced in submesothelial tissue (I) and in the interstitial tissue (L).

Arrows = co-localization.
Table 1. Histological and immunohistochemical analyses of the peritoneum.

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<th>Sham</th>
<th>Sul</th>
<th>Con</th>
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<td></td>
<td>/</td>
<td>PD + Sulodexide</td>
<td>PD</td>
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<tr>
<td>vWF staining (% of surface)</td>
<td>0.276 ± 0.165</td>
<td>0.483 ± 0.263</td>
<td>1.05 ± 0.644&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>blood vessels (n/mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>23.6 ± 9.27</td>
<td>31.8 ± 12.6</td>
<td>48.1 ± 16.2&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Submesothelial thickness (μm)</td>
<td>10.0 ± 1.57</td>
<td>15.5 ± 4.98&lt;sup&gt;d&lt;/sup&gt;</td>
<td>19.9 ± 7.26&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>EMT-score (0-3)</td>
<td>0.141 ± 0.185</td>
<td>0.704 ± 0.422&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.30 ± 0.628&lt;sup&gt;bc&lt;/sup&gt;</td>
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<sup>a</sup>P < 0.01 versus Sham  
<sup>b</sup>P < 0.01 versus Sul  
<sup>c</sup>P < 0.001 versus Sham  
<sup>d</sup>P < 0.05 versus Sham  
<sup>e</sup>P < 0.05 versus Sul

**VEGF pathway**

VEGF levels in the dialysate were, after correction for diffusion, higher in the Con and Sul group as compared to the Sham group (9.3 ± 6.1 versus 7.8 ± 4.9 versus 1.5 ± 1.2, P = 0.02), but the difference between Con and Sul animals did not reach significance.

**Relationships**

Significant correlations were observed between the number of blood vessels and the functional PM parameters after 30 min of the PET: D/P<sub>creatinine</sub> (r = 0.575, P < 0.01), D/D<sub>glucose</sub> (r = - 0.525, P < 0.01) and D/P<sub>protein</sub> (r = 0.598, P < 0.01).

No correlation was found between the vessel count and VEGF levels in the dialysate (r = 0.381, P = 0.060), but after excluding the Sul group, a significant correlation was observed (r = 0.635, P < 0.05). VEGF correlated positively with the high transport rate of D/P<sub>creatinine</sub> (r = 0.551, P < 0.01) and correlated negatively with D/D<sub>glucose</sub> (r = - 0.420, P < 0.05) after 240 min of PET. There was a weak correlation between the number of blood vessels and submesothelial thickness (r = 0.344, P < 0.05). EMT was not correlated with the number of blood vessels, but there was a correlation with submesothelial thickness (r = 0.405, P < 0.01). A weak negative correlation was detected between ultrafiltration and submesothelial thickness (r = - 0.393, P < 0.05).
2.1. Discussion

In this study, an oral formulation of sulodexide was tested in a rat model for PD, based on previous observations of a beneficial impact of this GAG on the PM during PD, and in the hypothesis that sulodexide could inhibit the activity of VEGF in the PM [24, 25, 29, 37].

We found a significantly worse peritoneal performance in the Con group by 12 weeks of exposure to PDFs, as evidenced by the increase of transport rate for creatinine, loss of ultrafiltration capacity and peritoneal tissue remodelling. Although no differences in net ultrafiltration were seen between the group with and without oral sulodexide administration, transport rates for creatinine and glucose and sodium sieving were better preserved in the sulodexide group. This functional improvement induced by sulodexide was associated with less neo-angiogenesis and reduced submesothelial thickening but not with a reduction of VEGF up-regulation in the dialysate.

As we observed higher VEGF levels in the dialysate of animals treated with peritoneal perfusion versus Sham but no differences between the animals on peritoneal perfusion treated versus not treated with sulodexide, we hypothesize that sulodexide inhibits VEGF activity either by binding it or by inhibiting the interaction with its receptor.

The role of VEGF in neo-angiogenesis during PD has been well established [34, 38]. Also in the current experiments, VEGF levels were higher in the animals submitted to peritoneal perfusion as compared to Sham animals. Structure–function relationship studies have shown that, both in rat models as in patients on long-term PD, loss of ultrafiltration capacity is associated with submesothelial thickening and the presence of neo-angiogenesis in the PM [39, 40]. For most people, exposure to glucose and especially glucose degradation products are the driving factors of the changes observed. However, also other factors can play a role [41]. Many additives and substances have been studied to counteract these alterations.

In the present study, oral administration of sulodexide resulted in lower peritoneal transport rate of creatinine and glucose and more sodium sieving as compared to the control group, suggesting better preservation of the PM. This was in line with a lower microvascular density in the sulodexide group. Vessel count and functional membrane parameters were significantly correlated, which was also demonstrated by Vrtovsnik et al. [42].

Low-molecular weight heparin can inhibit the binding of different growth factors to their receptors, and hyaluronan, another GAG, is a known important regulator of growth and
migration of vascular endothelial cells and neoangiogenesis. Heparin fragments of < 18 saccharides inhibit the activity of FGF and VEGF [13, 43, 44]. Pyda et al. [45] reported complexation of VEGF by heparin in patients presenting with acute myocardial infarction. In a rat model of oral administration of labelled sulodexide, fluorescent material was observed in the endothelium of veins and arteries [26, 46]. It can thus be hypothesized that oral sulodexide inhibits neo-angiogenesis by locally blocking the enhanced VEGF activity produced during PDF exposure. We observed that oral administration of sulodexide resulted in significantly lower neo-angiogenesis, but not in lower upregulation of VEGF at the protein level. These findings are compatible with the hypothesis that sulodexide interacts with the vascular effects of VEGF by either blocking the binding of VEGF to its receptor or by binding the VEGF molecule itself. This hypothesis is supported by our observation that VEGF levels and vessel count appeared to be significantly correlated, but only after excluding the sulodexide group. The data of the sulodexide group blur the correlation between VEGF and neo-angiogenesis, as the VEGF is present, but not active. Further experiments to explore the exact nature of this inhibition mechanism are warranted.

Several authors have already tried to modify outcome of PD using GAGs, with conflicting results [24, 47–50]. Using a similar rat model of peritoneal perfusion as ours, Schilte et al. [51] failed to demonstrate a beneficial impact of heparin. However, Schilte et al. administered heparin intraperitoneally, while systemic activity of GAGs might be crucial to obtain the described pleiotropic effects. Indeed, the beneficial effects on PM damage after acute peritonitis were only observed when sulodexide was administered systemically by intramuscular injection and not when administered in the PDF [52]. Finally, the half-life of heparin is much shorter than that of sulodexide, so a once daily injection of heparin might be ineffective [19]. Sjoland et al. [53] demonstrated a reduction in D/P creatinine, with an improvement in ultrafiltration capacity in a small human study of intraperitoneal heparin administration during 3 months. There was, however, a very high peritonitis rate in this study, making the results difficult to interpret.

Oral administration of sulodexide did not result in differences in ultrafiltration capacity in the present study, despite a (beneficial) difference in angiogenesis and D/P creatinine. Breborowicz et al. [54] also found changes in transperitoneal permeability after intraperitoneal administration of hyaluronan, however, with an opposite pattern. Wang et al. [55] reported that intraperitoneal administration of hyaluronan reduced peritoneal fluid absorption without changing transperitoneal fluid transport in a rat model. All these observations raise the
2.1. Sulodexide and Peritoneal Dialysis

Suspicion that also changes in the physicochemical properties of the peritoneal interstitium take place when GAGs are administered and that the obtained effect depends upon the physicochemical properties of the substance used. Sulodexide is a negatively charged molecule, interstitial accumulation might hamper transport of water differently from that of non-polarized small solutes. It is, however, extremely difficult to visualize the presence of sulodexide in peritoneal tissue, as it is cleansed out during the normal processing of the tissue, so it was impossible to confirm this accumulation in our experiments.

Breborowicz et al. demonstrated that hyaluronan had cytoprotective effects in an *in vitro* model of peritoneal mesothelial cells [54, 55]. Sulodexide also has anti-inflammatory properties. It is well-known that in animal models for PD, the presence of an indwelling catheter induces microtraumatization and inflammation [56]. It could thus be that sulodexide only inhibits this mechanical stress on the PM induced by the presence of the catheter. As we have no negative control group with an indwelling catheter, but without peritoneal perfusion, we cannot make any conclusion from our current study in that regard, however.

We also observed a reduction of EMT indirectly, using a score for co-localization of α-SMA and cytokeratin and directly, by the lower degree of submesothelial thickening, itself a result of EMT. Although, for technical reasons, we were not able to further elaborate this, our observations fit with those of Breborowicz et al., who demonstrated a dose-dependent protective effect on healing of cultured monolayer cells after mechanical trauma, when sulodexide was added to the culture medium [57]. Others have demonstrated that certain GAGs inhibit Transforming Growth Factor (TGF)-β (over)expression at the transcription level, and it has been well established that up-regulation of TGF-β is one of the driving mechanisms of EMT and fibrosis [33, 58].

Although it can be seen as a limitation, we did not include a uraemic rat model in the current study. As uraemia per se can induce changes in peritoneal morphology [42, 51, 59] and, at least in our hands, a nephrectomy model can create large differences in states of uraemia, additional bias in the interpretation of the results would have resulted, which we wanted to avoid. It would be interesting to repeat the experiments in a uraemic model as described by Vrtovsnik et al.

In conclusion, we have confirmed that PD induces significant damage to peritoneal tissues within 12 weeks, and in animals that received orally administered sulodexide, these changes
were less present. Neo-angiogenesis, submesothelial fibrosis and EMT were less pronounced in the group treated with oral sulodexide. Our data are compatible with the hypothesis that sulodexide inhibits VEGF activity at the local level and support further investigation of the longterm administration of sulodexide as a potential protective agent during chronic PD treatment.

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**Conflict of interest statement.**
None declared.

### 2.1.6. References


CHAPTER 3

EXPOSURE TO A FORGOTTEN URAEMIC TOXIN: EFFECT OF SALT INTAKE ON THE PERITONEAL MEMBRANE
3.1. **SALT INTAKE INDUCES EPITHELIAL-TO-MESENCHYMAL TRANSITION OF THE PERITONEAL MEMBRANE IN RATS**

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3.1.1. Abstract

Dietary salt intake has been linked to hypertension and cardiovascular disease through volume mediated effects. Accumulating evidence points to direct negative influence of salt intake independent of volume overload, such as cardiac and renal fibrosis, mediated through transforming growth factor beta (TGF-β). Epithelial-to-mesenchymal transition (EMT) has been implicated as a key process in chronic fibrotic diseases, such as chronic kidney disease or heart failure. The potential role of dietary salt intake on cell transdifferentiation has never been investigated.

This study analysed the effect of dietary salt intake on EMT and fibrosis in the peritoneal membrane (PM) in a rat model.

Twenty-eight Wistar rats were randomized to a normal salt (NS) or a high salt (HS) intake. NS and HS rats had free access to tap water or NaCl 2% as drinking water, respectively. After 2 weeks, samples of peritoneum were taken, and TGF-β₁, Interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF) mRNA expression were quantified with qRT–PCR. Fibrosis and submesothelial PM thickness were scored. EMT was evaluated using fluorescence staining with cytokeratin and alpha smooth muscle actin (α-SMA).

Dietary salt intake caused peritoneal fibrosis and thickening of the submesothelial layer and induced EMT as identified by colocalization of cytokeratin and α-SMA in cells present in the submesothelial layer. Peritoneal TGF-β₁ and IL-6 mRNA expression were upregulated in the HS group.

High dietary salt intake induces EMT and peritoneal fibrosis, a process coinciding with upregulation of TGF-β₁.

3.1.2. Introduction

Salt has been linked to hypertension for many years [1,2]. Initially, it was thought that the volume overload, induced by salt retention, was the main underlying mechanism and that this in turn was the driving factor for left ventricular hypertrophy. Recently, evidence has accumulated that dietary salt intake by itself, even without causing hypertension or volume overload, might be deleterious, resulting in cardiac remodelling, fibrosis and left ventricular hypertrophy [3,4]. Also in the kidney, it has been demonstrated that salt intake leads to enhanced glomerulosclerosis and deterioration of residual renal function [5–7]. It has been
postulated that upregulation of transforming growth factor beta 1 (TGF-β₁) might be one of the underlying mechanisms. The effects of TGF-β₁ seem to be diminished in the presence of nitric oxide (NO) [5], implying that salt mediated upregulation of TGF-β₁ might be even more deleterious in subjects with NO deficiency [8], such as in uraemia [3].

TGF-β is a potent inducer of epithelial-to-mesenchymal transition (EMT) [9,10], a process which has recently been linked to chronic fibrotic diseases, such as chronic kidney disease, heart failure, lung and hepatic fibrosis [11–15]. In these diseases, EMT results in transdifferentiation of epithelial cells to myofibroblasts, which invade the interstitial space by transgression of the basal membrane and expand the extracellular matrix. The link between dietary salt intake and cellular transdifferentiation has, to our knowledge, never been investigated.

In long-term peritoneal dialysis (PD) patients, both functional [16,17] and morphological [18,19] deterioration of the PM have been described. The morphological changes consist of neovascularisation, fibrosis [19] and EMT [20]. These negative effects have been related to exposure to glucose and glucose degradation products contained in the peritoneal dialysate, and to uraemia per se [16,21,22].

TGF-β₁ has been linked to glucose-induced enhanced senescence of mesothelial cells [23], peritoneal fibrosis and induction of EMT in the PM [24,25]. De Vriese et al. [21] have demonstrated that interaction of advanced glycation end products (AGE) with their cell surface receptor for AGE (AGE–RAGE interaction) in uraemia induces upregulation of TGF-β₁, a process which they could also link to EMT.

The role of dietary sodium intake on the deterioration of the PM has however never been investigated. It is conceivable that patients who have a high dietary salt intake will also need more hypertonic glucose exchanges because they will drink more. Additionally, if high salt intake would lead to upregulation of TGF-β₁ in the PM, in parallel to what has been shown for the heart and the kidneys, or an upregulation of vascular endothelial growth factor (VEGF), leading to neoangiogenesis as has been shown in the PM of PD patients, this would result in synergistic mechanisms leading to a rapid deterioration of the PM.

The present study has been undertaken to explore the effects of dietary salt loading on the PM in normal rats, with specific focus on EMT and fibrosis, and the role of TGF-β₁, VEGF and interleukin 6 (IL-6).
3.1.3. Materials and methods

Laboratory animals

Experiments were performed in 28 female Wistar rats (Iffa Credo, Brussels, Belgium), receiving care in accordance with the national guidelines for care and use of laboratory animals. The protocol was approved by the Ethical Committee of Experimental Animal Studies at the Faculty of Medicine and Health Sciences, Ghent University, Belgium.

Study protocol

The rats were randomly assigned to two groups: NS (normal salt intake) and HS (high salt intake). Each group was housed in separate cages. The NS group received normal rat chew (rat and mice maintenance chew, Carfil, Oud-Turnhout, Belgium) with a 0.1% salt content and free access to tap water. The HS group received the same rat chew, but had only free access to NaCl 2% as drinking water. Rats were weighed daily. After 2 weeks, rats were sacrificed. They were anaesthetized with thiobutabarbital (Inactin®, 100mg/kg s.c., Sigma, St. Louis, MO). The trachea was intubated to facilitate breathing, a carotid artery was cannulated for monitoring of arterial blood pressure and the abdomen was opened by a midline incision for tissue sampling. Samples of visceral peritoneum (VP) and parietal peritoneum (PP) were immediately fixed in a 4% phosphate buffered formaldehyde solution (pH = 7) (Klinipath, Olen, Belgium) and embedded in paraffin. The VP of the small and large bowel was entirely resected and together with biopsies of the PP, snapped frozen in liquid nitrogen and maintained at −80°C until analysis.

(Immunohistochemistry

From all tissue samples, 5 μm sections were cut with a Leica RM 2145 sliding microtome (Leica Microsystems, Nussloch, Germany) for histology and immunohistochemistry. A Sirius Red staining (Klinipath, Geel, Belgium) was used to evaluate fibrosis in the VP. Sections of the VP were deparaffinized, rehydrated and stained with Giemsa. Subsequently, sections were washed and stained with 0.1% Sirius Red, resulting in a brick red staining of all fibrillary collagen.

To determine the thickness of the PP, the sections were sliced perpendicularly to the peritoneal surface, and a classic Masson's trichrome staining was performed. Sections of the
PP were deparaffinized, rehydrated and stained with Haematoxylin Gill (Merck, Brussels, Belgium). Successively, sections went through a series of fluids [1% HCl (VWR, Leuven, Belgium), a ponceau (Sigma, Bornem, Belgium)-fuschin (VWR) mixture, phosphomolybdene acid and aniline blue (VWR)] to obtain the desired colour.

Immunofluorescence stainings for alpha smooth muscle actin (α-SMA) and cytokeratin, as well as a double staining, were performed. Sections of VP were deparaffinized, rehydrated and pre-treated for antigen retrieval in Tris/EDTA (Tris[hydroxymethyl]aminomethane and [Ethylenedinitrilo]tetraacetic acid) (Acros Organics, Geel, Belgium) epitope retrieval solution (pH 9.0) at 96°C for 30 min. After cooling down, free aldehyde groups were blocked with NH₄Cl to block cross-linking of the antibodies (Ab) to inappropriate structures, and 1% BSA/TBS was used to block aspecific binding of the Ab. Subsequently, sections were incubated with the primary antibody: either a mouse monoclonal anti-human α-SMA Ab (M0851, Dako, Heverlee, Belgium) and/or a polyclonal rabbit anti-cow cytokeratin Ab (Z0622, Dako), followed respectively by a secondary goat anti-mouse Ab labelled with a green fluorescent dye (A-11017, Invitrogen, Merelbeke, Belgium) and/or a goat anti-rabbit Ab labelled with a red fluorescent dye (A-11072, Invitrogen). Sections were incubated shortly with a 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (Invitrogen) and finally mounted with Vectashield mounting medium (Labconsult, Brussels, Belgium).

Morphometric analysis

Morphometric measurements of the stainings were made by a blinded operator with an Olympus BX41 microscope (Olympus, Aartselaar, Belgium) at magnification x400. From each experimental animal, three peritoneal samples were analysed. For each sample, three sections were analysed quantitatively with a computerized image analysis system (CellD software, Olympus). For the Sirius Red staining, the total amount of connective tissue (%) in the VP was determined. For the Masson's trichrome staining, the thickness of the PP was measured. The double α-SMA/cytokeratin staining was viewed with a fluorescence microscope (Axioscooop, Zeiss, Germany) and pictures were taken using CellF Software (Olympus Soft Imaging Solutions, Germany). A semi-quantitative assessment was performed independently by two blinded operators. Each section was screened to estimate the extent and distribution of colocalization of α-SMA and cytokeratin. Staining results were classified from 0 to 3: 0 = no, 1 = mild-, 2 = moderate- and 3 = pronounced colocalization and migration of mesothelial cells into the interstititium. The results were calculated as the mean of the individual scores of the two operators for each sample.
3.1. Dietary salt intake and peritoneal membrane morphology

**IL-6/TGF-β1/VEGF mRNA determination**

Tissues were homogenized in TRI-Reagent (AB, Applied Biosystems, Foster City, CA, USA) on ice using a PowerGen 125 Tissue Homogenizer (Fisher Scientific). An aliquot of homogenate was separated into aqueous and organic phases by chloroform (Sigma) addition and centrifugation. RNA was precipitated from the aqueous phase by addition of isopropanol (Sigma), washed with ethanol (MERCK) and solubilized. Concentration and purity of the extracted RNA were determined by spectrophotometry (UV-DU64 spectrophotometer, Beckman). Each sample was confirmed for integrity using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription was performed by using the High Capacity cDNA Reverse Transcription kit (AB). Thermal cycling conditions were 25°C (10'), 37°C (120'), 85°C (5") and 4°C (∞). All cDNA samples were stored at −20°C until analysis.

The expression of IL-6, TGF-β1 and VEGF mRNA was quantified by using the 7900HT Fast Real Time PCR System (AB). The thermal profile consisted of two hold steps, one at 50°C (2') and one at 95°C (10'), followed by 40 cycles × [95°C (15''), 60°C (1')]. RT–PCR efficiencies for each assay were calculated using the formula: Efficiency = [10(1/slope)] − 1. Samples and endogenous control were amplified in separate wells in a 96-well plate. The samples were run in triplicate and normalized to actin glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels, which was used as the endogenous control (reference gene). Pre-designed and labeled primer/probe sets were purchased from AB (TaqMan® Gene Expression Assays). The relative expression compares mRNA expression levels of the genes of interest (IL-6, TGF-β1, VEGF) to the expression levels of the endogenous reference gene (Ref; GAPDH) according to the ΔΔCt method. In this method, the cycle in which the fluorescence level crosses a threshold value of fluorescence, during the exponential phase of amplification, is determined. As the fluorescence is directly correlated to the amount of double-stranded DNA present in each amplification cycle, the number of cycles needed to reach this level can therefore be used to calculate relative amounts of starting transcript mRNA. These values are expressed as relative values to an endogenous reference (= an internal control gene) to correct for differences in transcription rate and sample size between animals. Normalized relative quantity (NRQ) values were calculated using the following formula, as described previously by Pfaffl [26]:

\[
NRQ = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}} (\text{control-sample})}{(E_{\text{ref}})^{\Delta Ct_{\text{ref}}} (\text{control-sample})}
\]
NRQ values from HS rats were compared to those from NS rats and expressed as relative increases (fold increase) between groups.

Statistical analysis

Data analysis was performed with SPSS version 15.0 (SPSS Inc., Chicago, USA). Normal distribution of data was tested using Kolmogorov–Smirnov testing. Data are accordingly presented as mean ± standard deviation (SD). Normally distributed data were compared with the Student's t-test for independent samples. P-values < 0.05 (two-tailed probability) were considered as significant.

The increase in relative mRNA expression between NS and HS was calculated, and 95% confidence intervals (CI) were determined. Hereto, the natural log transformation of the NRQ was calculated, the difference of the mean between the test group and the control group was calculated, and this value was used as the exponent of 2, resulting in the fold increase with its corresponding 95% CI. For the NS group, data of the NS were used both for test and control, which theoretically should result in a mean difference of zero, and thus a 1-fold increase in mRNA expression.
3.1.4. Results

**General data of laboratory animals (Table 1)**

Mean body weight of the rats was 213 ± 7 g. After 2 weeks, rats of the HS group had a lower weight compared with the rats of the NS group. Blood pressure after 2 weeks was not different between the HS and NS groups. Haematocrit levels were significantly lower in the HS group after 2 weeks. Mean daily water intake in the HS group was 62.5 ml/rat, resulting in an extra dietary salt load of 1.25 g/day/rat.

<table>
<thead>
<tr>
<th></th>
<th>Normal Salt</th>
<th>High Salt</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial Body Weight (g)</strong></td>
<td>211.9 ± 9.1</td>
<td>214.0 ± 5.9</td>
<td>p = NS</td>
</tr>
<tr>
<td><strong>Body Weight (g) after two weeks</strong></td>
<td>234.9 ± 19.1</td>
<td>217.2 ± 11.5</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td><strong>Blood Pressure after two weeks (mmHg)</strong></td>
<td>140.4 ± 22.0</td>
<td>137.5 ± 15.3</td>
<td>p = NS</td>
</tr>
<tr>
<td><strong>Haematocrit level (%)</strong></td>
<td>46.3 ± 1.7</td>
<td>44.2 ± 1.8</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

**Peritoneal morphology**

Sirius Red staining of collagen was significantly more pronounced in the HS rats than in the NS rats. This was evident both in the submesothelial compact zone and in the interstitial tissue (18.8 ± 3.5 vs 24.7 ± 5.8% of total tissue in the NS vs HS group, respectively; P < 0.01) (Figures 1 and 2). The Masson's trichrome staining showed a significant thickening of the submesothelial layer of PP in the HS group (13.7 ± 3.2 vs 18.7 ± 3.7 μm in the NS vs HS group, respectively; P < 0.001) (Figures 3 and 4).

Staining for the epithelial marker cytokeratin was confined to the mesothelial cell layer in all NS animals. In the HS animals, an extensive additional staining was observed in the submesothelial tissue. Staining for α-SMA was limited to the muscularis of the blood vessels in all the NS rats, but was also found in submesothelial areas in HS rats (Figure 5). Double α-SMA/cytokeratin staining with fluorescence was virtually absent in all the NS animals, but was significantly prominent in HS animals (score 0.25 ± 0.25 vs 1.22 ± 0.32 in the NS vs HS group, respectively; P < 0.001) (Figure 6).
Figure 1.
Sirius Red Staining of collagen in the visceral peritoneal membrane (magnification x400). Prominent submesothelial and interstitial fibrosis was observed in the ‘high salt’ (C & D) animals, compared with the ‘normal salt’ animals (A & B).

Figure 2.
The amount of connective tissue in the visceral peritoneal membrane was significantly different between the ‘normal salt’ (18.8 ± 3.5%) group and the ‘high salt’ (24.7 ± 5.8%) group (*P < 0.01).
Data are expressed as mean ± SD for groups of 14 rats.
3.1. Dietary salt intake and peritoneal membrane morphology

Figure 3.
Masson’s trichome staining of the parietal peritoneal membrane (magnification x400). The submesothelial thickness of the parietal peritoneum was increased in the ‘high salt’ (HS, panel B) group vs the ‘normal salt’ group (NS, panel A).

Figure 4.
Submesothelial thickness of the parietal peritoneal membrane was significantly different between the ‘normal salt’ (13.7 ± 3.2 µm) group and the ‘high salt’ (18.7 ± 3.7 µm) group (P < 0.001).
Data are expressed as mean ± SD for groups of 14 rats.
Chapter 3: Exposure to a forgotten uraemic toxin

Figure 5. α-SMA-, Cytokeratin- and a double staining for α-SMA and Cytokeratin (magnification: x400). Serial sections of the visceral peritoneum from ‘normal salt’ (controls) rats (A-C) and ‘high salt’ rats (D-I) were stained for α-SMA (A,D,G), cytokeratin (B,E,H) and double stained for α-SMA and cytokeratin (C,F,I). In the control rats, only vascular smooth muscle cells stain for α-SMA (A), only mesothelial cells stain for cytokeratin (B) and no α-SMA/cytokeratin colocalization (C) occurs. In the peritoneal membrane of the ‘high salt’ animals, α-SMA staining is found not only in the vascular smooth muscle layer of blood vessels, but also in submesothelial tissue (G). Cytokeratin staining (E) is visible in the mesothelial cells and is additionally found in the interstitial tissue. Co-localization of α-SMA and cytokeratin (F,I) is pronounced in submesothelial tissue. Thick arrow, colocalization.

Figure 6. Semi-quantitative scoring for colocalization of α-SMA and cytokeratin. Double α-SMA/cytokeratin staining was virtually absent in the NS animals, but was significantly prominent in the HS animals (score 0.25 ± 0.25 vs 1.22 ± 0.32, in the NS vs HS group respectively, P < 0.001). Scoring was done by two independent blinded observers for three samples of each experimental animal as 0, no colocalization; 1, mild colocalization; 2, moderate colocalization; 3, strong colocalization.
3.1. Dietary salt intake and peritoneal membrane morphology

**mRNA expression of IL-6, TGF-β₁ and VEGF**

Expression of IL-6 mRNA was upregulated 4.25 (95% CI 2.22–8.13) times in the HS versus the NS group in the VP, and 1.94 (95% CI 1.37–2.75) times in the PP. Expression of TGF-β₁ mRNA was upregulated 2.10 (95% CI 1.31–3.37) times in the HS vs the NS group in the VP, and 1.32 (95% CI 1.08–1.60) times in the PP. Expression of VEGF mRNA was not upregulated in the VP nor in the PP (Figure 7).

![Figure 7](image)

**Figure 7.** mRNA expression of IL-6, TGF-β₁ and VEGF: fold change high salt (HS) versus normal salt (NS). ‘Normal salt’ group = control group, taken as a standard. Fold change of the NS group = 1. In the ‘high salt’ group, expression of IL-6 mRNA was 4.25 times [CI: (2.22, 8.13)HS vs (0.60, 1.65)NS] more upregulated in the visceral peritoneum (VP) and 1.94 times [CI: (1.37, 2.75)HS vs (0.71, 1.39)NS] more upregulated in the parietal peritoneum (PP). TGF-β₁ mRNA expression was 2.1 times [CI: [1.31, 3.37]HS vs [0.79, 1.26]NS] more upregulated in the VP and 1.32 times [CI: (1.08, 1.60)HS vs (0.75, 1.31)NS] more upregulated in the PP. VEGF mRNA expression was 1.74 times [CI: (0.97, 3.12)HS vs (0.81, 1.22)NS] higher in the VP, but was not different between the two groups in the PP [CI: (0.84, 1.00)HS vs (0.83, 1.19)NS].

3.1.5. Discussion

This study demonstrates that dietary salt intake by itself induces EMT of mesothelial cells as identified by colocalization of cytokeratin and α-SMA in the submesothelial layer, fibrosis of the PM as documented by Sirius Red staining and a thickening of the submesothelial layer on Masson's trichrome staining. Concordant with this, peritoneal TGF-β₁ and IL-6 mRNA expression were increased, but not VEGF mRNA expression. These observations suggest that dietary salt loading induces EMT and peritoneal fibrosis, potentially by upregulation of TGF-β₁ and IL-6 mRNA.

It is now progressively accepted that EMT underlies epithelial degeneration and fibrogenesis in some chronic degenerative, fibrotic disorders, in particular of the heart [15], the kidney [11], the lung [14] and the liver [13]. TGF-β₁ is able to induce all the basic steps of EMT: loss
of epithelial adhesion properties, de novo α-SMA expression and actin reorganization, disruption of basement membrane and enhanced cell migration and invasion capacity [27].

Also in the PM, there are several lines of evidence that TGF-β1 plays a pivotal role in EMT and enhanced fibrosis. Margetts et al. used an adenoviral vector to increase TGF-β1 expression in a rat model of PD [24]. By day 28, a substantial thickening of the PM was observed. Further experiments indicated that overproduction of TGF-β1 resulted in an increase in expression of genes associated with EMT and fibrosis, such as those regulating type I collagen A2, α-SMA and the zinc finger regulatory protein Snail [25]. Seven to fourteen days after exposure to TGF-β1, appearance of epithelial cells in the submesothelial zone could be demonstrated. This phase was associated with disruption of the basement membrane and increased expression of matrix metalloproteinase 2.

In our experiments, there was clear EMT of the peritoneal membrane in the rats fed with a high salt diet, as we observed colocalization of cytokeratin and α-SMA, as a hallmark of transdifferentiation of mesenchymal cells. In addition, these cells were localized in the submesothelial cell layer, as a sign of their transgression through the basal membrane with beginning invasion of the extracellular matrix.

To our knowledge, this is the first observation of a link between dietary salt intake and EMT of the peritoneal membrane.

So far, it is unclear how salt upregulates TGF-β1 expression. Ying and Sanders suggested that salt-induced shear stress in glomeruli activates tetraethylammonium-sensitive potassium channels, resulting in enhanced TGF-β1 production [7]. More recently, the same group demonstrated that dietary salt induces the activation of proline-rich tyrosine kinase-2 (Pyk2) and identified c-Src as an important binding partner of Pyk2 in dietary salt-mediated production of TGF-β1. Their data support the hypothesis that activation of Pyk2 recruits and activates c-Src and that this complex participates integrally in the vascular production of TGF-β1 in response to dietary salt in the rat [28]. Others have made a link with digitalis-like substances, such as marinobufagenin, which tend to be upregulated by salt loading [29,30] and result in enhanced formation of procollagen in the heart [31]. Digitalis-like substances block the Na/K-ATPase pumps, and thus increase the intracellular Ca^{2+} concentration, which can activate calcium-dependent and downstream pro-fibrotic pathways.

Angiotensin II stimulates extracellular matrix protein synthesis through induction of TGF-β in rat mesangial cells [32]. In addition, there is accumulating evidence that intracellular
angiotensin II plays an important role in renal cellular growth and fibrotic responses by activating NF-κB signalling, which is also on the final common pathway of the TGF-β1 pathway [33]. Several studies have demonstrated that high salt intake decreases circulating levels of angiotensin II, but activates the tissue renin-angiotensinaldosterone system (RAAS) [34,35]. In salt-sensitive rats, a high salt intake resulted in increased intrarenal RAAS activity, associated with renal hypertrophy, fibrosis and damage [34]. Liang and Leenen demonstrated that fibrosis under these conditions of salt loading and high intrarenal RAAS activity could be prevented by angiotensin I converting enzyme (ACE)-inhibiting drugs [35]. This might explain why use of ACE inhibitors has a positive impact on PM morphology [36].

Another potential mechanism is that the creation of local hyperosmolality in the gut activates a tonicity-responsive enhancer binding protein (TonEBP)-mediated response. TonEBP activates osmoprotective genes to ensure cell function in hostile environments with increased interstitial tonicity, such as the renal medulla [37] and the lymphatic system [38]. A recent paper [39] showed that high salt intake increases protein expression of e.g. VEGF in macrophages in the subcutaneous tissue through activation of TonEBP. If such a hyperosmolarity-driven response would exist in the gut, it would be conceivable that this can be one of the mechanisms of EMT and the changing peritoneal morphology during long-term PD, where the peritoneum is exposed to hypertonic solutions.

Further elaboration of this exciting hypothesis is certainly warranted, as it would imply that changing glucose for another hyperosmolar osmotic agent will not avoid the long-term peritoneal damage observed during PD.

Next to TGF-β1, IL-6 expression is also linked to PM degeneration and fibrosis [40] and this was the case as well in the present study.

It is not clear how salt intake upregulates IL-6 expression. It might be that upregulation of TGF-β1 leads to upregulation of IL-6. In vitro experiments already showed that TGF-β1 can induce IL-6 production in human myoblasts in a dose- and time-dependent manner [41]. This finding is in agreement with studies which report similar in vitro results in other cell types: TGF-β1 increases IL-6 mRNA levels in cultured thymus epithelial cells [42] and astrocytes [43] and IL-6 protein secretion in bone marrow stromal cells [44]. A recent paper by Leung et al. showed no clear association between upregulation of TGF-β and IL-6 in cultured human peritoneal mesothelial cells (HPMCs)[40]. However, the subtle and complex interplay of different cell types in vivo cannot be completely mimicked in vitro with one single cell type. In our experiments, there was a slight upregulation of VEGF mRNA in the visceral, but not in
the parietal membrane. This could point to a mechanism where the upregulation of TGF-β₁ and IL-6 induces upregulation of VEGF, as in the study by Margetts et al., [45] rather than to a direct upregulation of VEGF by the enhanced dietary salt intake. Also here, upregulation of TonEBP, by creation of a hypertonic environment in the gut, might be involved [39].

Adipocytes are ubiquitous in peritoneal tissue, and it is hypothesized that they can be an important source of cytokine secretion, including IL-6 and TGF-β [46,47]. In our study, salt intake induced a more pronounced upregulation of IL-6 and TGF-β₁ expression in the VP, where adipocytes are abundant, as compared to the PP, where less adipocytes are present.

Many experiments considering well-defined signaling pathways, e.g. in an *in vitro* setting, use purified stimulating factors in high concentrations, which do not represent the biological situation where a complex interplay of different pathways and cells is possible.

In our model, the only intervention was an increased dietary salt intake, but nevertheless, the resulting effects on EMT were still as impressive.

Of course, the pathways leading to these observations need further elaboration. Potential interventions are the use of inhibitors of the renin-angiotensin system, of TGF-β₁ and/or of TonEBP. Also the potential role of adipocytes and of macrophages infiltrating adipose tissue needs further exploration.

It is surprising that the effects of salt intake appeared so rapidly, after only 2 weeks of exposure. However, Ying and Sanders [7] also demonstrated enhanced renal fibrosis and glomerulosclerosis linked to upregulation of TGF-β after 15 days of salt loading in rats, and effects on the vasculature even after 4 days [48]. Machnik et al. [39] found important changes in the subcutaneous tissue after 2 weeks of salt loading in rats. All these experiments point out that effects of high salt intake seem to appear very rapidly.

Finally, the question arises in how far our findings impact the application of PD which is a well-established renal replacement modality.

Unfortunately, its longevity as a technique is restricted by functional and morphological deterioration of the PM over time [16,18,19,49]. It has always been puzzling why some patients do and others do not develop such morphological alterations. Although deterioration of the PM is mostly attributed to the exposure to PD fluids and peritonitis [16,50–52], large differences in PM structure and function can be found already at the start of PD, as is apparent both from morphological studies, such as the PD biopsy registry [19], and from functional
3.1. Dietary salt intake and peritoneal membrane morphology

studies [16,53]. So far, this has been explained by differences in comorbidities such as diabetes, genetic background and/or uraemia [54–56].

Hence, the changes over time of the PM in PD patients are not exclusively induced by the exposure to PD fluids and inflammation. Our study adds dietary salt intake to the underlying mechanisms, even without exposure to PD fluids. This finding becomes even more relevant in view of the need for more hypertonic glucose, which by itself is damaging to the PM, to counter volume overload in the case of high salt intake, thus initiating a synergistic pathway to a faster deterioration of the PM. This synergism needs further investigation.

As such, our findings of peritoneal fibrosis and EMT of mesothelial cells induced by dietary salt intake are of direct clinical relevance for patients on PD. Dietary salt restriction is thus of importance in patients with chronic kidney disease, even during the pre-dialysis phase.

In conclusion, dietary salt intake in non-uraemic rats induced EMT and peritoneal fibrosis. This was correlated with an upregulation of TGF-β1 and IL-6 mRNA, which could be the link between dietary salt intake and EMT.

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Conflict of interest.

The results presented in this paper have not been published previously in whole or part, except in abstract form.

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3.1. Dietary salt intake and peritoneal membrane morphology


CHAPTER 4

ACUTE PERITONEAL EXPOSURE TO URAEMIC TOXINS USING THE INTRAVITAL MICROSCOPE TECHNIQUE
4.1. **AN UPDATE ON PROTEIN-BOUND URAEMIC RETENTION SOLUTES**

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4.1. Update on protein-bound uraemic retention solutes

4.1.1. Abstract

Although protein-bound uremic retention solutes are recognized as 1 of the 3 main categories of uremic retention solutes, they only recently have been submitted to thorough analysis. *In vitro* and *ex vivo* data link both p-cresyl sulfate and indoxyl sulfate, two of the main compounds of this solute group, to negative impact on the cardiovascular system and progression of kidney failure. Recent *in vivo* observational data also relate concentration of these compounds to survival outcome, inflammation, and vascular disease in different, even moderate, stages of chronic kidney disease.

Removal by different dialysis strategies, even high-flux hemodialysis, is difficult, and only by applying convection, some improvement of removal has been obtained. The other strategy with the potential to decrease concentration is by influencing intestinal generation and/or absorption. The sorbent Kremezin (AST-120) has been shown in controlled studies to decrease protein-bound solute concentration. In pilot controlled studies, AST-120 has been shown to be superior on outcome parameters to placebo. Results from large randomized trials are awaited, before these data can be considered as solid enough to warrant the recommendation to use these compounds for overall therapeutic purposes.

4.1.2. Introduction

For reasons of convenience, uremic retention solutes are subdivided into 3 groups, depending on the physicochemical characteristics influencing their removal by dialysis: (1) the small water-soluble compounds, with urea as a prototype; (2) the larger peptides (molecular weight > 500 Da), also named middle molecules, with β2-microglobulin as a prototype; (3) the proteinbound solutes, with the groups of indolic and phenolic compounds as prototypes. Among these, the protein-bound solutes have been the ones to receive the least attention until lately, possibly because strategies enhancing their removal were scanty and became identified only recently.

In this review, we will summarize the most important *in vitro* and *in vivo* data suggesting their toxicity; in the second part of this text, we will discuss therapeutic options for enhancing their removal and/or decreasing their concentration.
4.1.3. *In Vitro and In Vivo* Data

A host of protein-bound retention solutes have been identified, mostly during recent years, and many of these interfere with essential biochemical functions (Table 1). In this section, we will essentially focus on p-cresyl sulfate and indoxyl sulfate, the two protein-bound compounds that have recently received most attention.

### Table 1. Protein-Bound Solute Bioactivity

<table>
<thead>
<tr>
<th>Solute</th>
<th>Biological Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGEs</td>
<td>Inflammation, vascular disease</td>
</tr>
<tr>
<td>CMPF: protein binding of drugs, detoxification, neuropathy, anemia</td>
<td></td>
</tr>
<tr>
<td>Cytokines</td>
<td>Inflammation, malnutrition, anemia</td>
</tr>
<tr>
<td>Dimethylguanidine</td>
<td>Inhibition calcium ATPase</td>
</tr>
<tr>
<td>Hippuric acid: protein binding of drugs, glucose intolerance</td>
<td></td>
</tr>
<tr>
<td>Homocysteines: vessel disease, detoxification</td>
<td></td>
</tr>
<tr>
<td>Indole-3-acetic acid: protein binding of drugs, neuropathy, cytotoxicity</td>
<td></td>
</tr>
<tr>
<td>Indoxyl sulfate: declines renal function, thyroid function, protein binding of drugs, detoxification, and endothelial function and repair: induces oxidative stress, osteoblast resistance to PTH, smooth muscle cell proliferation, and aortic calcification</td>
<td></td>
</tr>
<tr>
<td>Kinurenine: neuropathy</td>
<td></td>
</tr>
<tr>
<td>Leptin: malnutrition</td>
<td></td>
</tr>
<tr>
<td>p-cresyl sulfate: inflammation, free radical production, endothelial microparticle release</td>
<td></td>
</tr>
<tr>
<td>Phenols: immune dysfunction, neuropathy</td>
<td></td>
</tr>
<tr>
<td>Phenylacetic acid: inhibition of nitric oxide synthesis</td>
<td></td>
</tr>
<tr>
<td>Quinolinic acid: neuropathy</td>
<td></td>
</tr>
</tbody>
</table>

AGE, advanced glycation end product; CMPF, carboxymethyl-propyl-furanpropiolic acid; PTH, parathyroid hormone.

**Note:** These protein-bound solutes are at the same time middle molecules (molecular weight >500 Da) and protein bound.

#### 4.1.3.1. p-Cresyl Sulfate

Until recently, studies on cresol and its derivatives had been focusing on p-cresol because it was believed to be the main compound of this family circulating in the body in uremia. A few years ago, it became apparent that the generation of p-cresol in uremic plasma was an artifact, due to hydrolysis of more complex conjugates as a consequence of sample acidification for deproteinization.

In reality, conjugates were present, of which p-cresyl sulfate was the most important one. While p-cresol had been shown to be an immune inhibitor, p-cresyl sulfate essentially activates leukocytes, pointing to the potential of this compound to contribute to vascular
4.1. Update on protein-bound uraemic retention solutes

damage. Further studies also revealed a role of p-cresyl sulfate in endothelial microparticle release, another indicator of vascular damage.\textsuperscript{9}

Observational trials demonstrated a relationship between the concentration of p-cresol, in that setting acting as a surrogate for p-cresyl sulfate, and clinical outcomes.\textsuperscript{10,11} Only recent studies showed a similar relationship with outcomes for the genuine compound, p-cresyl sulfate.\textsuperscript{12} A study by Wang \textit{et al.} also demonstrated a similar relation with coronary artery disease in patients with no or only moderate degrees of renal failure,\textsuperscript{13} suggesting that protein-bound solutes might play a role as well in populations not suffering of advanced stages of chronic kidney disease.\textsuperscript{14}

Interestingly, a recent study by Eloot \textit{et al.} demonstrated an extremely poor correlation between the concentration of p-cresyl sulfate and estimated glomerular filtration rate (eGFR), pointing to the fact that concentration might be influenced not only by glomerular filtration rate but also by other, essentially metabolic, factors (see later text).\textsuperscript{15}

4.1.3.2. Indoxyl Sulfate

The indole indoxyl sulfate has been linked \textit{in vitro} to almost every element at play in the generation of cardiovascular damage: the compound is proinflammatory\textsuperscript{16}; increases osteoblastic resistance to parathyroid hormone,\textsuperscript{17} which might contribute to vascular calcification; increases endothelial microparticle release\textsuperscript{18}; increases the adhesion of leukocytes to the endothelium\textsuperscript{19}; enhances the proliferation of smooth muscle cells\textsuperscript{20}; and increases cardiac fibrosis.\textsuperscript{21} \textit{Ex vivo}, it increases vascular calcification \textit{per se} in Dahl salt-sensitive hypertensive rats.\textsuperscript{22} Indoxyl sulfate has also been related to kidney damage and progression of renal failure.\textsuperscript{16}

Hence, these \textit{in vitro} and \textit{ex vivo} data point to an important impact of indoxyl sulfate on many aspects of cardiovascular disease. The question can be raised whether this is translated into clinical data \textit{in vivo}. Remarkably enough, this aspect has been studied only recently. In a study by Barreto \textit{et al.}, indoxyl sulfate was related both to aortic calcification and mortality.\textsuperscript{23} Lee \textit{et al.} demonstrated a correlation between indoxyl sulfate and interleukin-6.\textsuperscript{24}
4.1.4. Removal

4.1.4.1. Dialysis and Related Strategies

Up till recently, data on the removal of the protein-bound compounds have been deceiving. High-efficiency dialysis by high-flux membranes was not superior to standard low-flux dialysis, and long slow dialysis, which had a definite impact on the removal of so-called difficult-to-remove compounds, such as phosphate and $\beta_2$-microglobulin, had no impact on protein-bound molecules. On the contrary, daily dialysis resulted in a long-term decrease of predialysis concentration. Only recently it was demonstrated that convective strategies under optimal conditions could remove more protein-bound compounds than conventional or high-flux hemodialysis: compared with low-flux hemodialysis, predilution on-line hemofiltration was superior for decreasing the predialysis concentration of several protein-bound molecules; in acute experiments, convective strategies were superior to high-flux hemodialysis with regard to intradialytic reduction rates; postdilution hemodiafiltration was superior to predilution hemofiltration; and in the long term, postdilution hemodiafiltration decreased predialysis concentration of protein-bound molecules, as compared with high-flux hemodialysis. Although most of these effects were significant, the long-term impact on concentration was only moderate, leaving openings for alternative removal strategies.

4.1.4.2. Absorption/Adsorption

One aspect of protein-bound solute removal that has been assessed successfully during recent years has been related to methods pursuing elimination of these solutes through the intestine. In fact, both p-cresol and indole are generated in the intestine after metabolism of amino acids by the intestinal flora. Owing to changes in assimilation, generation of these mother compounds is even increasing as renal function regresses. They are then further conjugated in the intestinal wall and/or the liver to the currently known sulfates and glucuronides.

Protein-bound solute concentration can be decreased through this intestinal process by dietary protein restriction, by changing metabolic capacity of intestinal flora after administration of probiotics or prebiotics, and by therapy with specific sorbents. Among these therapies, protein restriction has been shown to be efficient; however, because of the risk of malnutrition, it is considered with care. More appealing approaches are
4.1. Update on protein-bound uraemic retention solutes

prebiotics and probiotics.\textsuperscript{36,37} All these approaches decrease the concentration of protein-bound solutes, but none of them have been demonstrated to improve hard outcome parameters.

However, the sorbent AST-120 (Kremezin, Kureha, Tokyo, Japan) not only impacts on the concentration of indoxyl sulfate\textsuperscript{38} and of other protein-bound solutes,\textsuperscript{39} but, more importantly, also was shown to postpone the start of dialysis,\textsuperscript{40} the progressive decline of eGFR,\textsuperscript{41} and survival outcomes after dialysis was started.\textsuperscript{42} Although most of these studies are of limited extent, and some of them may be flawed, they all point into the same direction; therefore, results of more extended controlled studies are anxiously awaited.

It is conceivable that extracorporeal adsorptive strategies could also play an important role in this context. Pilot studies on fractionated plasma separation and adsorption (Prometheus [Fresenius Medical Care, Bad Homburg, Germany]—an artificial liver setup) in patients with chronic kidney disease showed a strong adsorptive impact on protein-bound uremic solutes by the resins involved in this strategy.\textsuperscript{43} However, this effect was jeopardized by a strong risk for thrombotic complications, probably due to simultaneous elimination of antithrombotic factors.\textsuperscript{44}

4.1.4.3. Peritoneal Dialysis

In peritoneal dialysis, absolute removal of protein-bound molecules such as indoxyl sulfate and p-cresyl sulfate is substantially lower than with hemodialysis.\textsuperscript{45,46} Nevertheless, plasma concentrations are the same (indoxyl sulfate)\textsuperscript{45} or even lower (p-cresyl sulfate),\textsuperscript{45,46} suggesting that elements other than dialysis removal are at play, such as intestinal or metabolic factors.\textsuperscript{47}
4.1.5. Conclusions

More and more data suggest a cardinal role of protein-bound uremic toxins in uremic toxicity and especially in cardiovascular damage. Translating these in vitro and ex vivo findings into the clinical situation also provides more and more data suggesting an association between protein-bound solutes and outcome.

Removal by dialysis strategies is limited but can be optimized by applying convection. Alternative options to be considered are altering intestinal metabolism and/or applying adsorption. Especially, controlled data obtained with the sorbent Kremezin suggest a positive impact of adsorption of protein-bound solutes on outcome parameters; therefore, results of more extended controlled trials are anxiously awaited.

Metabolic factors play a remarkable role in influencing concentration, as also suggested by the highly different degrees of correlation between the concentration of these solutes and eGFR.

4.1.6. Practical Application

Identification of protein-bound uremic retention solutes and their pathophysiologic role has resulted in their classification according to importance. The next step is to evaluate whether improved removal of the most relevant solutes would result in better outcomes.

4.1.7. References


4.1. Update on protein-bound uraemic retention solutes


4.1. Update on protein-bound uraemic retention solutes


4.2. In vivo effects of the protein-bound uraemic toxins indoxylsulfate, p-cresylsulfate, and p-cresylglucuronide on the crosstalk between leukocytes and the vessel wall

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4.2. In vivo effects of protein-bound uraemic toxins

4.2.1. Abstract

Both leukocyte activation and endothelial damage contribute to cardiovascular disease, the principal cause of morbidity and mortality in chronic kidney disease. In vitro and in vivo data link several protein-bound uremic retention solutes to cardiovascular damage. However, these models never took into account the complicated cross-talk between endothelium and leukocytes. This study evaluated the effects of acute exposure to indoxylsulfate (IS), p-cresylsulfate (pCS) and p-cresylglucuronide (pCG) on the recruitment of circulating leukocytes in the rat peritoneal vascular bed, using intravital microscopy.

The visceral peritoneum of rats was superfused by a physiological solution (HBSS) with or without a uremic toxin at concentrations in the high uremic range. Leukocyte recruitment and hemodynamic parameters were analyzed in postcapillary venules.

IS induced very strong leukocyte adhesion and extravasation and interrupted blood flow. pCS caused an immediate increase in rolling. The combination of both pCS and pCG had no additive effect to pCS alone on leukocyte recruitment, but caused impaired blood flow and leakage of the vessels.

These results provide for the first time clear in vivo evidence that IS, pCS and pCG exert proinflammatory effects that could contribute to vascular damage by stimulating cross-talk between leukocytes and vessels. In addition, IS and the combination of pCS and pCG induced unusual patterns of blood flow.
4.2.2. Introduction

Cardiovascular disease (CVD) remains an important cause of death among patients with chronic kidney disease (CKD)\(^1\) and is associated with a baseline inflammatory status.\(^2,3\) Atherosclerosis is highly prevalent and advances more rapidly in individuals with renal dysfunction compared to the general population.\(^4,6\) A key role in the development of atherosclerosis is played by leukocyte-endothelial interactions.\(^2\)

CKD is characterized by the progressive retention of a host of solutes. A substantial number of these compounds is protein-bound.\(^7,8\) The indole indoxylsulfate (IS) and the phenolic conjugates p-cresylsulfate (pCS) and p-cresylglucuronide (pCG) are prototype members of this group. In vitro and in vivo data link these solutes to several isolated molecular mechanisms associated with cardiovascular damage and progression of kidney failure.\(^8,12\) Although for all three compounds two key mechanisms of vascular damage, leukocyte activation and endothelial dysfunction, have been demonstrated in separate in vitro models, they were to the best of our knowledge never directly assessed by evaluating the complicated cross-talk between endothelium and leukocytes in vivo. Intravital microscopy permits in vivo visualization of leukocyte recruitment in translucent tissues in real time. This technique was already applied successfully by our group to study harmful effects of peritoneal dialysis solutions on peritoneal membrane physiology.\(^13\)

The present study evaluates the effects of an acute exposure to the protein-bound uremic compounds IS, pCS or pCG on the recruitment of circulating leukocytes in the rat peritoneal vascular bed, using this intravital microscopic method.
4.2.3. Results

Leukocyte recruitment in response to lipopolysaccharides (LPS)

Before observing leukocyte recruitment in response to uremic solutes, we first evaluated the effect of lipopolysaccharide (LPS) as a positive control. As represented in figure 1, there were no differences in baseline values between the control-group and the LPS-group.

Stimulation with LPS (n=8) dramatically and progressively increased the number of rolling leukocytes (A-1) compared to control (n=12), from 30 minutes on (36.6 ± 9.4 vs 14.6 ± 2.3, p<0.01), reaching a number of 90.4 ± 9.0 rolling leukocytes after 120 minutes exposure to LPS (p<0.01 vs control). The number of adhering leukocytes (B-1) was significantly higher versus control after 60 min (3.7 ± 0.8 vs 1.8 ± 0.3, p<0.05), reaching a number of 7.6 ± 1.4 after 120 min exposure to LPS (p<0.05 vs control). The number of extravasating leukocytes (C-1) increased progressively from 30 min on (425 ± 59 vs 247 ± 36, p<0.05) resulting in a total number of 1574 ± 237 at 120 min (p<0.05 vs control).

Figure 2 illustrates the increasing number of rolling, adhering and extravasated leukocytes after exposure to LPS at different time periods.

Leukocyte recruitment in response to IS

Baseline values were not different between the control-group and the IS-group, as represented in figure 1.

Exposure to IS (n=9) provoked similar effects as with the LPS-induced recruitment, except for the number of rolling leukocytes (A-2). A progressive increase in the number of adhering leukocytes (B-2) was observed (9.9 ± 2.7 vs 3.5 ± 0.3 at 120 min, p<0.05). A pronounced and progressive effect of IS on the number of extravasating leukocytes (C-2) was observed, reaching significance after 30 min exposure (440 ± 79 vs 247 ± 36, p<0.05), and further increasing until 120 min (1120 ± 89 vs 655 ± 147, p<0.05).

Leukocyte recruitment in response to pCS and the combination of pCS and pCG

No difference in baseline values between the control group and the experimental groups was observed, as shown in figure 1 and illustrated in a representative experiment in figure 2.
Compared to control (n=12), superfusion with pCS (n=8) caused an almost immediate but moderate increase in the number of rolling leukocytes (A-3) after 10 min (25.5 ± 5.4 vs 13.2 ± 2.6, p<0.05) with a maximal effect after 60 min exposure (44.3 ± 12.7 vs 14.3 ± 2.2, p<0.01); subsequently rolling stagnated. The combination of pCS and pCG (n=6) induced a similar effect on rolling, be it not significant; thus, no additive effect of combining the two compounds was observed. No effect was seen on the number of adhering leukocytes (B-3) in the presence of pCS, nor in the presence of the combination of pCS and pCG. Although a trend towards an increase in the number of extravasating leukocytes (C-3) in the presence of pCS and pCSpCG was observed, the increase was not significant.

Figure 1: Leukocyte recruitment in response to LPS (1), IS (2), pCS and pCSpCG (3)

The number of rolling (A), adhering (B) and extravasated (C) leukocytes at different time points during superfusion by HBSS (■, full black line, n=12), HBSS with LPS (●, full grey line, n=8), HBSS with IS (♦, dotted line, n=9), HBSS with pCS (▲, broken line, n=8) and HBSS with pCS & pCG (▼, dotted line, n=6). *p<0.05; **p<0.01; ***p<0.001 versus HBSS.
Figure 2: Images of intravital microscopy

Leukocyte rolling (black arrows), adhesion (white arrows) and extravasation (white circles) in response to LPS, pCS or the combination of pCS and pCG at baseline (t=0 min) and after 60 and 120 min of exposure.

Remark: The amount of symbols is not representative for the exact number of leukocytes with corresponding characteristics present in the panels. Moreover, it is impossible to see the difference between rolling and adhering leukocytes on these static pictures. A short movie illustrating the different elements is posted online.
**Effects on blood flow**

In 6 out of 9 IS treated rats, an interruption of blood flow was observed, as illustrated in figure 3 and figure 4-A. In 4 rats, flow stopped permanently after 30 - 60 min (figure 3-A). A strong decrease in red blood cell velocity (\(V_{\text{RBC}}\)) was detected after 10-20 min in 2 out of 9 rats. In one of these two, the blood flow disappeared completely but returned 30 min later (figure 3-B).

In addition, a strong blood flow decrease was also observed in 4 out of 6 pCSpCG rats (figure 4-C). The combination of pCS and pCG caused a flow-stop in 1 of 4 rats after approximately 60 min, but flow was restored again 30 min later. The flow in the 3 other rats showed a clear decline, but remained present.

![Figure 3: Evolution of red blood cell velocity (\(V_{\text{RBC}}\)) in IS treated rats](image)

Panel A is representative for 4 rats with permanent stop of \(V_{\text{RBC}}\) after 30-60 min. Panel B is representative for 2 rats with transient stop of VRBC, and return 30 min later.

Leukocyte rolling (black arrows), adhesion (white arrows) and extravasation (white circles) in response to IS at baseline (\(t=0\) min) and after 30, 60 and 120 min of exposure.

The disappearance of a visible signal for the vessel corresponds to the disappearance of moving erythrocytes and thus for a defective (A) or temporary (B) stop of flow.

**Remark:** The amount of symbols is not representative for the exact number of leukocytes with corresponding characteristics present in the panels. Moreover, it is impossible to see the difference between rolling and adhering leukocytes on these static pictures. A short movie illustrating the different elements is posted online.
Figure 4: Impaired red blood cell velocity subsequent to IS and pCSpCG exposure

In 6 out of 9 IS treated rats, an interruption of flow was observed in the period between the 30th and the 60th minute of exposure (A, red lines). In 4 rats, flow stopped permanently after 30-60 min (A, full red lines). A strong decrease in red blood cell velocity ($V_{RBC}$) was detected after 10-20 minutes in 2 other rats (A, dotted red lines). The remaining 3 animals showed less dramatic decreases of flow pattern (A, black lines).

A strong blood flow decrease was observed in 4 out of 6 pCSpCG rats (C, dotted red lines). The combination of pCS and pCG caused a flow-stop in 1 rat after 60 min, but flow was restored. The remaining 2 animals showed only minor declines of $V_{RBC}$ (C, black lines). Comparing the lowest detected values, both for IS (B) and pCSpCG (D) lower values were found compared to control lowest values. *p<0.05 vs controls
Capillary leakage

Capillary leakage was significantly higher compared to control in both the LPS-group and pCSpCG-group (p<0.05). (figure 5)

Hemodynamic parameters

Hemodynamic parameters are shown in table 1.

Although baseline venular diameters of the pCSpCG group were significantly lower compared to the pCS group, they remained stable throughout the evaluation period. No difference was observed with the control group.
Unexpectedly, the flow problems observed after exposure to IS and pCSpCG were not reflected by significant changes in $V_{RBC}$ if the analysis was performed per time point. However, comparing the lowest detected values per animal for $V_{RBC}$ independently of time, for both IS (figure 4-B) and pCSpCG (figure 4-D) lower values were found than for the nadir of controls (resp. $0.5 \pm 0.25$ and $0.5 \pm 0.17$ vs $1.1 \pm 0.11$, $p<0.05$).

Like for $V_{RBC}$, a decreasing trend in venular wall shear rate (VWSR) was observed in the IS and pCSpCG rats, but the effect was not significant.

**Table 1**

<table>
<thead>
<tr>
<th>Hemodynamic parameters</th>
<th>Blood Pressure (mmHg)</th>
<th>Venular Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Controls</td>
<td>132 ± 3</td>
<td>133 ± 3</td>
</tr>
<tr>
<td>IS</td>
<td>136 ± 4</td>
<td>131 ± 4</td>
</tr>
<tr>
<td>pCS</td>
<td>126 ± 2</td>
<td>122 ± 3</td>
</tr>
<tr>
<td>pCSpCG</td>
<td>129 ± 6</td>
<td>129 ± 6</td>
</tr>
</tbody>
</table>

| Blood Pressure (mmHg), Venular Diameter (µm), Red Blood Cell Velocity (mm/s), Venular Wall Shear Rate (s$^{-1}$), are shown as means ± SEM. Controls (n=12), IS (n=9), pCS (n=8), pCSpCG (n=6). | apl<0.05 versus control group, bp<0.05 versus pCS-group |

**Evaluation of IS for vasoactive effects**

Because detectable changes in vessel diameter were absent throughout the experiments with IS, vasoconstrictive properties of IS were tested *in vitro* by a wire myograph in segments of both aorta and femoral artery. IS did not elicit a contractile effect even when present for 2 hours.

**Circulating leukocytes and hematocrit**

Total number of leukocytes and hematocrit values were not significantly different between the groups at any of the experimental time points. (data not shown)
Quantification of uremic solute concentrations in HBSS solutions and sera by HPLC

The concentrations of the uremic toxins in the HBSS solutions conformed with those observed in CKD patients. IS-HBSS solutions had a mean IS level of 232 ± 4.4 mg/l, the pCS-HBSS solutions contained a mean pCS level of 100 ± 2.3 mg/l and the pCSpCG-HBSS solutions a pCS level of 85 ± 1.5 mg/l and a pCG level of 26 ± 0.33 mg/l. The plasma levels, which were low (total) to undetectable (free) at the start of the experiment, steadily increased, but never exceeded 15 % of the HBSS concentration. (table 2)

<table>
<thead>
<tr>
<th></th>
<th>Time (min)</th>
<th>Total solute concentration (mg/l)</th>
<th>Free solute concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS-group</td>
<td>IS</td>
<td>2.1 ± 0.23</td>
<td>0.4 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>IS 60</td>
<td>23.1 ± 1.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9 ± 0.78&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>IS 120</td>
<td>28.5 ± 2.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6 ± 2.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pCS-group</td>
<td>pCS</td>
<td>0.7 ± 0.17</td>
<td>&lt; 0.25&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>pCS 60</td>
<td>9.9 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>pCS 120</td>
<td>11.3 ± 1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9 ± 0.95</td>
</tr>
<tr>
<td>pCS/pCG-group</td>
<td>pCS</td>
<td>0.5 ± 0.16</td>
<td>&lt; 0.25&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>pCS 60</td>
<td>9.9 ± 1.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>pCS 120</td>
<td>12.7 ± 2.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>pCG</td>
<td>1.5 ± 0.39</td>
<td>1.3 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>pCG 60</td>
<td>1.8 ± 0.33</td>
<td>1.6 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>pCG 120</td>
<td>1.9 ± 0.40</td>
<td>1.9 ± 0.41</td>
</tr>
</tbody>
</table>

Uremic solute concentrations are expressed as means ± SEM.  
<sup>a</sup>p<0.05 vs baseline (= 0 min), <sup>*</sup>below detection limit

Chemotaxis experiments

In comparison to control, pCS and pCG (alone or in combination) and IS had no effect on the fMLP induced chemotaxis of polymorphonuclear leukocytes (PMNL); neither had they a chemotactic potential on their own in this assay.
4.2.4. Discussion

In this study, the effects of the protein-bound uremic solutes indoxylsulfate (IS), p-cresylsulfate (pCS) and p-cresylglucuronide (pCG) on leukocyte recruitment were evaluated for the first time in an in vivo rat model, using intravital microscopy.

IS caused an immediate firm adhesion and extravasation of the circulating leukocytes (figure 1). In addition, IS had a dramatic effect on blood flow, resulting in an interruption or even a complete flow stop (figure 3 & 4). On the other hand, pCS induced a moderate activation only resulting in an increased number of rolling leukocytes (figure 1). The combination of both pCS and pCG had no additive effect to pCS alone on leukocyte recruitment. However, the addition of pCG to pCS caused an impaired blood flow and leakage of albumin (figure 4 & 5).

Until now, experimental effects of IS, pCS and pCG have mainly been evaluated in an in vitro setting. IS plays a role in inflammation-related processes by inducing ROS production in endothelial cells\textsuperscript{14;15}, increasing endothelial microparticle release\textsuperscript{16}, enhancing the proliferation of vascular smooth muscle cells\textsuperscript{17} and inhibiting endothelial cell repair.\textsuperscript{18} IS increases cardiac fibrosis\textsuperscript{19} and osteoblastic resistance to parathyroid hormone\textsuperscript{20} which might contribute to vascular calcification. Also pCS was shown to have an immune stimulating effect due to the increase of leukocyte free radical production.\textsuperscript{21} In addition, a synergistic effect of pCS and pCG was demonstrated.\textsuperscript{22} Vascular damage of pCS is suggested by demonstration of endothelial microparticle release.\textsuperscript{23}

Clinical studies already showed a relation between p-cresol (acting as a surrogate for pCS) and clinical outcomes.\textsuperscript{24;25} Recent studies confirmed that pCS levels were predictive for mortality at different stages of CKD\textsuperscript{26} and for cardiovascular and all-cause mortality in hemodialysis patients.\textsuperscript{27} The relation between pCS and coronary artery disease in patients with no or only moderate degrees of CKD\textsuperscript{28} extends the association of this compound with cardiovascular outcomes beyond the scope of pronounced uremia.\textsuperscript{29} IS was linked to mortality and aortic calcification.\textsuperscript{30} In addition, a correlation between IS and plasma levels of interleukin-6 was demonstrated.\textsuperscript{31}

In clinical studies, it is difficult to determine whether a given uremic toxin intrinsically has deleterious effects or is merely an inert biomarker for the degree of renal function. The present in vivo approach enables us to evaluate the effects of specific uremic compounds on the cross-talk between the major cell types involved in vascular damage: the leukocytes and
the endothelium. Intravital microscopy permits direct in vivo visualization. Different parameters can be studied concomitantly in the same animal. This technique was already used by our group to study effects of peritoneal dialysis solutions on peritoneal membrane physiology.\textsuperscript{13}

The present in vivo data demonstrate a pro-inflammatory effect of pCS, as was already suggested in vitro.\textsuperscript{21,22} Increased production of free radicals by leukocytes can contribute to endothelial damage. The additive effect of pCG and pCS as demonstrated by Meert et al in vitro for leukocytes could not be confirmed in the present recruitment studies. However, addition of pCG to pCS induced leakage through the vessel wall and a drop in blood flow which was not observed in the presence of pCS alone.

In comparison to the p-cresyl conjugates, the effect of IS on recruitment was more pronounced, resulting in an immediate and strong adhesion and extravasation. The in vivo data strengthen previous in vitro findings demonstrating that IS influences expression of endothelial adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1)\textsuperscript{32} and E-selectin.\textsuperscript{33} In addition, Ito et al showed IS-induced leukocyte adhesion to the femoral artery after administering IS in the drinking water of nephrectomized mice for 10 days\textsuperscript{33}, which was significantly reduced by anti-E-selectin antibody treatment. The firm arrest of leukocytes, which was seen in our study after exposure to indoxylsulfate has already been observed for another tryptophan metabolite, kynurenic acid,beit in an in vitro model.\textsuperscript{34}

The present study also demonstrated a clear effect on blood flow in the presence of pCSpCG and an even more substantial impact in the presence of IS. The underlying mechanism of this disturbed blood flow is not clear. Since a hemodynamic effect could not be demonstrated neither by direct observation of vessel wall diameters, nor by wire myograph on thoracic and femoral arteries, a possible role of the coagulation cascade in this phenomenon might be an interesting line of thought. Low and oscillatory shear stress is well known to cause endothelial activation\textsuperscript{35-37}, which can shift the thromboresistant surface of the endothelium towards a prothrombotic state.\textsuperscript{38} This can result in the expression of tissue factor (TF), the main stimulus for thrombin generation. Gondouin et al demonstrated the ability of IS and indole acetic acid (IAA) to modulate TF production, via the aryl hydrocarbon receptor pathway.\textsuperscript{39} IS and IAA increased TF protein expression, which was associated with increased procoagulant activity. This might be an explanation for the strong decrease and even disappearance of blood flow after IS superfusion in our study. Furthermore, soluble tissue factor (sTF) is elevated in CKD.
patients and several uremic solutes raise TF production in different cell types: asymmetric dimethyl arginine (ADMA) increases monocyte TF antigen, and also kynurenin, which like IS belongs to the large group of the indoles, is associated with increased sTF levels and hypercoagulability in CKD patients. Moreover, endothelial microparticles (EMPs), considered as markers of endothelial dysfunction, are shed upon endothelial activation and are involved in the regulation of coagulation by activating the tissue factor pathway. Both IS and pCS have been shown to elicit EMPs release in vitro.

An attempt to explain the leukocyte recruitment characteristics of the tested uremic solutes was made by evaluating their chemotactic properties. Since these compounds did not affect the chemotaxis process, nor were chemotactic by themselves, this suggest a possible direct role on the expression of leukocyte or endothelial surface molecules or an indirect role by inducing the release of substances from other cell types like the mesothelial cells, which on their turn could have an influence on leukocyte recruitment.

The present in vivo data support the hypothesis that protein-bound uremic toxins are not only involved in the progression of CKD, but also in the promotion of cardiovascular disease. Although pCS, pCG and IS all are protein bound molecules, they seem to exert divergent effects. The large group of protein-bound uremic solutes cannot be considered as a homogenous entity since pathophysiology, degree and strength of protein binding, protein-binding sites, retention pattern, and even the nature of the binding protein(s) are unlikely to be the same for all of them.

In conclusion, the present study provides in vivo evidence that IS, pCS, and pCG exert proinflammatory effects that could contribute to vascular damage by stimulating cross-talk between leukocytes and vessels. In addition, IS and the combination of pCSpCG induced unusual blood flow patterns. A possible role of the coagulation cascade in this phenomenon should be considered and further evaluated. The next step would be to set up infusion studies in animals, examining biological effects of one single toxin over a prolonged period of time.
4.2.5. Concise methods

Laboratory animals

The study was performed in 43 healthy female Wistar rats (Charles River Laboratories, L'Abresle, France). They were handled in accordance to the NIH guide for the care and use of laboratory animals, avoiding the influence of environmental stress. The ethical committee for animal experiments at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocol.

Intravital microscopy

Rats were anesthetized subcutaneously in the neck with thiobutabarbitral (Inactin® 100mg/kg, Sigma, St. Louis, MO, USA). After intubation of the trachea (polyethylene (PE) 240 catheter; Becton Dickinson (BD), Erembodegem, Belgium) to ensure open airways throughout the experiment, a jugular vein was cannulated (PE50, BD) for continuous infusion of isotonic saline (3ml/h), and a carotid artery was cannulated (PE50, BD) for continuous monitoring of arterial blood pressure and blood sampling. Cromoglycate (Cromolyn®, 10mg/kg, intravenously (IV), Sigma, St. Louis, MO, USA) was administered 15 min before surgery to block degranulation of mast cells induced by surgical manipulation. Body temperature was kept at 37°C during the whole experiment by a heating pad with feedback control. A small midline abdominal incision was made and a short segment of the small bowel was exteriorized. Carefully avoiding stretching, the visceral peritoneum was spread over a glass plate and superfused continuously with Hank’s Balanced Salt Solution (HBSS; Gibco, Life Technologies Europe B.V., Gent, Belgium) maintained at 37°C.

In each experimental animal, a single unbranched venule with a diameter of 15–25 μm and a length of 150-200 μm was selected for investigation. After completion of surgery and exposure of the venule, the preparation was allowed to stabilize for 30 minutes. The exposed intestine was moistened with sterile soaked gauze to prevent tissue dehydration.

Observations were made with an Axiotech Vario 100 HD microscope (Zeiss, Jena, Germany) using water immersion objectives (Achroplan 10X, 40X). The microscopic stage was operated by using a joystick (Lang, Hüttenberg, Germany). The tissue was transilluminated via a fiberoptic using a light source (KL 1500, Schott, Wiesbaden, Germany) equipped with a 150 W halogen lamp. The resulting image was displayed on a television monitor by a TK-1281.
camera (Victor Company of Japan LTD-JVC, Tokyo, Japan) or a high-speed video camera (Kodak Motioncorder Analyzer, Eastman Kodak Company, San Diego, CA, USA) and video recorded (S-VHS Panasonic AG-7350, Matsushita, Japan) for off-line analysis with an image analysis software program (Cap-Image®, Ingenieurbüro Zeintl, Heidelberg, Germany), as previously described by De Vriese et al. At the end of the experiment, the animal was euthanized by an overdose of anaesthetic. If hemodynamic instability occurred, the experiment was ended prematurely.

**Study of leukocyte recruitment**

Rolling leukocytes were assessed by counting the number of rollers per minute crossing an imaginary line perpendicular to the axis of the vessel. They were defined as those cells moving at lower velocity than red blood cells and being in contact with the endothelial surface. Adhering leukocytes were defined as white cells not moving during a 30 second period; they were counted as the number per 100 µm vessel length. Extravasated leukocytes were determined as the number counted within a predefined area of perivenular tissue. Preactivation of the tissue was minimized by considering for further analysis only vessels in which baseline leukocyte rolling was < 30 cells/min and baseline adhesion was < 3 cells/100 µm of vascular endothelium. A short video fragment with the different steps of leukocyte recruitment is posted online as supplemental material.

**Experimental protocol: LPS-induced or uremic toxin-induced leukocyte recruitment**

*a. Inflammatory stimulus*

As a positive control for leukocyte stimulation, 1 µg/ml lipopolysaccharide (LPS) derived from E. coli (Escherichia coli serotype 0127:B8; Phenol extracted, L3129, Sigma, Bornem, Belgium) was used.45-47

*b. Uremic toxins*

The uremic toxins were tested at concentrations in the uremic range, as applied in preceeding in vitro experiments: 236 mg/l for IS, 100 mg/l for pCS and 24 mg/l for pCG.22 pCS was synthesized according to Feigenbaum and Neuberg as a potassium salt.48 pCG was synthesized from protected glucuronyl-trichloroacetimidate and p-cresol using a protocol similar to the one described by Van der Eycken et al.49 Indoxylsulfate was purchased from Sigma-Aldrich (Bornem, Belgium). Because recent in vitro findings pointed out synergistic
effects of pCS and the other conjugate of p-cresol, p-cresylglucuronide\textsuperscript{22}, also this combination was tested.

c. *Endotoxin concentration*

All experimental solutions were checked for endotoxin contamination, by means of the Limulus Amebocyte Lysate (LAL) QCL-1000-test\textsuperscript{®}, a quantitative kinetic and chromogenic assay (Cambrex Bio Science, Walkersville, MD, USA). All HBSS solutions with and without uremic toxins were endotoxin free (<0.003 EU/ml).

d. *Study design*

After a 30 min stabilization period, the peritoneal membrane was superfused with either the control solution (HBSS, alone), or with (a) LPS (positive control) or (b) one or two uremic solutes dissolved in HBSS. After establishing baseline levels (0 min), measurements were repeated after 2, 10, 20, 30, 60, 90 and 120 min exposure to HBSS with or without solved LPS or uremic solute(s). The number of rolling, adhering and extravasated leukocytes, leukocyte rolling velocity, red blood cell velocity ($V_{RBC}$), and vessel diameter (D) were measured three times at each time point. Venular wall shear rate ($\gamma_w$) was calculated as $\gamma_w = 8 \times V_{RBC}/D$.

*Capillary leakage*

FITC-labeled albumin (Sigma-Aldrich) was injected (200 mg/kg, IV) at the end of each experiment to check for capillary leakage. A visual score was allocated (0: no leakage at all, 1: suggestive for weak leakage at a few spots, 2: evidence of leakage at many spots, 3: heavy leakage covering the whole area).

*Studies of vasoactive effect of indoxyl sulfate (IS) in vitro*

Because of the unusual flow-patterns seen during the IS-superfusion experiments, vasocontractile effect of IS on the vessel was studied by using a wire myograph, as described by Nimmgeers *et al.*\textsuperscript{50}.

Briefly, three female Wistar rats were used. After cervical dislocation, the thoracic aorta and femoral artery were carefully removed from each animal and transferred to cooled Krebs-Ringer bicarbonate (KRB) solution. Ring segments of the isolated arteries were mounted in a small-vessel myograph with a tissue chamber filled with 10 ml of KRB solution and were
cleansed from adhering tissue. Two stainless steel wires were guided through the lumen of the segments. One was fixed to a force transducer, the other was connected to a micrometer. After mounting, the preparations were allowed to equilibrate for 30 min in the KRB solution bubbled with 95% O₂-5% CO₂ (pH 7.4) at 37 °C. After applying the optimal resting tension, preparations were contracted 3 times with a KRB solution containing 30 mmol/L K⁺ and 10⁻⁶ M norepinephrine (NOR), washed and allowed to relax to basal tension. To evaluate endothelial integrity, precontraction was elicited with 10⁻⁶ M NOR and when a stable contraction plateau was obtained the relaxing influence of adding 10⁻⁶ M acetylcholine (Ach) was evaluated. After washing the ring segments so that they were again at optimal basal tension, cumulative concentrations of IS were added to the KRB solution (10⁻⁷-10⁻³ M). The latter concentration (concentration tested in the intravital experiment) was left in contact with the preparations for 2 hours.

**Circulating leukocytes and hematocrit**

Arterial blood samples were taken at baseline and after 1 and 2 hours. Twenty-five µl blood was added to 475 µl 2% orthophosphoric acid (VWR International, Leuven, Belgium) to lyse the red blood cells. Peripheral leukocytes were counted in a Bürker chamber and expressed as number/mm³. To estimate the hydration status of the rats, micro hematocrit capillaries (Brand, Wertheim, Germany) were used.

**Quantification of uremic toxins in HBSS solutions and sera by HPLC**

The solute (pCS, pCG and IS) concentrations in the experimental solutions and in serum at different time points was checked by HPLC analysis, as previously described. A detection limit of 0.25 mg/l was applied for pCS and pCG and 0.15 mg/L for IS.

**Chemotaxis**

Polymorphonuclear leukocyte (PMNL) chemotaxis was determined by the under-agarose method. Formyl-Methionine-Leucine-Phenylalanine (fMLP, Sigma Aldrich) dissolved in PBS at a final concentration of 4x10⁻⁷ M was used as chemoattractant. Five x 10⁵ PMNLs were suspended in 10 µl control or an experimental solution (containing pCS, pCS+pCG or IS at the above mentioned concentrations). After a 2 h incubation period at 37 °C, the cells were fixed with methanol and paraformaldehyde and stained with Giemsa (Merck, Darmstadt,
Germany). The distance migrated under the agarose was measured under the microscope. In addition, the chemotactic capacity of the uremic solutes by themselves was also tested by using them in the assay instead of fMLP.

Statistical Analyses

Data analysis was performed with SPSS Statistics version 19 (IBM, Armonk, New York, US), using the non parametric Mann Whitney U test or Kruskall Wallis test for comparison between 2 or more groups. Post hoc analysis was performed using Dunn’s multiple comparison test. The results are expressed as mean ± standard error of the mean (SEM). A p-value < 0.05 was considered statistically significant.

Acknowledgements

The authors thank Julien Dupont and Marie-Anne Waterloos for their expert technical assistance. The results presented in this paper have not been published previously in whole or part, except in abstract form.
4.2. In vivo effects of protein-bound uraemic toxins

4.2.6. References


4.3. **Vascular damage by ADMA and SDMA is mediated by strong leukocyte-endothelium interaction**

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In preparation
4.3. Vascular damage by ADMA and SDMA

4.3.1. Abstract

Rationale
Asymmetric dimethylarginine (ADMA) and its structural counterpart symmetric dimethylarginine (SDMA) are elevated in patients with chronic kidney disease (CKD). ADMA is considered a mediator of vascular damage through inhibition of endothelial nitric oxide synthase (eNOS) irrespective of kidney function. SDMA has been linked to proinflammatory leukocyte effects. However, the complicated in vivo cross-talk between endothelium and leukocytes was never directly assessed for these two compounds.

Objective
The present intravital microscopy study evaluated the effects of acute exposure to ADMA or SDMA on the recruitment of circulating leukocytes in the rat peritoneal vascular bed.

Methods and Results
The visceral peritoneum of rats was superfused by a physiological solution (HBSS = control), HBSS with ADMA or SDMA at concentrations in the CKD range or HBSS with LPS as a positive control. Leukocyte recruitment, capillary leakage and hemodynamic parameters were analyzed.

Rolling of leukocytes was activated by ADMA (54.2 ± 8.9 vs 20.2 ± 3.9 at 90 min, p<0.05) and even more by SDMA (80.2 ± 20.5 vs 14.3 ± 2.2 at 60 min, p<0.001), compared to controls. This activation was similar to the reaction elicited by 1 µg/ml LPS (36.6 ± 9.4 vs 14.6 ± 2.3 at 30 min, p<0.01). Leakage scores were significantly higher (p<0.05) after ADMA-, SDMA-, and LPS-superfusion, compared to the control group, revealing endothelial damage.

Conclusions
In conclusion, the present study indicates for the first time directly in vivo that both ADMA and SDMA are important simultaneous inducers of leukocyte activation, interaction of leukocytes with endothelium and severe vascular leakage.
4.3.2. Introduction

Endothelium-derived nitric oxide (NO) plays a critical role in endothelial cell relaxation.\(^1\) Endothelial dysfunction caused by reduced availability of NO is an early step in the course of atherosclerotic vascular disease. Inhibition of NO synthesis by endogenous inhibitors of the NO synthase (NOS) pathway is causally involved in this process.\(^2\) Next to endothelial dysfunction, also leukocyte activation and the interaction between activated leukocytes and endothelial cells has been stressed to play a role in atherosclerosis.\(^3-5\)

In 1992, Vallance et al. first reported markedly elevated levels of asymmetric dimethylarginine (ADMA) and its structural counterpart symmetric dimethylarginine (SDMA) in patients with chronic kidney disease (CKD) and demonstrated inhibition of NO production by ADMA \textit{in vitro} and \textit{in vivo}.\(^6\) More recently, also SDMA was shown to inhibit NO synthesis by competing with the transport of L-arginine, the substrate of NOS.\(^7\)

Nowadays, ADMA is accepted as a general marker for endothelial dysfunction and a strong predictor of cardiovascular disease (CVD) in the general population.\(^8-12\) ADMA correlates well with established clinical markers of cardiovascular burden such as intima-media thickness of the carotid artery in the general population\(^13\) or left ventricular mass in hemodialysis patients.\(^14\) ADMA is demonstrated \textit{in vitro} to affect vascular structure as well as vascular reactivity.\(^15-17\) Of note, the majority of observations allocate the biological activity of ADMA to endothelial effects, whereas the role of leukocytes has been barely explored.

SDMA, for a long time considered to be inert, was recently shown \textit{in vitro} to enhance the production of reactive oxygen species (ROS) in monocytes by a mechanistic pathway involving Ca\(^{2+}\) influx.\(^18\) Additionally, activation of NF-kB resulting in the enhanced expression of IL-6 and TNF-\(\alpha\) by monocytes was demonstrated, next to a strong correlation of SDMA with IL-6 and TNF-\(\alpha\) in patients at different CKD-stages.\(^19\) In the case of SDMA, the only study considering the endothelial aspect showed an increased ROS production together with an inhibited NO synthesis in endothelial cells.\(^7\)

Hence, the role of ADMA in endothelial dysfunction and of SDMA in leukocyte activation has been studied, but to the best of our knowledge, no studies assessed the complex cross-talk between endothelium and leukocytes \textit{in vivo} as a basic mechanism of vascular damage with these two compounds. Intravital microscopy permits real time \textit{in vivo} visualization of leukocyte recruitment in translucent tissues.\(^20\)
4.3. Vascular damage by ADMA and SDMA

The present study evaluated for the first time the in vivo effects of acute exposure to ADMA or SDMA on the recruitment of circulating leukocytes in the rat peritoneal vascular bed, using an intravital microscopic model.

4.3.3. Materials and methods

Laboratory animals

The study was performed in 33 healthy female Wistar rats (Charles River Laboratories, L'Abresle, France). They were treated in accordance to the NIH guide for the care and use of laboratory animals, avoiding the influence of environmental stress. The ethical committee for animal experiments at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocol.

Intravital microscopy

Rats were anesthetized subcutaneously in the neck with thiobutabarbital (Inactin® 100mg/kg, Sigma, St. Louis, MO, USA). After intubation of the trachea (polyethylene (PE) 240 catheter; Becton Dickinson (BD), Erembodegem, Belgium) to ensure open airways throughout the experiment, a jugular vein was cannulated (PE50, BD) for continuous infusion of isotonic saline (3ml/h), as well as a carotid artery was cannulated (PE50, BD) for continuous monitoring of arterial blood pressure and blood sampling. Cromoglycate (Cromolyn®, 10mg/kg, intravenously (IV), Sigma, St. Louis, MO, USA) was administered 15 min before surgery to block degranulation of mast cells induced by surgical manipulation. Body temperature was kept at 37°C during the whole experiment by a heating pad with feedback control. A small midline abdominal incision was made and a short segment of the small bowel was exteriorized. Carefully avoiding stretching, the visceral peritoneum was spread over a glass plate and superfused continuously with Hank’s Balanced Salt Solution (HBSS; Gibco, Life Technologies Europe B.V., Gent, Belgium) maintained at 37 °C. (Figure 1)

In each experimental animal, a single unbranched venule with a diameter of 15–25 μm and a length of 150-200 μm was selected for investigation. After completion of surgery and exposure of the venule, the preparation was allowed to stabilize for 30 minutes. The exposed intestine was moistened with sterile gauze soaked in HBSS to prevent tissue dehydration.
Chapter 4: Peritoneal Exposure to uraemic toxins

Observations were made with an Axiotech Vario 100 HD microscope (Zeiss, Jena, Germany), using water immersion objectives (Achroplan 10X, 40X). The microscope stage was operated by using a joystick (Lang, Hüttenberg, Germany). The tissue was transilluminated via a fiber-optic using a light source (KL 1500, Schott, Wiesbaden, Germany) equipped with a 150 W halogen lamp. The resulting image was displayed on a television monitor by a TK-1281 camera (Victor Company of Japan LTD-JVC, Tokyo, Japan) or a high-speed video camera (Kodak Motioncorder Analyzer, Eastman Kodak Company, San Diego, CA,USA) and recorded by a video recorder (S-VHS Panasonic AG-7350, Matsushita, Japan) for off-line analysis with an image analysis software program (Cap-Image®, Ingenieurbüro Zeintl, Heidelberg, Germany), as previously described by De Vriese et al.20, 21 At the end of the experiment, the animal was euthanized by an overdose of anaesthetic. If hemodynamic instability occurred, the experiment was ended prematurely.

Study of leukocyte recruitment

Rolling leukocytes were assessed by counting the number of rollers per minute crossing an imaginary line perpendicular to the axis of the venule. They were defined as those cells moving at lower velocity than red blood cells and being in contact with the endothelial surface. Adhering leukocytes were defined as white cells not moving during a 30-s period; they were counted as the number per 100 µm length of venule. Extravasated leukocytes were determined as the number counted within a predefined area of perivenular tissue. Preactivation of the tissue was minimized by considering for further analysis only vessels in which baseline leukocyte rolling was < 30 cells/min and baseline adhesion was < 3 cells/100 µm of vascular endothelium.

Experimental protocol: dimethylarginine- or LPS-induced leukocyte recruitment

a. Dimethylarginines

SDMA and ADMA were purchased from Merck (Darmstadt, Germany). Separate stock solutions were prepared in HBSS (Gibco) and stored at -20°C. Just before starting the experiment, they were diluted in HBSS resulting in a concentration of 36.0 µmol/l ADMA or 6.1 µmol/l SDMA22, corresponding to the values observed in CKD. HBSS per se was used as control condition.
4.3. Vascular damage by ADMA and SDMA

b. Inflammatory stimulus

As a positive control for leukocyte stimulation, 1 µg/ml lipopolysaccharide (LPS) derived from E. coli (Escherichia coli serotype 0127:B8; Phenol extracted, L3129, Sigma, Bornem, Belgium) was used.

c. Endotoxin concentration

All experimental solutions were checked for endotoxin contamination, by means of the Limulus Amebocyte Lysate (LAL) QCL-1000-test, a quantitative kinetic and chromogenic assay (Cambrex Bio Science, Walkersville, MD, USA). All HBSS solutions with and without dimethylarginines were endotoxin free (<0.003 EU/ml).

d. Study design

After a 30 min stabilization period, the peritoneal membrane was superfused with either the control solution (HBSS, alone), or with (a) ADMA or SDMA or (b) LPS (positive control) dissolved in HBSS. After establishing baseline levels (0 min), measurements were repeated after 2, 10, 20, 30, 60, 90 and 120 min exposure to HBSS with or without dissolved LPS or dimethylarginines. The number of rolling, adhering and extravasated leukocytes, leukocyte rolling velocity, red blood cell velocity (VRBC), and venular diameter (D) were measured three times at each time point. Venular wall shear rate (VWSR) was calculated as VWSR = 8 x VRBC/D.

Capillary leakage

FITC-labeled albumin (Sigma-Aldrich) was injected (200 mg/kg, IV) at the end of each experiment to check for capillary leakage. A visual score was allocated (0: no leakage at all, 1: suggestive for weak leakage at a few spots, 2: evidence of leakage at many spots, 3: heavy leakage covering the whole area).
Circulating leukocytes and hematocrit

Arterial blood samples were taken at baseline, after 1 and 2 hours. Twenty-five µl blood was added to 475 µl 2% orthophosphoric acid (VWR International, Leuven, Belgium) to lyse the red blood cells. Peripheral leukocytes were counted in a Bürker chamber and expressed as number/mm³. To estimate the hydration status of the rats, micro hematocrit capillaries (Brand, Wertheim, Germany) were used.

Statistical Analyses

Data analysis was performed with SPSS Statistics version 19 (IBM, Armonk, New York, US), using the non parametric Mann Whitney U test or Kruskall Wallis test for comparison between respectively 2 or more groups. Post hoc analysis was performed using Dunn’s multiple comparison test. The results are expressed as mean ± standard error of the mean (SEM). A p-value<0.05 was considered statistically significant.

Figure 1: Intravital Microscopy

A segment of the small bowel is exteriorized, spread over a glass plate, and superfused with Hank’s Balanced Salt Solution (HBSS), HBSS mixed with LPS or HBSS mixed with ADMA or SDMA, which allows the study of leukocyte recruitment in postcapillary venules.
4.3.4. Results

Leukocyte recruitment

Leukocyte recruitment in response to ADMA and SDMA was assessed with LPS as a positive control. At baseline, no differences were found between the control group and the experimental groups. Figure 2-A shows images from representative experiments at different time periods. Illustrative movies can be found online as supplemental material.

a. Rolling leukocytes

As shown in figure 2-B, ADMA activated rolling with significantly higher numbers compared to the control group from 90 min on (54.2 ± 8.9 vs 20.2 ± 3.9, p<0.05).

Superfusion with SDMA caused an even stronger activation of rolling. The number of rolling leukocytes tended to increase from 30 min on (28.8±4.7 vs 14.6±2.3, p=NS), and then further increased to reach a plateau at 60 min (80.2 ± 20.5 vs 14.3±2.2, p<0.001 vs control).

Stimulation with LPS induced a strong increase, similar to that of SDMA, in the number of rolling leukocytes from 30 minutes on (36.6 ± 9.4 vs 14.6 ± 2.3, p<0.01 vs control), reaching a number of 90.4 ± 9.0 rolling leukocytes after 120 minutes exposure (p<0.01 vs control).

b. Adhering and extravasated leukocytes

No effect on adhesion and extravasation was seen in the presence of ADMA or SDMA, in contrast to LPS which was associated with a higher number of adhering leukocytes versus control after 60 (3.7 ± 0.8 vs 1.8 ± 0.3, p<0.05) and 120 min exposure (7.6 ± 1.4 vs 3.5 ± 0.3; p<0.05 vs control), and with an increased number of extravasating leukocytes from 30 min on (425 ± 59 vs 247 ± 36, p<0.05).

Hemodynamic parameters

Hemodynamic parameters are shown in table 1. Blood pressure, venular diameter and red blood cell velocity were not different between the groups.
Figure 2: Representative pictures (A) of intravital microscopy. Rolling leukocytes in response to ADMA, SDMA or LPS (B).

A: Leukocyte rolling (arrows) in response to ADMA, SDMA or LPS, at baseline (t=0 min), after 60 and 120 min of exposure.

B: The number of rolling leukocytes at different time points during superfusion by HBSS (=controls), ADMA (▼, n=6), SDMA (▲, n=7) or LPS (●, n=8).

*p<0.05; **p<0.01; ***p<0.001 versus HBSS.
### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Blood Pressure (mmHg)</th>
<th>Venular Diameter (μm)</th>
<th>Red Blood Cell Velocity (mm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>30 min</td>
<td>60 min</td>
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<tr>
<td>Controls</td>
<td>132 ± 3</td>
<td>133 ± 3</td>
<td>126 ± 3</td>
</tr>
<tr>
<td>ADMA</td>
<td>131 ± 4</td>
<td>125 ± 5</td>
<td>123 ± 4</td>
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<tr>
<td>SDMA</td>
<td>133 ± 3</td>
<td>131 ± 4</td>
<td>129 ± 5</td>
</tr>
<tr>
<td>LPS</td>
<td>131 ± 4</td>
<td>130 ± 4</td>
<td>126 ± 5</td>
</tr>
</tbody>
</table>

Blood Pressure (mmHg), Venular Diameter (μm), Red Blood Cell Velocity (mm/s) are shown as means ± SEM.

Controls (n=12), ADMA (n=6), SDMA (n=7), LPS (n=8)
Capillary leakage

Capillary leakage, as demonstrated in figure 3, was more pronounced in the presence of ADMA and SDMA compared to control, and the leakage score was comparable to that caused by LPS (p<0.05). Leakage was observed in 3/12 (25%) rats in the control group, 6/8 (75%) rats in the LPS group, 6/7 (86%) rats in the SDMA group and 5/6 (83%) rats in the ADMA group.

Figure 3: Capillary leakage

Mean leakage scores of the fluorescent isothiocyanate labeled albumin at the end of the experiments, expressed as means ± SEM. Representative pictures are shown (A: 400x, B: 100x).

*p<0.05 versus controls
Circulating leukocytes and hematocrit

The total number of leukocytes and hematocrit values were not significantly different between the groups at any of the experimental time points. (data not shown)

4.3.5. Discussion

The present study investigates for the first time the effects of the dimethylarginines ADMA and SDMA on leukocyte recruitment, a critical step for inflammation and atherosclerosis, in an in vivo rat model, using intravital microscopy.

The main findings were as follows: (1) rolling was activated by ADMA, (2) SDMA induced even earlier a more pronounced increase in rolling of the circulating leukocytes, similar to the reaction elicited by 1 µg/ml LPS; (3) both ADMA and SDMA caused strong leakage through the vessel wall.

Until now, biological effects of ADMA have only been evaluated in an in vitro setting and mainly in cultured endothelial cells. Exposure to ADMA reduces endothelial NO synthesis, increases endothelial superoxide generation and increases adhesiveness of endothelial cells for monocytes by upregulation of MCP-1 and an increased endothelial NF-κB activity. In addition, angiotensin II induces endothelial inflammatory reactions via the NF-κB pathway by stimulation of ADMA production. Monocytes become hyperadhesive when co-cultured with endothelial cells that have been exposed to ADMA. While the above-mentioned in vitro studies demonstrated endothelial effects of ADMA, only few focused on direct effects on leukocytes, and if so they reported contradictory results. Zhang et al demonstrated the ability of ADMA to increase intracellular monocytic TNF-α in a cultured THP-1 cell line, concomitantly with activation of NF-κB and intracellular reactive oxygen species (ROS) production. Schepers et al could only confirm the increase of ROS production but not the effect on NF-κB. Our data on the other hand, definitely and directly underscore the involvement of leukocytes in the vascular impact of ADMA in vivo.

While the impact on leukocytes has largely been neglected previously for ADMA, a reverse situation is seen for SDMA, where in vitro focus has been laid on leukocytes, overlooking a potential interference with the endothelium. SDMA stimulates ROS production in monocytes through a mechanistic pathway involving Ca^{2+} influx. Stimulated ROS production is related
to oxidative stress, which is generally considered as one of the key factors causing vascular damage.\textsuperscript{29} Furthermore, SDMA has been shown to activate NF-κB in monocytes, resulting in an increased intracellular expression of TNF-α and IL-6.\textsuperscript{19} This was confirmed by a highly significant correlation between SDMA on one hand and IL-6 and TNF-α on the other in a clinical study in patients with different stages of CKD.\textsuperscript{19} SDMA also increases ROS production and inhibits NO synthesis in endothelial cells,\textsuperscript{7} but the latter effect is probably due to the fact that SDMA causes a decreasing availability of L-arginine to NOS. In contrast to ADMA, which inhibits NO production by directly interfering with eNOS, SDMA acts as a competitor for the uptake of L-arginine, the substrate for eNOS, into the cells by inhibition of the γ\textsuperscript{+} class of cationic amino acid transporters.\textsuperscript{30}

Our data show that both leukocytes and the endothelium interfere in the presence of SDMA, and that this effect is even more pronounced than in the presence of ADMA, which in general is considered as the more vasoactive compound of the two dimethylarginines. To the best of our knowledge, no previous studies have considered the cross-talk between the major cell types involved in vascular damage: leukocytes and endothelial cells, in the presence of ADMA and SDMA. Intravital microscopy by direct \textit{in vivo} visualization enables to study and quantify these effects. This technique was already used by our group to study effects of peritoneal dialysis solutions on leukocyte recruitment.\textsuperscript{20}

Activation of circulating leukocytes is an early event in the inflammatory process. By releasing ROS, they have the potential to damage the endothelium.\textsuperscript{31, 32} For a long time, leukocyte adhesion has been implicated as a prerequisite for leukocyte induced endothelial injury.\textsuperscript{33, 34} However, other studies evidenced that sites of albumin leakage are often different from those of leukocyte adhesion and migration, suggesting that other mechanisms are critical in the regulation of vascular leakage and barrier dysfunction during inflammation.\textsuperscript{35-38} More recently, activated leukocytes have been shown to increase vascular permeability in the absence of adhesion.\textsuperscript{39} In addition, the release of respiratory burst products upon leukocyte activation was found to increase microvessel permeability and endothelial intracellular calcium, independently from leukocyte adhesion and the migration process.\textsuperscript{32} This could explain the fact that rolling in the absence of adhesion or extravasation of leukocytes, as observed in the presence of ADMA and SDMA, is associated with distinct vascular leakage. These data then show that both rolling and leakage independently of adhesion can be linked to vascular damage.
Increased capillary permeability is a hallmark of functional microangiopathy and is associated with local interstitial edema in specific organs, such as the heart and the kidney, in arterial hypertension and diabetes mellitus. It is likely that alteration in the interstitial matrix composition contributes to organ damage. Leakage induced by the dimethylarginines was suggested earlier in vitro for ADMA and in vivo for SDMA, be it at higher concentrations than in the present study, respectively more than two-fold for ADMA and even more than 10-fold for SDMA.

It is conceivable that induction of rolling is linked to altered expression of adhesion molecules. However, the information on the effects of ADMA and SDMA on surface molecules is scanty. An in vitro study showed that both ADMA and SDMA decrease CD62L expression on granulocytes, a leukocyte selectin that mediates rolling. This could be caused by a downregulation of L-selectin by proteolytical shedding in response to cell activation, as previously demonstrated by Kansas et al. In addition, SDMA was shown to upregulate expression of the adhesion molecules CD11a and CD11b on monocytes. To the best of our knowledge, there are no data on the effects on endothelial adhesion molecules.

In conclusion, the present study indicates for the first time in vivo that both ADMA and SDMA are important inducers of leukocyte activation and their recruitment towards the endothelium, and that they can cause severe vascular leakage, comparable to leakage induced by LPS. These findings might have important pathophysiologic consequences, necessitating focus on leukocytes next to endothelium in the search for neutralization of the activity of ADMA on the vessels. In addition, conform clinical and experimental data from our group, the role of SDMA was at least as important as that of ADMA, stressing that among the dimethylarginines next to ADMA, also SDMA should as such be taken into account when assessing cardiovascular damage or risk.
Acknowledgements

The authors thank Julien Dupont and Marie-Anne Waterloos for their expert technical assistance.

Disclosures

None

4.3.6. references


4.3. Vascular damage by ADMA and SDMA


4.3. Vascular damage by ADMA and SDMA


CHAPTER 5

GENERAL CONCLUSIONS & FUTURE PERSPECTIVES
Peritoneal dialysis (PD) is a life saving renal replacement therapy with several advantages compared to haemodialysis (HD) such as an increased flexibility and a better survival in the first years of application. Moreover, it is more cost-effective compared to predominantly hospital based HD. The most important limiting factor for its long-term use is the progressive decline of dialytic efficacy, especially ultrafiltration, as a result of progressive degradation of peritoneal membrane structure and function.

This deterioration of the quality of the peritoneal membrane in patients on peritoneal dialysis has been described as a combination of neoangiogenesis and fibrosis, the latter especially due to an increase in submesothelial thickness.

The presence of glucose and glucose degradation products (GDPs) in the dialysate fluid, and the unphysiological low pH of the dialysate solution have attracted most attention as culprits in these processes, finally leading to the development of solutions low in GDPs and with a physiological pH.

Although these solutions perform clearly better in terms of biocompatibility in an in vitro setting and in animal models, their benefit in clinical conditions on preservation of peritoneal membrane integrity so far remains unproven.

This discrepancy can be explained by the fact that, besides GDPs and low pH, many other factors impact on the properties of the peritoneal membrane. These factors are often neglected in clinical studies, potentially leading to unnoticed differences in case-mix and blurring of the potential impact of the novel solutions. On the other hand, many of these factors are modifiable, and attention should be paid to them also in everyday clinical practice to maintain peritoneal membrane integrity.

The present thesis focused on factors potentially influencing peritoneal membrane integrity, with exclusion of those associated to the PD fluid (PDF) itself. In the first part, we assessed the effect of potentially protective interventions to slow down deterioration of the peritoneal membrane. In the second part, we investigated the impact of individual uraemic retention solutes on the behaviour of leukocytes in vivo to unravel the association between uraemia, inflammation and cardiovascular damage at vascular level.
In chapter 2, it was demonstrated that an oral formulation of sulodexide (Biofer SpA, Medolla, Italy) preserved the peritoneal membrane integrity in a chronic rat PDF perfusion model.

Sulodexide is a highly purified mixture of glycosaminoglycans (GAGs) containing 80% low-molecular weight heparin and 20% dermatan sulphate. It has the advantage that it is available as an oral preparation, in contrast to e.g. hyaluronan and low-molecular weight heparins. Sulodexide has been approved for human use.

Of interest, peritoneal membrane changes during long-term PD are similar to those observed in diabetes and both conditions have been related to the presence of high glucose concentrations. Sulodexide has been investigated as an oral drug for prevention of diabetic nephropathy, and the preliminary investigations were promising. However, in a recent large randomized controlled trial in diabetics with micro-albuminuria, oral sulodexide did not result in any difference in evolution of either albuminuria or serum creatinine. In an accompanying editorial, a warning was given that, although surrogate endpoints were not changed, one should be careful not to miss harder endpoints, such as morphology. In addition, the authors raised the point that sulodexide could potentially be protective through mechanisms not related to albuminuria.

Although in our study no difference in net ultrafiltration between the PD-groups with and without sulodexide was seen, peritoneal transport rates for creatinine and glucose, sodium sieving and protein loss were better preserved in the sulodexide treated PD-group. This functional improvement was associated with less neoangiogenesis and reduced submesothelial thickening and epithelial-to-mesenchymal transition (EMT). However, no reduction of VEGF in the dialysate was observed. We hypothesize that sulodexide inhibits VEGF activity either by binding it or by inhibiting the interaction with its receptor.

These data at least support further investigation of oral sulodexide administration as a potentially interesting strategy in PD patients.

As we did not use diabetic rats in our model, it could be interesting to repeat the experiments to see whether the effect of sulodexide is reproducible in diabetic animals. Furthermore, it could be interesting to evaluate the effect of chronic oral sulodexide administration on capillary leakage, using intravital microscopy.
We also believe it could be of interest to investigate renal morphology of diabetic rats treated with oral sulodexide, to investigate the effects on neoangiogenesis under these conditions in the kidney.

Additional experiments with longer study periods, testing different doses of sulodexide is necessary before performing clinical trials. Until today, no large clinical trial to examine potential positive effects of sulodexide on peritoneal membrane morphology and function has been executed. However, sulodexide has already been extensively investigated as a drug for prevention of diabetic nephropathy, as described above. The reason for this is practical rather than scientific. The pharmaceutical market for diabetic nephropathy is larger than that for peritoneal dialysis, which makes it unfortunately more difficult to start such large clinical trials in the field of PD.

In Chapter 3, we focused on the potentially harmful effects of dietary salt intake on the peritoneal membrane. There is abundant evidence about deleterious effects of high salt intake in the general population, ranging from hypertension to inflammatory events. Increased salt intake is related to extracellular fluid expansion, resulting in an increased blood pressure and left ventricular hypertrophy. Furthermore, increased salt intake by itself is directly associated to cardiovascular complications, even without volume overload.

End-stage renal disease patients are particularly susceptible to the negative consequences of sodium loading. While individuals with normal kidney function are able to regulate their sodium balance through adaptation of the renal sodium excretion, dialysis patients have to rely virtually entirely on the dialysis procedure for the elimination of their dietary sodium. For PD-patients, high salt intake and subsequent higher fluid intake are detrimental because of the need to use more hypertonic dialysate solutions to maintain volume balance, which by itself is a cause of deterioration of the peritoneal membrane.

Our animal study demonstrated that dietary salt intake by itself induces EMT of the peritoneal membrane, as identified by co-localization of cytokeratin and alpha-smooth muscle actin (α-SMA), together with an increase in interstitial fibrosis and thickening of the submesothelial layer.

Concordant with this, peritoneal Transforming Growth Factor beta 1 (TGF-β1) and interleukin-6 (IL-6) mRNA expression were increased, suggesting that dietary salt loading induces EMT and peritoneal fibrosis, by upregulation of TGF-β1 and IL-6.
It is surprising that the effects of salt intake appeared so rapidly, after only 2 weeks of exposure. However, previous experiments of salt loading from other groups already pointed out that effects of high salt intake seem to appear very rapidly.²⁹-³¹

The underlying mechanisms leading to these observations need further elaboration.

Interesting ways to unravel the pathways could be to block TGF-β₁ using anti-TGF-β₁ antibodies or using ACE inhibitors to block angiotensin II, which is able to activate TGF-β.³² The latter mechanism explains why use of ACE-inhibitors has a positive impact on peritoneal membrane morphology.³³ The activation of TGF-β through angiotensin hypothetically could even explain the discordant results found in studies on the detrimental effects of sodium restriction in diabetic patients.³⁴ In patients with type 2 diabetes, lower 24-h urinary sodium excretion was paradoxically associated with increased all-cause and cardiovascular mortality.³⁵;³⁶ An association between reduced sodium intake and an activation of metabolic and neurohormonal pathways, including the renin-angiotensin-aldosterone (RAAS) system has been reported, so it could be hypothesized that sodium restriction leads to upregulation of RAAS, and sodium loading can thus give an effect comparable to that of ACE-inhibition.

The possible role of the hyperosmolar environment created in the gut by the ingestion of dietary salt could be also interesting to investigate. It has been demonstrated that local hyperosmolarity leads to osmotic stress and upregulation of tonicity-responsive enhancer binding protein (TonEBP). TonEBP activates osmoprotective genes to ensure cell function in hostile environments with increased interstitial tonicity,³⁷;³⁸ but also stimulates macrophages and upregulates VEGF production.³¹

Another interesting pathway is to investigate the adipocytes, which are ubiquitous in peritoneal tissue. It is hypothesized that they can be an important source of cytokine secretion, including IL-6 and TGF-β₁.³⁹-⁴¹ In our study, salt intake induces more pronounced upregulation of IL-6 and TGF-β₁ expression in the visceral peritoneal membrane, where adipocytes are abundant as compared to the parietal peritoneal membrane, where they are rather scarce. Again, it could be that the local hyperosmolarity induced by peroral salt intake leads to irritation of the adipocytes. Finally, it would be interesting to know if peritoneal changes induced by high sodium diet are reversible.
In chapter 4, we analysed the impact of the uraemic milieu itself on the integrity of the endothelium of the venules in the peritoneal membrane.

When kidney function is lost and renal failure develops, a host of compounds which under normal conditions are excreted into the urine by the healthy kidneys, accumulate in the body. These compounds cause a gradual state of endogenous intoxication, named uraemia, after the most abundant, but relatively inert compound urea. This uraemic intoxication results in the malfunctioning of most body organs and organ systems. One of the most worrying problems is the accelerated vascular damage, which is partially a result of the increased inflammation in uraemia.

The last decade, an increasing number of uraemic solutes with direct vasotoxic effects are identified\textsuperscript{42}, and a lot of uraemic solutes can be held responsible for the micro-inflammation in the uraemic patient. Besides cardiovascular disease, another important cause of death in chronic kidney disease patients is infection.\textsuperscript{43-45} It has been postulated that disturbances in leukocyte function due to accumulation of uraemic retention solutes are the common pathway of these two processes.

On the one hand, leukocytes at baseline are chronically activated in an uraemic milieu. This results in chronic inflammation, on itself one of the underlying reasons of cardiovascular disease and malnutrition, a syndrome coined as “malnutrition inflammation arteriosclerosis” (MIA) syndrome.\textsuperscript{46-48} On the other hand, they do not respond adequately upon stimulation, resulting in an impaired immune response and increased risk of (bacterial) infection. Chronic infection in its turn has been associated with enhanced cardiovascular risk.\textsuperscript{49} Thus, these two apparently conflicting phenomena can explain the chronic inflammation as well as the defective immune response causing an increased susceptibility for infection, observed in uraemia.

Chronic inflammation of the peritoneal membrane leads to fibrosis, neoangiogenesis, and EMT.\textsuperscript{50} The prevention of leukocyte activation caused by pro-inflammatory uraemic toxins, could thus be an interesting pathway to prevent damage of the peritoneum. For example, it has been demonstrated that administration of inhibitors of the prostaglandin synthase pathway can result in an improved preservation of the peritoneal membrane.\textsuperscript{51}

Our laboratory has done extensive research on the \textit{in vitro} impact of individual uraemic retention products. Such an \textit{in vitro} setting has the major advantage that all known and
unknown factors involved can be well controlled. However, the interaction between different factors and the responsible mechanisms can better be evaluated in an *in vivo* system.

As described in chapter 4.2. and chapter 4.3., we used an intravital video microscopy model to evaluate leukocyte function in postcapillary venules of the peritoneal membrane under exposure to representatives of 2 major subgroups of uraemic retention solutes: 1) the protein-bound compounds (indoxylsulfate, p-cresylsulfate and p-cresylglucuronide) and 2) the water-soluble compounds (ADMA and SDMA).

The major advantage of intravital microscopy is the possibility to evaluate leukocytes in their normal environment. In this model, we could easily add well defined doses of individual uraemic toxins to the superfusion solution, to explore the impact of this addition on the behaviour of the leukocytes.

*In vitro* and *ex vivo* data link both p-cresylsulfate and indoxylsulfate to negative impact on the vascular system. Recent *in vivo* observational data also relate serum concentrations of these compounds to mortality, inflammation, vascular disease and progression of renal disease in different, even moderate stages of chronic kidney disease.52-58

As described in chapter 4.2., indoxylsulfate caused an immediate firm adhesion and extravasation of the circulating leukocytes and had a dramatic effect on blood flow, resulting in an interruption, or even a complete stop of the flow. P-cresylsulfate induced a moderate activation, resulting in an increased number of rolling leukocytes. The combination of both p-cresylsulfate and p-cresylglucuronide had no additive effect to p-cresylsulfate alone on leukocyte recruitment. On the other hand, the addition of both compounds caused an impaired blood flow and enhanced capillary leakage.

Although all three compounds investigated in this study are protein-bound molecules, they seem to exert divergent effects, but all are proinflammatory in a way that they could contribute to vascular damage by stimulating cross-talk between leukocytes and vessels. In addition, indoxylsulfate and the combination of p-cresylsulfate and p-cresylglucuronide induced unusual blood flow patterns.

Next to the group of the protein-bound compounds, the effects of the water-soluble compounds asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are described in chapter 4.3., ADMA and SDMA appeared to be important inducers of
leukocyte rolling and were able to cause severe vascular leakage, in a degree comparable to the vascular damage induced by LPS.

This is, to our knowledge, the first time that the association between ADMA and inflammation through activation of leukocytes has been demonstrated in an in vivo setting. Neutralizing ADMA will thus not only have beneficial effects on the endothelium directly through NO mediated vasodilation, but also indirectly through its impact on leukocyte activity and thus inflammation. In addition, the role of SDMA was at least as important, stressing that next to ADMA, SDMA should also be taken into account when assessing cardiovascular damage or risk, and especially when developing removal or neutralization strategies.

Future work will be conducted to elucidate the underlying mechanism responsible for blood flow interruption after exposure to indoxylsulfate. It might be interesting to investigate the impact of these uraemic toxins on the coagulation pathway, more especially the role of tissue factor, and certainly to evaluate cross-links between coagulation and the inflammatory pathways.

It is also of interest to analyze the effects of individual uraemic toxins on leukocyte function in a more chronic setting. For this goal, chronic infusion studies with individual uraemic toxins can be set up in healthy animals. Alternatively, acute and chronic infusion of these toxins could be performed in uraemic animals, to explore the impact of the individual compounds on top of the uraemic state. These type of experiments could shed light in the question whether selective removal of individual uraemic retention solutes could result in improved outcomes. Finally, we think it would be also useful to investigate the effects of different solutes simultaneously.

At the moment, technology for direct adsorption from blood by haemoperfusion with bead columns is available for β2-microglobulin and interleukin-1β, and systems for protein-bound solutes are under development.

To unravel the underlying pathways by which uraemic toxins exert their effects, specific blockers can be used. In the case of SDMA for example, in vitro experiments from our group showed that N-acetyl-cysteine (NAC) and the ACE-inhibitor captopril are able to inhibit respectively the SDMA-induced NF-κB activation and the SDMA-enhanced ROS production. Also the in vivo effect of such substances could be analyzed.
Although we agree that using a uraemic rat model would be extremely interesting, we did not use it in the studies included in this doctoral thesis, because of different reasons:

1) We wanted to investigate the impact on peritoneal membrane morphology and function of the different compounds (salt, sulodexide, uremic toxins,...) *per se* and in isolated fashion. Inducing uraemia would cause more interfering factors, as it has been well described that uraemia *per se* can cause changes in peritoneal morphology.\(^{64-66}\)

2) A problem of the nephrectomy rat model is that it creates large differences in degrees of uraemia obtained, at least in our hands, but also in other reported models. This would create additional bias in the interpretation of the results, as then the different degrees of baseline uraemia would be a major confounder, potentially blurring effects. We wanted to first establish the separate effect of individual components.

3) The initial mortality rate of a peritoneal dialysate exposure model in uraemic animals is high. This creates a problem of “selection bias” (survival of the fittest), and most likely also of survival of those animals who have the lowest degree of uraemia. Again, this would severely hamper the interpretation of the results.

4) In the case of the salt study, we asked ourselves the question why some uraemic patients do and others do not develop morphological and functional alterations of the peritoneal membrane, already before the start of peritoneal dialysis. Of course, these differences are partially explained by differences in comorbidities such as diabetes, genetic background and/or uraemia. However, salt intake on itself has already been shown to induce cardiac fibrosis\(^ {67}\) and glomerulosclerosis in the kidney.\(^ {29}\) Because we hypothesized salt intake could induce similar effects on the peritoneal membrane, we used healthy Wistar rats. However, in the future, it would indeed be interesting to perform the same experiments in uraemic and/or PD rats.

5) For the intravital microscopy experiments, we preferred to evaluate the acute effects of one single toxin, excluding the effect of the whole uraemic milieu. For clinical use, a classification of uraemic solutes according to their unique biological effect is needed to permit a clinically and economically justified choice of target molecules in set-ups of selective toxin removal.
In summary, the main conclusions of the studies performed in this thesis are:

1) Excessive salt administration induces peritoneal membrane changes, even without the presence of glucose-containing dialysis fluids. Therefore, dietary salt restriction in the diet of CKD patients should be advocated already in pre-dialysis patients.

2) The glycosaminoglycan sulodexide in its oral formulation is a good candidate for the protection of the peritoneal membrane during peritoneal dialysis.

3) *In vivo*, indoxylsulfate, p-cresylsulfate and p-cresylglucuronide exerted pro-inflammatory effects through activation of leukocytes, while indoxylsulfate and the combination of p-cresylsulfate and p-cresylglucuronide induced a dramatic decrease of blood flow.

4) *In vivo* data show that both ADMA and SDMA are important inducers of leukocyte activation, thereby causing severe vascular damage.
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5. General Conclusions & future perspectives


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5. General Conclusions & future perspectives


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ALGEMENE CONCLUSIES & TOEKOMSTPERSPECTIEVEN
Peritoneale dialyse (PD) is een levensreddende, nierfunctievervangende therapie met verscheidene voordelen ten opzichte van hemodialyse (HD) zoals een grotere flexibiliteit en een betere overleving gedurende de eerste jaren. Bovendien is ze goedkoper, vergeleken met HD die overwegend in het ziekenhuis wordt uitgevoerd. De belangrijkste limiterende factor voor het langdurig gebruik van PD is de progressieve achteruitgang van de doeltreffendheid van de dialyse, voornamelijk wat betreft de ultrafiltratie, als gevolg van progressieve achteruitgang van de structuur en functionaliteit van de peritoneale membraan.

Deze achteruitgang van de kwaliteit van de peritoneale membraan bij PD-patiënten bestaat uit een combinatie van neovascularisatie en fibrose, meer in het bijzonder een toename van de submesotheliale zone.

De aanwezigheid van glucose en glucose degradatieproducten (GDPs) in het dialysevocht, en de onfysiologisch lage pH van het dialysevocht hebben tot nog toe steeds het meest de aandacht getrokken als oorzaken voor deze processen, wat uiteindelijk geleid heeft tot de ontwikkeling van dialysaatoplossingen met lage GDP concentraties en een fysiologische pH.

Ondanks het feit dat deze oplossingen het beter doen op vlak van biocompatibiliteit in een in vitro setting en in dierenmodellen, is hun voordeel qua behoud van de integriteit van de peritoneale membraan tot nog toe nog niet bewezen in klinische omstandigheden. De tegenstrijdige resultaten bekomen met deze nieuwe dialysaatoplossingen kunnen verklaard worden door het feit dat, behalve GDPs en een lage pH, ook vele andere factoren een impact hebben op de eigenschappen van de peritoneale membraan. Deze factoren worden vaak over het hoofd gezien in klinische studies, wat kan leiden tot onopgemerkt verschillen tussen de studiepopulaties die de mogelijke impact van nieuwe oplossingen kunnen doen vervagen. Aan de andere kant zijn vele van deze factoren modificeerbaar en zou er rekening mee moeten gehouden worden in de klinische praktijk om de integriteit van de peritoneale membraan te behouden.

Deze thesis heeft zich toegelegd op factoren die mogelijk de integriteit van de peritoneale membraan beïnvloeden, met uitsluiting van componenten aanwezig in het dialysaatvocht zelf. In het eerste deel hebben we de gevolgen nagekeken van interventies die de achteruitgang van de peritoneale membraanfunctie zouden kunnen vertragen. In het tweede deel onderzochten we de impact van individuele uremische retentiestoffen op het gedrag van leukocyten in vivo.
om het verband tussen uremie, inflammatie en cardiovasculaire schade te ontrafelen op vasculair niveau.

In *hoofdstuk 2* werd in een chronisch ratmodel met perfusie van peritoneale dialysaatvloeistoffen aangetoond dat de integriteit van de peritoneale membraan beschermd kan worden door orale toediening van sulodexide (Biofer SpA, Medolla, Italië).

Sulodexide is een zuivere mengeling van glycosaminoglycanen (GAGs) bestaande uit 80% laag-moleculair heparine en 20% dermatan sulfaat. Het heeft als groot voordeel beschikbaar te zijn in een orale vorm, in tegenstelling tot bijvoorbeeld hyaluronan en laag-moleculair gewicht heparines. Sulodexide werd goedgekeurd voor humaan gebruik.

Veranderingen in de peritoneale membraan tijdens lange-termijn toepassing van PD zijn dezelfde als bij diabetes terwijl beide condities aan mekaar verwant zijn door de aanwezigheid van hoge glucose concentraties. In zijn orale vorm is sulodexide al onderzocht in het kader van preventie van diabetische nefropathie met veelbelovende preliminaire resultaten. In een recente grote gerandomiseerde gecontroleerde studie in diabetic met micro-albuminurie kon daarentegen geen effect van orale sulodexide in de evolutie van albuminurie of serum creatinine aangetoond worden.

In een bijhorend editoriaal werd er op gewezen dat, hoewel geen veranderingen in surrogaat eindpunten werden gevonden, de resultaten toch met omsichtigheid moesten worden geïnterpreteerd. Inderdaad werden geen harde eindpunten zoals bijvoorbeeld morfologie onderzocht in deze studie. Het is daarom niet uit te sluiten dat een positief effect van sulodexide in deze studie werd gemist indien de beschermende werking niet via een reductie van albuminurie zou gaan.

Alhoewel we in onze rattenstudie geen verschil zagen in ultrafiltratie tussen de groepen met en zonder sulodexide, werden het peritoneaal transport voor creatinine en glucose, de ‘sodium sieving’ (sodium dip) en het eiwitverlies wel beter bewaard in de sulodexide groep. Deze functionele verbetering was geassocieerd met minder neovascularisatie, minder epitheliale naar mesenchymale transitie van de mesothecelcellen (EMT) en een mildere toename van de submesotheliale zone. Merkwaardigerwijs werd echter geen daling van ‘Vascular Endothelial Growth Factor’ (VEGF) in het dialysaat geobserveerd, zodat we kunnen veronderstellen dat sulodexide de VEGF activiteit kan inhiberen door ofwel VEGF te binden, ofwel door de interactie van VEGF met zijn receptor te beletten.
Deze gegevens zijn een stimulans om verder onderzoek te doen naar orale sulodexide toediening als een potentieel interessante strategie in PD patiënten.

Aangezien we geen diabetische ratten in ons model hebben gebruikt, zou het interessant kunnen zijn deze experimenten te herhalen om te kijken of het effect van sulodexide reproduceerbaar is in dieren met diabetes.

Bovendien kan het interessant zijn om het effect van chronisch orale sulodexide toediening op capillaire lekkage te bekijken, gebruik makende van intravitaalmicroscopie.

We geloven ook dat het van belang kan zijn om de morfologie van de nieren van diabetische ratten, behandeld met sulodexide, te bestuderen om de vorming van nieuwe bloedvaten in de nier te onderzoeken.

Extra dierexperimenten, met een langere studieduur, en waarbij verschillende dosissen van sulodexide worden getest, zijn noodzakelijk vooraleer er kan worden overgegaan naar klinische studies. Tot op vandaag zijn er geen klinische studies waarbij potentieel positieve effecten van sulodexide op de morfologie en functie van de peritoneale membraan zijn bestudeerd. Sulodexide is wel reeds uitgebreid geëvalueerd als medicijn voor de preventie van diabetische nefropathie, zoals hierboven beschreven. Uiteraard dient hierbij in rekening te worden gebracht dat de farmaceutische markt voor diabetische nefropathie veel groter is dan die voor peritoneale dialyse, wat het minder waarschijnlijk maakt dat dergelijke grootschalige gerandomiseerde klinische studies in een populatie onder behandeling met PD effectief zullen plaatsvinden.

In hoofdstuk 3 richtten we ons op de mogelijke nadelige effecten van zoutinname op de peritoneale membraan. Er is al heel wat gekend over de schadelijke effecten van een hoge zoutinname in de algemene populatie, gaande van hypertensie tot inflammatie. Een verhoogde zoutinname is gerelateerd aan een toename van het extracellulaire volume, resulterend in een verhoging van de bloeddruk en linkerventrikelhypertrofie. Verder is verhoogde zoutinname op zich rechtstreeks geassocieerd aan cardiovasculaire aandoeningen, zelfs zonder dat er sprake is van volume overbelasting (pleiotrope effecten).

Patiënten met eindstadium nierfalen zijn bijzonder vatbaar voor de negatieve gevolgen van een te hoge inname van natriumchloride. In tegenstelling tot mensen met een normale nierfunctie, die in staat zijn om hun natriumbalans te regelen door aanpassing van de renale
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natrium excretie, hangen dialysepatiënten zo goed als volledig af van de dialyseprocedure om hun natrium dat aanwezig is in hun dieet te elimineren. Voor PD patiënten is een hogere zoutinname en een daaruit volgende hogere vloeistofinname extra nadelig omdat er dan meer hypertone dialysaatoplossingen nodig zijn om de volumebalans in stand te houden, wat op zichzelf dan weer beschadiging kan veroorzaken aan de peritoneale membraan.

Ons proefdieronderzoek toonde aan dat een verhoogde zoutinname via het voedsel interstitiële fibrose in het interstitium veroorzaakt, en een verdikking van de submesotheliale zone. We observeerden ook EMT van de peritoneale membraan, aangetoond door colokalisatie van cytokeratine en ‘alpha-smooth muscle actin’ (α-SMA). We vonden ook een toegenomen expressie van peritoneaal ‘Transforming Growth Factor beta 1’ (TGF-β₁) en interleukine-6 (IL-6) mRNA, wat zou kunnen wijzen op het feit dat zoutinname fibrose en EMT in het peritoneum induceert via opregulatie van TGF-β₁ en IL-6.

Het is opmerkelijk dat de effecten van zoutinname zo snel optraden, na slechts 2 weken blootstelling. Echter, voorgaande experimenten van andere onderzoeksgroepen met zoutinname wezen ook uit dat effecten van een hoge zoutinname zeer snel optraden.29-31

De onderliggende mechanismen verantwoordelijk voor deze veranderingen moeten nog verder worden onderzocht. Het zou interessant zijn deze te ontrafelen door TGFβ₁ te blokkeren door middel van hetzij anti-TGFβ₁ antilichamen, hetzij ‘Angiotensin Converting Enzyme’ inhibitoren (ACEI), die de productie remmen van angiotensine II, een TGF-β activator.32 Het laatste mechanisme zou kunnen verklaren waarom het gebruik van ACE-inhibitoren een positieve invloed heeft op de morfologie van de peritoneale membraan.33 Hypothetisch gezien kan het zelfs de tegenstrijdige resultaten, gevonden in studies omtrent schadelijke effecten van zoutrestrictie in diabetespatiënten, verklaren.34 In patiënten met diabetes type II is een lagere 24-uurs urine natrium excretie paradoxaal geassocieerd met een toegenomen mortaliteit.35,36 Het is aangetoond dat een gereduceerde zoutinname geassocieerd is met een activatie van metabolie en neurohormonale assen, inclusief het renine-angiotensine-aldosterone systeem (RAAS); het zou dus kunnen dat zoutrestrictie bij diabetespatiënten leidt tot een opregulatie van het RAAS, en zoutinname in dat geval dus een effect vergelijkbaar met dat van ACE-inhibitie kan hebben.

De invloed van de hyperosmolaire omgeving die in de darm gecreëerd wordt door de inname van zout via de voeding, is ook interessant om te bestuderen. Het is namelijk al aangetoond dat hyperosmolariteit leidt tot osmotische stress en opregulering van het ‘tonicity-responsive
enhancer binding protein’ (TonEBP). TonEBP activeert osmoprotectieve genen die de celfunctie in een vijandige omgeving met een toegenomen interstitiële toniciteit verzekeren\textsuperscript{37,38}, maar stimuleert ook macrofagen en VEGF productie.\textsuperscript{31}

Een ander interessant aspect om te onderzoeken, is de rol van de adipocyten, welke veelvuldig aanwezig zijn in het peritoneale weefsel. Zij vormen een belangrijke bron van cytokine secretie, inclusief IL-6 en TGF-β\textsubscript{1}.\textsuperscript{39-41} In onze studie zagen we dat zoutinname een meer uitgesproken opregulering van IL-6 en TGF-β\textsubscript{1} expressie gaf in het visceraal peritoneum, waar adipocyten overvloedig aanwezig zijn in vergelijking met het pariëtaal peritoneum, waar adipocyten eerder schaars zijn. Het is ook mogelijk dat de lokale hyperosmolariteit, geïnduceerd door orale zoutinname, leidt tot irritatie van de adipocyten.

Tenslotte zou het interessant zijn om te weten of de veranderingen in de peritoneale membraan veroorzaakt door een verhoogd zoutdieet, reversibel zijn.

In hoofdstuk 4 analyseerden we de impact van het uremisch milieu op de integriteit van het endotheel van de venules in de peritoneale membraan.

Wanneer de nierfunctie achteruit gaat en nierfalen ontstaat, accumuleren een groot aantal stoffen in het lichaam welke normaal gezien door de nieren uitgescheiden worden in de urine. Deze stoffen veroorzaken een geleidelijke staat van endogene vergiftiging, uremie genaamd, wat resulteert in een dysfunctie van de meeste organen en orgaansystemen. Eén van de meest zorgwekkende problemen is de versnelde ontwikkeling van vaatschade die deels een gevolg is van de toegenomen inflammatie in uremie.

Het voorbije decennium werd er een groot aantal uremische retentiestoffen met directe vasotoxische effecten geïdentificeerd\textsuperscript{42}, waarvan een aanzienlijk deel ook in verband kon worden gebracht met de micro-inflammatie in de uremische patiënten. Naast cardiovasculaire ziekten is infectie een andere grote oorzaak van sterfte in patiënten met nierfalen.\textsuperscript{43-45} Accumulatie van uremische toxines resulteert in een verstoring van de functies van de witte bloedcellen, die zowel in cardiovasculaire ziekten als in infectie een rol speelt.

Enerzijds worden witte bloedcellen chronisch basaal geactiveerd in een uremisch milieu. Dit resulteert in chronische inflammatie, op zichzelf één van de onderliggende redenen van cardiovasculaire ziekte en malnutritie, een syndroom benoemd als het “malnutritie
inflammatie arteriosclerose” (MIA) syndroom.46-48 Anderzijds reageren de witte bloedcellen van uremische patiënten niet adequaat op stimulatie, wat resulteert in een verzwakte immuunrespons en een toegenomen risico op (bacteriële) infectie. Chronische infectie is op zijn beurt geassocieerd met een groter cardiovasculair risico.49 Op deze manier kunnen in uremie zowel de chronische inflammatie als de defectieve immuunrespons met verhoogde vatbaarheid voor infectie tot gevolg, worden verklaard.

Chronische inflammatie van de peritoneale membraan leidt tot fibrose, neovascularisatie en EMT.50 Het afremmen van de pro-inflammatoire eigenschappen van de uremische toxines die een activatie van de witte bloedcellen veroorzaken, zou dus een interessante manier kunnen zijn om de peritoneale membraan te beschermen. Het is bijvoorbeeld al aangetoond dat toediening van inhibitoren van de prostaglandine synthese resulteert in een beter behoud van de peritoneale membraan.51


Zoals beschreven in hoofdstuk 4.2. en hoofdstuk 4.3., maakten we in dit werk gebruik van een intravitaal videomicroscopie model om de functie van de witte bloedcellen te bestuderen in postcapillaire venules van de peritoneale membraan. We stelden in dit model de witte bloedcellen bloot aan representatieve vertegenwoordigers van twee grote subgroepen uremische retentiestoffen: 1) de eiwitgebonden stoffen (nl. indoxylsulfaat, p-cresylsulfaat en p-cresylglucuronide) en 2) de water-oplosbare stoffen (nl. ADMA en SDMA).

Het grote voordeel van intravitaalmicroscopie is de mogelijkheid om witte bloedcellen te observeren in hun normale omgeving. Met dit model konden we relatief eenvoudig goed gedefinieerde concentraties van individuele uremische toxines toevoegen aan de superfusie oplossing, om zo de impact van deze toxines op het gedrag van de witte bloedcellen te bestuderen.

In vitro en ex vivo gegevens tonen aan dat zowel p-cresylsulfaat als indoxylsulfaat een negatieve vasculaire impact kunnen hebben. Recente in vivo observationele gegevens associëren serum concentraties van deze componenten met mortaliteit, inflammatie,
vaatziekten en de progressie van nierziekte in verschillende, zelfs milde stadia van chronisch nierfalen.52-58

In hoofdstuk 4.2. beschrijven we de sterke adhesie en extravasatie van de circulerende witte bloedcellen die uitgelokt wordt door indoxylsulfaat, met een dramatisch effect op de bloeddoostroming in de capillairen, zelfs resulterend in een onderbreking van de flow. P-cresylsulfaat veroorzaakte een matige activatie van de witte bloedcellen, hetgeen resulterde in een toegenomen aantal rollende witte bloedcellen. De combinatie p-cresylsulfaat en p-cresylglucuronide veroorzaakte geen additief effect op de recrutering van witte bloedcellen in vergelijking met p-cresylsulfaat alleen, maar veroorzaakte daarentegen wel een verzwakte doorbloeding en toegenomen capillaire lekkage.

De drie eiwitgebonden moleculen die werden onderzocht in deze studie zijn allemaal pro-inflammatoir, maar lijken ook uiteenlopende effecten te kunnen veroorzaken. Ze kunnen bijdragen tot vaatschade door de interferentie tussen witte bloedcellen en bloedvaten te stimuleren. Daarenboven veroorzaakten indoxylsulfaat en de combinatie van p-cresylsulfaat en p-cresylglucuronide ook nog eens ongewone patronen van capillaire doorbloeding.

De effecten van de wateroplosbare stoffen asymmetrisch dimethylarginine (ADMA) en symmetrisch dimethylarginine (SDMA) staan beschreven in hoofdstuk 4.3. ADMA en SDMA bleken de witte bloedcellen sterk te activeren en veroorzaakten ernstige vaatschade, waarvan de mate vergelijkbaar is met de vaatschade veroorzaakt door LPS. Het is voor zover we weten de eerste keer dat er een associatie is aangetoond tussen ADMA en inflammatie door activatie van witte bloedcellen in een *in vivo* opstelling. Het neutraliseren van ADMA zou dus niet enkel een rechtsrechts voordelig effect hebben op het endotheel door beter in stand houden van NO gemedieerde vasodilatatie, maar ook onrechtstreeks door het bestrijden van de impact van ADMA op witte bloedcel activatie en dus inflammatie. Daarnaast blijkt SDMA een minstens even belangrijke rol te spelen. Naast ADMA moet dus ook met SDMA rekening worden gehouden wanneer men cardiovasculaire schade of risico gaat inschatten, en vooral wanneer men selectieve strategieën voor verwijdering- of neutralisatie wenst te ontwikkelen.

In de toekomst zullen er experimenten worden opgezet om de mechanismen op te helderen die verantwoordelijk zijn voor de onderbreking van de bloedstroom na blootstelling aan indoxylsulfaat. Het kan interessant zijn om het effect van dit uremisch toxine op de
stollingscascade te bestuderen, en dan meer specifiek de rol van tissue factor, en om verbanden tussen stolling en ontsteking op te helderen.

Het is ook van belang om de effecten van de individuele toxines op witte bloedcel functie te bestuderen in een chronisch experiment. Om dit doel te bereiken zullen chronische infusie-experimenten met individuele toxines in gezonde dieren worden opgezet. Bijkomend zouden ook acute en chronische infusie-experimenten met deze toxines moeten worden uitgevoerd in uremische dieren, om op die manier de impact van een individuele component bovenop het volledig uremisch milieu te bekijken. Deze experimenten zouden een licht kunnen werpen op de vraag of selectieve verwijdering van een individueel uremisch toxine kan resulteren in een verbeterde ‘outcome’. Ten slotte denken we dat het ook nuttig zou zijn om het effect van verschillende stoffen simultaan te bestuderen.

Hemoperfusietechnologie voor directe adsorptie van retentiestoffen uit het bloed met kolommen die gevuld zijn met korrels, is momenteel al beschikbaar voor β₂-microglobuline\(^59\) en interleukine-1β\(^60\). Systemen voor eiwitgebonden stoffen zijn in ontwikkeling.\(^61\) Het zou interessant zijn om dergelijke technieken uit te testen voor een eventueel gunstig effect op de pathologische mechanismen die we nu hebben geobserveerd.

Om de onderliggende elementen waardoor de uremische toxins hun effect uitoefenen, te ontrafelen, kunnen specifieke blockers worden gebruikt. In het geval van SDMA bijvoorbeeld, hebben *in vitro* experimenten uitgevoerd door onze onderzoeksgroep aangetoond dat N-acetyl-cysteine (NAC) en de ACE-inhibitor captopril respectievelijk de SDMA-geïnduceerde NF-κB activatie\(^62\) en de SDMA-verhoogde ROS productie\(^63\) kunnen inhiberen. Ook het *in vivo* effect van dergelijke stoffen zou kunnen worden geanalyseerd.
Alhoewel het zeer interessant zou zijn om een uremisch rattenmodel te gebruiken, hebben we dit niet gedaan in de studies beschreven in deze doctoraatsthesis, omwille van verschillende redenen:

1) We wilden de impact van de verschillende componenten (zout, sulodexide, uremische toxines,...) *per se* op de morfologie en functie van de peritoneale membraan onderzoeken. Een uremische status zou hiermee sterk kunnen interfereren aangezien het goed beschreven is dat uremie op zichzelf veranderingen kan veroorzaken in de peritoneale morfologie.\(^{64-66}\)

2) Volgens onze eigen ervaring, maar ook volgens andere gerapporteerde modellen is het belangrijkste nadeel bij een rattenmodel de variabele graad van uremie die met nefrectomie wordt bekomen. Dit kan een extra bias creëren in de interpretatie van de resultaten. De gradatie in uremie wordt dan een grote confounder, die mogelijks andere effecten kan doen vervagen. Daarom wilden we eerst de individuele effecten van de verschillende componenten aantonen.

3) Het sterftecijfer van een peritoneaal dialyse model bij uremische ratten is zeer hoog. Dit creëert een “selectie-bias” (survival of the fittest), en waarschijnlijk ook overleving van die dieren met de laagste graad van uremie. Ook dit kan de interpretatie van de resultaten sterk beïnvloeden.

4) In het geval van de zoutstudie vroegen we ons af waarom sommige uremische patiënten wel en andere geen morfologische en functionele veranderingen van de peritoneale membraan ontwikkelen, reeds vóór de start van peritoneale dialyse. Deze verschillen zijn natuurlijk gedeeltelijk te verklaren door verschillen in comorbiditeit zoals diabetes, genetische achtergrond en/of uremie. Zoutinname kan echter ook op zichzelf cardiale fibrose\(^{67}\) en glomerulosclerose in de nier induceren.\(^{29}\) Omdat we dachten dat zoutinname op zichzelf gelijkaardige effecten zou kunnen veroorzaken in de peritoneale membraan, gebruikten we gezonde Wistar ratten, om de onafhankelijke impact te evalueren. Niettemin zou het interessant zijn om in de toekomst dezelfde experimenten te herhalen in uremische ratten en/of ratten behandeld met peritoneale dialyse.

5) Voor de experimenten met de intravitaalmicroscoop, verkozen we om de acute effecten van één enkel toxine te evalueren, zonder rekening te houden met het volledige uremisch milieu. Bij het klinische gebruik van selectieve bloedzuiveringstechnieken is het
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nodig de uremische retentiestoffen te klasseren volgens hun biologisch effect, zodat de keuze van de doelmoleculen klinisch en economisch verantwoord is.

Samenvattend zijn de belangrijkste conclusies van de studies die uitgevoerd zijn in het kader van deze thesis:

1) Overdadig zoutverbruik veroorzaakt veranderingen in de peritoneale membraan, zelfs in afwezigheid van glucose-bevattende dialysaatvloeistoffen. Daarom is zoutrestrictie in het dieet van patiënten met CKD aangewezen, zelfs nog voor de start van dialyse en in patiënten die andere nierfunctie vervanging ondergaan dan PD.

2) Het glycosaminoglycan sulodexide is in de orale vorm een goede kandidaat om verder onderzocht te worden om na te gaan of het bescherming biedt voor de peritoneale membraan tijdens peritoneale dialyse.

3) In vivo onderzoek toont aan dat indoxylsulfaat, p-cresylsulfaat en p-cresylglucuronide een pro-inflammatoire effect hebben door activering van witte bloedcellen, terwijl indoxylsulfaat en de combinatie van p-cresylsulfaat en p-cresylglucuronide een dramatische daling in bloedstroom veroorzaken.

4) In vivo data tonen aan dat zowel ADMA als SDMA belangrijke activatoren zijn van witte bloedcellen, waarbij ze ook sterke vaatschade veroorzaken.
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If we knew what it was we were doing, it would not be called research, would it.

Albert Einstein.
DANKWOORD

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  22-04-2010, Ghent
Publicaties

A. Artikels


**B. Hoofdstukken in boeken**


**C. Abstracts/Poster presentaties**


12. **Anneleen Pletinck**, Eva Schepers, Griet Glorieux, Maria Van Landschoot, Johan Van De Voorde, Wim Van Biesen, Raymond Vanholder. The effects of the uremic toxins para-cresyl


**Mondelinge presentaties**


Oral presentation at the 10th European Peritoneal Dialysis Meeting 2011, 21st-24th October, ICC, Birmingham, United Kingdom.


**Prizes and Awards**

1. Awarded for one of ‘the best abstracts presented by young authors’ on the XLV Congress of the European Renal Association European Dialysis and Transplant Association (ERA-EDTA), May 10-13, Stockholm, Sweden, 2008
   ‘Impact of dietary salt intake on peritoneal membrane transport and structure in rats.’

2. Poster prize on the 4th Meeting “Uremic toxins and Cardiovascular Disease”, University Medical Center Groningen, The Netherlands, May 20-22, 2011
   ‘The effects of the uremic toxins p-cresylsulfate and indoxylsulfate on the cross-talk between leukocytes and the vessel wall in an *in vivo* rat model.’