DISRUPTED CELLULAR BARRIERS IN KIDNEY FAILURE:
the endothelium and the peritoneal membrane

Marc Vila Cuenca
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1. **CHRONIC KIDNEY DISEASE**

Chronic kidney disease (CKD) is a highly prevalent condition (10% of the population in Europe) and is caused by a wide range of kidney-specific (glomerulonephritis) or systemic conditions (hypertension and diabetes) that can disturb the structure and function of the kidney irreversibly\(^1\). The diagnosis of CKD rests on (i) establishing the estimated amount of fluid filtered through all the functioning nephrons per unit time defined as glomerular filtration rate (GFR) and (ii) quantifying the amount of proteinuria\(^2\). According to international guidelines, when GFR is less than 60ml/min/1.73m\(^2\) for at least three months (category G3a, Figure 1), a patient is diagnosed with kidney damage. At GFR <10-15ml/min·1.73m\(^2\) (G5), dialysis or kidney transplantation is usually considered.

Of particular concern, derangement in mineral homeostasis due to impaired renal function emerged as a universal problem in CKD, also in more early stages\(^3\). The healthy kidney tightly regulates serum concentrations of phosphate and calcium by controlling the circulating levels of calciphiphotropic hormones\(^4\). As an early event in CKD, α-Klotho protein levels in kidney and blood decrease and are progressively reduced along with the decline of renal function (Figure 2). Reduced renal α-Klotho expression precedes Fibroblast Growth Factor 23 (FGF23) overexpression to maintain phosphorous homeostasis\(^4\). Subsequently, serum FGF23 suppresses the synthesis of the active vitamin D (1,25(OH)\(_2\)D) which is followed by elevation of parathyroid hormone (PTH)\(^4\). Despite the action of FGF23, another major determinant of low 1,25(OH)\(_2\)D levels is the reduction of renal mass with decreased 1α-hydroxylase available for converting 25-dihydroxyvitamin D (25(OH)D) into its active
form by the proximal tubular cells\textsuperscript{5}. Additionally, hyperphosphatemia, inflammatory mediators, oxidative stress and other uremic toxins may also suppress 1\(\alpha\)-hydroxylase activity and 1,25(OH)\(_2\)D synthesis. Already in CKD stage 2 patients, 1,25(OH)\(_2\)D levels decline to the lower limit of the normal concentrations and further declines in patients with stage 3 and 4 CKD\textsuperscript{5}.

\textbf{2. VITAMIN D}

Vitamin D can be obtained from the diet and by the action of sunlight on the skin\textsuperscript{6}. Exposure of the skin to the UV rays of sunlight induces the photolytic conversion of 7-dehydrocholesterol to pre-vitamin D\(_3\) followed by thermal isomerization to vitamin D\(_3\)\textsuperscript{6} (Figure 3). This endogenous synthesis is the main source of vitamin D to the body and accounts for approximately 80\% of the vitamin D supply\textsuperscript{7}. The first step in the metabolic activation of vitamin D is the hydroxylation of carbon 25, which occurs primarily in the liver. The levels of 25(OH)D increase in proportion to vitamin D intake and cutaneous production, but in serum, only a fraction of 25(OH)D is converted to its active metabolite 1,25(OH)\(_2\)D. For this reason, plasma 25(OH)D levels are commonly used as an indicator of vitamin D status\textsuperscript{7}. The values between 20 and 30 ng/mL (50 nmol/l to 60 nmol/l) are considered insufficient, and <20 ng/mL (<50 nmol/l), deficient\textsuperscript{6}. Predominantly in the kidneys, the second step in vitamin D bioactivation involves a second hydroxylation (on carbon 1) to produce renal-activated end-product 1,25(OH)\(_2\)D\textsuperscript{8}. In addition, other cell types can contribute to circulating levels of extra-renally produced 1,25(OH)2D which primarily serves as an autocrine/paracrine factor with cell-specific functions.
Figure 3: Vitamin D3 is synthesized from 7-dehydrocholesterol by UV radiation in the epidermis of the skin. Vitamin D originating from either the diet (both vitamin D2 and D3) or the skin (D3) is first delivered to the liver where it is metabolized by vitamin D 25-hydroxylase (CYP2R1 and CYP27A1) to 25(OH)D, which is the major circulating form of vitamin D in serum. 25(OH)D is further metabolized by 25(OH)D 1α-hydroxylase (CYP27B1) mainly in the proximal tubule of the kidney to 1,25(OH)2D which is the most biologically active form of vitamin D. In target tissues, 1,25(OH)2D binds to VDR, a member of the nuclear receptor family of ligand-activated transcription factors which dimerizes with the retinoid X receptor (RXR) and induces both genomic and non-genomic regulation of downstream targets involving diverse biological functions.

The traditional dogma was that the effects of the kidney-derived end-product 1,25(OH)2D were restricted to the regulation of bone and mineral metabolism. However, new evidence has now established that the role of 1,25(OH)2D is no longer solely limited to this classical functions\textsuperscript{9}. In particular, a more expanded function was suggested by the wide distribution of the vitamin D receptor (VDR) in almost all body cells\textsuperscript{6}. Upon VDR binding, 1,25(OH)2D can control gene transcription and non-genomic actions regulating cellular proliferation and differentiation, inflammation, and the endocrine system\textsuperscript{6,9}. The discovery of these non-classical, or pleiotropic effects widened the spectrum of pathology in CKD associated with vitamin D deficiency\textsuperscript{10}. In this regard, alterations in the immune response\textsuperscript{11}, abnormal epithelial barrier function\textsuperscript{12}, vascular dysfunction\textsuperscript{13}, and cardiomyopathy\textsuperscript{14} are all strongly associated with vitamin D deficiency in these patients. Hence, the beneficial effects of active vitamin D treatment may be of importance in patients with impaired renal function to reverse these abnormalities.
2.1. The beneficial effect of vitamin D on cell function and behavior in uremic conditions

2.1.1 Endothelial barrier function:

Cardiovascular disease is the leading cause of death in CKD, and its prevalence increases with declining kidney function\(^1\). Since traditional cardiovascular risk factors (e.g., advanced age, hypertension and diabetes mellitus) are insufficient to explain the high incidence of CVD among CKD patients, the mechanism underlying the increased risk of cardiovascular events in these patients has not been well defined. Several hypotheses have been proposed for understanding the molecular basis for the link between CKD and cardiovascular disease. The non-traditional risk factors, which are uremic specific, are able to provide some explanation in terms of increased risk of cardiovascular abnormalities in CKD patients. These factors include inflammation, hyperparathyroidism, oxidative stress, increased levels of FGF23, and α-Klotho and vitamin D deficiency.

Given the potentially widespread beneficial effects of active vitamin D treatment in a setting of vitamin D deficiency, it is conceivable to expect a paramount positive impact on the incidence and severity of cardiovascular complications associated with CKD. Emerging studies suggest that vitamin D has important indirect effects via traditional and possibly nontraditional cardiovascular disease risk factors as well as direct effects on cardiac and vascular cells. Experimental data have shown that treatment with active vitamin D protects from vascular calcifications in uremic rats\(^15\), mitigates disturbed uremic-induced aortic gene expression\(^16\) and decreases left ventricular hypertrophy and fibrosis secondary to uremia\(^17, 18\). Despite these encouraging preclinical studies, in the PRIMO and OPERA randomized placebo-controlled trial, active vitamin D treatment failed to reduce left ventricular hypertrophy in CKD patients\(^19, 20\).

Beyond heart disease and vascular calcification, dysfunctional endothelium also plays an important role in the development of cardiovascular complications in CKD. Remarkably, the effects of CKD on the endothelium are not studied as extensively as its effects on the heart or its role in medial calcification. However, in uremic conditions, also the endothelium undergoes functional and structural alterations, eventually resulting in loss of its role as a protective barrier\(^21\). The vast majority of clinical and experimental studies on endothelial dysfunction in CKD have concentrated on determining the impaired vascular function (vasodilation or constriction) as a result of renal disease while structural changes remain insufficiently studied. Similar to other cardiovascular complications in CKD, there is a close connection between the degree of kidney failure and the development of endothelial dysfunction\(^21\). In this regard, active vitamin D has been implicated as a potentially important treatment to restore the uremia-mediated vascular function\(^22, 23\). However, little is known about how vitamin D mediates its vascular protection during CKD.
2.1.2 Epithelial barrier function:

A growing body of evidence suggests that vitamin D is essential for the correct functioning and maintenance of epithelial barriers, which include the intestinal mucosal barrier, corneal, pulmonary and kidney epithelial barriers. Indeed, vitamin D deficiency has been reported to induce barrier dysfunction and to increase permeability\(^\text{24-27}\). Recently, the protective effect of vitamin D on the stability of the proximal tubule epithelial monolayer in uremic conditions was demonstrated\(^{12}\). As previously observed for the intestinal barrier\(^{24}\), vitamin D attenuated the uremia mediated kidney epithelial barrier dysfunction by a redistribution of tight junction proteins between cells.

Also, the peritoneal membrane may be compromised during CKD\(^{28}\). The peritoneal membrane consists of a monolayer of mesothelial cells underneath which submesothelium, fibroblasts, collagen fibrils, and capillaries are present\(^{28}\). Peritoneal mesothelial cells are specialized epithelial cells that line the peritoneal cavity. In end-stage kidney disease, renal replacement therapy is required, one widely applied method of which is Peritoneal dialysis (PD). The therapy is based on the ability of the peritoneal membrane to function as dialyzing membrane, allowing the exchange of solutes and waste products between the peritoneal dialysis solutions and the circulation. This dialysis technique, however, is dependent on optimal functioning of the peritoneal membrane. However, the constant exposure of mesothelial cells to peritoneal dialysis solutions is associated with deleterious changes to the structural integrity of the peritoneal membrane \(^{28}\). As a consequence, long-term PD is associated with peritoneal fibrosis and ultrafiltration failure\(^{29}\). In a quest to preserve the functional properties of the peritoneal membrane and avoid the peritoneal dialysis technique failure, new pharmacologic interventions are needed. In this regard, the combination of therapeutic interventions such as vitamin D together with more biocompatible PD solutions may offer a solution to protect against peritoneal injury in the setting of PD. To study this in detail novel experimental studies mimicking the clinical situation of CKD and peritoneal dialysis are necessary.

2.1.3 Immune cells function:

Inflammatory state contributes in several ways to peritoneal malfunction upon PD fluid exposure. The production and secretion of profibrotic cytokines by inflammatory cells such as monocytes/macrophages and T cells create a fibrogenic microenvironment\(^{30, 31}\). Thus, modulating the immune cells functioning could be of relevance for protecting against the development of peritoneal membrane damage during PD. Given the expression of VDR in immune cells\(^{32}\), the therapeutic effects of active vitamin D may not be limited in preserving the mesothelial barrier function but also in modulating the inflammation. In particular, active vitamin D therapy has been shown to protect from peritoneal membrane deteriora-
tion by reducing the Interleukin-17 (IL-17) inflammatory cytokine production in the peritoneal cavity. Besides this, vitamin D is also known to modulate the macrophage-related pro-inflammatory cytokine release. As an interesting and novel therapeutic approach, Alanyl-Glutamine (Ala-Glu) was shown to be effective in restoring the cytoprotective stress proteome mediated by the exposure of PD solutions resulting in improved mesothelial cell resistance. Similarly as vitamin D, the protective effects of Ala-Glu could extend into the modulation of IL-17 pathway as previously observed in a lipopolysaccharide-induced acute lung injury. This immunomodulatory effects might be of importance in the PD-induced chronic peritoneal inflammation.
OUTLINE OF THE THESIS

The studies in this thesis aim to protect two disrupted cellular barriers during CKD: the endothelium (Part I) and the peritoneal membrane (Part II). In Part I, we investigated the uremia-mediated endothelial dysfunction and the protective effects of vitamin D on this pathological feature. In order to optimize current treatment of Peritoneal Dialysis during CKD, in the Part II we investigated the benefits of active vitamin D together with other interventions in the protection of the peritoneal membrane by modulating the inflammatory environment during PD.

Part I:

- Protective effects of active vitamin D in the CKD-induced endothelial dysfunction.

In the present dissertation, a special focus was placed on the question of how uremia-specific factors contribute to the dysfunction of vascular endothelium and if there is a therapeutic potential of active vitamin D regarding this feature. In Chapter 1, the impact of kidney disease on the endothelial layer of the vascular system is reviewed and the main uremia-related factors of the development of endothelial abnormalities are highlighted. We specifically focused on the impact of the disturbance of the mineral metabolism regulators such as α-Klotho and vitamin D during CKD. The importance to restore the abnormal levels of these two metabolic factors in order to prevent endothelial dysfunction in CKD was suggested. Encouraged by the versatile role of active vitamin D in CKD not only by protecting the cardiovascular system but also for its assumed potential to increase circulatory α-Klotho, in Chapter 2 we studied the direct effects of active vitamin D and α-Klotho on structural changes of the vascular endothelium in a uremic rat model. Specifically, we studied the endothelial-protective properties of both factors in vitro. Looking for a better understanding of the uremia-induced endothelial damage, in Chapter 3 we studied in vitro novel structural and functional changes mediated by plasma of CKD patients. Further, we compared the effects of uremic plasma together with two uremic toxins associated with endothelial dysfunction. Also, we aimed to test the protective effects of active vitamin D in this experimental approach. Finally, in Chapter 4, we investigated how the microvasculature in the heart is affected in a uremic rat model of CKD by detecting the Nε-Carboxymethyllysine depositions as an integrative biomarker of microvascular damage. In addition, we investigated the protective effects of active vitamin D in this feature.
Part II:

- Protecting the peritoneal membrane in Peritoneal Dialysis models through anti-inflammatory interventions.

Some studies have shown the importance of T cells in regulating inflammatory responses in fibrotic tissue disorders. Furthermore, macrophages are considered to be key cells linking inflammation and fibrosis. Recently, both CD4+ T cells and pro-fibrotic M2 macrophages have been suggested to dominate the peritoneal infiltrate in patients with encapsulating peritoneal sclerosis which is a severe complication of PD treatment. Importantly, IL-17 produced by Th17 (a subset of CD4+ T cells) has been reported to be a key player of peritoneum damage.

These different mediators of the chronic inflammation offer a window of opportunity to use immunomodulating therapy in the management of peritoneal dialysis patients in order to protect the epithelial lining of the peritoneal cavity. In Chapter 5 the different factors and cell types that contribute to peritoneal remodeling during PD are described. Furthermore, different therapeutic interventions to prevent and reduce the deleterious effects of peritoneal dialysis exposure on the mesothelial cell layer are suggested. In this dissertation, two additional compounds were studied as immunomodulating therapy. Considering the versatile role of active vitamin D, the protective effects on peritoneal remodeling were tested in a rat model of peritoneal dialysis (Chapter 6). In this study, a special focus was placed on the potential anti-inflammatory properties of active vitamin D treatment by modulating the macrophage population during peritoneal dialysis fluid exposure. In parallel to this, as an additional therapeutic strategy, Alanyl-Glutamine immunomodulatory effects in the IL-17 pathway were tested in uremic mice and rat model PD exposure models (Chapter 7). Finally, in Chapter 8, a comparison between a conventional and a more biocompatible peritoneal dialysis fluid was tested in a uremic mice model focusing on differences in cell population and peritoneal remodeling.
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PART I
CHAPTER 1

Most exposed: the endothelium in chronic kidney disease

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ABSTRACT

Dysfunctional endothelium coexists with inadequate restoration of the vascular function after endothelial injury in patients with chronic kidney disease (CKD). Accumulating evidence indicates that the pathological state of the endothelium may contribute to the development of cardiovascular complications in CKD. Non-traditional risk factors related to CKD are associated with the incidence of cardiovascular disease but their role in uremic endothelial dysfunction has often been disregarded. In this context, soluble α-klotho and vitamin D are of importance because they may preserve the integrity of the endothelium, but decline in CKD, thereby contributing to endothelial damage. These hormonal disturbances are accompanied by an increment of circulating FGF23 and phosphate, both exacerbating endothelial toxicity. Furthermore, impaired renal function leads to an increment of inflammatory mediators, reactive oxygen species and uremic toxins that further aggravate the endothelial abnormalities and inhibit the regeneration of disrupted endothelial lining. This article reviews the endothelial alterations during CKD and evaluates the direct implication of the disturbed mineral homeostasis together with the progressive increment of cytokines, oxidative elements and uremic toxins in vascular dysfunction. Finally, we discuss therapeutic strategies to restore the imbalanced mineral homeostasis and reducing serum concentrations of uremic retention molecules in order to ameliorate the deleterious effect of CKD on endothelial health.
INTRODUCTION

The vascular endothelium constitutes a monolayer of endothelial cells (ECs) forming the inner lining of the entire circulatory system. Among its many physiological functions are the regulation of vasomotor tone, leukocyte trafficking and maintenance of blood fluidity. The endothelium functions as a major barrier at the interface between the vessel lumen and surrounding tissue. Maintaining endothelial barrier function is crucial for the normal functioning of the vascular system and requires tightly regulated intercellular junctions and cell attachments to the extracellular matrix and basement membrane. Thus, endothelial cell dysfunction (ECD) is characterized by a compromised regulation of these vital properties and comprises structural changes in the cytoskeleton, disturbance in proliferative and migratory capacities, breakdown of endothelial cell-cell contacts and impairment of the barrier function. These changes in endothelial monolayer morphology and function are suggested to be key pathophysiological processes leading to cardiovascular disease.

Cardiovascular complications are frequent and severe in patients with chronic kidney disease (CKD) as compared to the general population with normal renal function. An increased proportion of deaths due to cardiovascular disease is observed as estimated glomerular filtration rate declines. This complex association cannot be fully explained by the presence of traditional risk factors such as hypertension, hyperlipidemia, and diabetes. Moreover, standard clinical interventions for managing cardiovascular complications that are advantageous in the general population, are inefficient in CKD patients. Alternatively, non-traditional risk factors related to a reduced renal function provide some insights in terms of increased risk of cardiovascular events and may explain the high cardiovascular incidence in CKD.

Some of these uremic-specific factors include disturbed mineral metabolism, inflammation, oxidative stress and the accumulation of uremic toxins and showed a strong association with reduced heart function, vascular thickness or calcification. Despite this strong relationship with cardiovascular abnormalities, the connection between some of these uremic-related risk factors and the incidence of impaired endothelial function in CKD is still controversial. Furthermore, research in vascular calcification of the medial layer or the decreased cardiac function in CKD has dominated the field, while dysfunctional vascular endothelium has been long neglected even though it has multiple consequences for the progression of cardiovascular disease. In this regard, dysfunctional endothelium occurs with a remarkable frequency in CKD patients on dialysis. However, also studies with more early-stage CKD patients reported alterations in the endothelium and suggested that ECD is indeed an instigator of cardiovascular complications in these early stages as well.

Despite the evidence that ECD may critically impact cardiovascular health, there is still limited knowledge about the mechanisms involved in the uremic pathophysiology driving
vascular dysfunction and there are insufficient preventive treatment strategies. Here, we aim to present available information on the morphological and functional abnormalities in the endothelial lining manifested during CKD and evaluate how kidney-related, non-traditional risk factors, critically impact endothelial integrity. Finally, we discuss some plausible therapeutic strategies by targeting these CKD-associated effectors in order to prevent the progression of endothelial injury and ameliorate its influence on cardiovascular disease.

ENDOTHELIAL DYSFUNCTION DURING CKD

Dysfunctional endothelium in patients with CKD has been demonstrated in both large and small arteries. The pathophysiology of ECD following uremia is complex and the exact mechanism is largely unknown. However, CKD patients frequently display some common adverse endothelial-related characteristics that provide a better understanding of the impact of uremia on this tissue (Figure 1). In this regard, impaired flow-mediated dilation (FMD), reflecting abnormal endothelium-dependent vasodilatory function, has been frequently reported in CKD patients and its impairment is associated with the severity of the renal damage. Under basal conditions, the endothelium maintains the vessel in a relatively dilated state. Thus, dysfunctional endothelium would encompass the cascade of events leading to an impaired vascular homeostasis.

Moreover, upon exposure to uremia, the endothelium loses its quiescent phase and exhibits an activated phenotype. Indeed, elevated levels of soluble cell adhesion molecules such as soluble Intercellular Adhesion Molecule 1 (sICAM-1), Vascular Cell Adhesion Molecule 1 (sVCAM-1), and sE-selectin (involved in the leukocyte trafficking into the subendothelial space) are frequently found in increased concentrations in serum from CKD patients and associated with a defective FMD. Enhanced transcription of these adhesion molecules requires the activation of the nuclear factor-kB (NF-kB) signalling pathway. NFkB participates in pro-inflammatory responses by regulating the expression of several genes and is activated in ECs following human uremic plasma exposure. Importantly, additional target genes of NF-kB also include regulators of cell growth, proliferation and cell survival. In experimental studies, the most prominent changes observed in ECs exposed to human uremic...
serum are suggested to be mediated by NF-κB signalling, substantiating its key role in the development of ECD during uremia\textsuperscript{34-37}. However, the harmful effects of uremic media are not limited to activation of NF-κB pathway but extend to structural alterations such as lower expression of Vimentin and Annexin A2 that are both involved in cell-cell and cell-matrix interactions\textsuperscript{38}. In line with this, it was shown that uremia modulates the expression of Matrix Metalloproteinases (MMPs) leading to a remodeling of the extracellular matrix promoting endothelial detachment and loss\textsuperscript{39}. Overall, several pathways are affected differentially in ECs exposed to uremic conditions and more experimental research of these characteristics is urgently required to identify the molecular mechanisms underlying ECD in the clinical situation of human CKD.

Different from difficulties in assessing structural changes of the vascular endothelium in patients with CKD, the evaluation of endothelial-specific circulating markers has become a reliable and clinically applicable indicator of vascular damage which has contributed to a better understanding of uremia-induced ECD. In this regard, the analysis of circulating endothelial microparticles (EMPs), released into the extracellular space by EC-membrane disruption after endothelial injury or activation, became a non-invasive approach to explore pathological consequences of CKD\textsuperscript{40, 41}. Indeed, in patients with End-stage Renal Failure, EMPs levels are associated with loss of FMD and increased pulse wave velocity\textsuperscript{40}. Importantly, the same study demonstrated that patient-derived EMPs decreased the Wistar rat thoracic aorta endothelial-dependent relaxations induced by acetylcholine and reduced the release of NO\textsuperscript{40}. As an alternative circulating marker of vascular damage, patients with different degrees of impaired renal function also display increased levels of circulating endothelial cells (CECs)\textsuperscript{42, 43}. This subpopulation of cells -originating from the blood vessel wall- is detached due to endothelial damage and reflects ongoing injury\textsuperscript{42}. Finally, CKD reduces the number of circulating endothelial progenitor cells (EPCs)\textsuperscript{44-46}, a bone marrow-derived, circulating EC population that can be recruited to sites of endothelial injury and then mature, playing a major role in vascular repair and protection against the development of ECD\textsuperscript{47}. In this regard, uremia not only dampens the availability of circulating EPCs but also impacts on the normal functioning of this subset resulting in abnormal colony formation together with impaired progenitor cell function, adhesion and incorporation, worsening the repair capacity of the vascular system\textsuperscript{44, 45}. Taken together, uremia-induced disturbances of the vascular endothelium are complex and involves a large number of mechanisms including impaired cell-cell and cell-matrix interaction which contributes to detachment from the vessel wall, increased endothelial cell activation and limited repair capacity of damaged endothelial surfaces, all leading to loss of endothelial barrier function.
THE INFLUENCE OF SPECIFIC CKD-RELATED FACTORS ON ENDOTHELI-AL HEALTH

Recently, many CKD specific factors such as disturbed mineral metabolism, accumulation of uremic retention solutes, inflammation and oxidative stress have been identified that may cause ECD in many ways (Figure 2). Indeed, the vascular pathological characteristics observed by these non-traditional risk factors in experimental uremic animal models or cell cultures resemble the clinical manifestations described in CKD patients, thus reinforcing that CKD-related factors may actually contribute to the pathogenesis of human ECD.

Disturbances in mineral metabolism:

Compelling evidence suggests that the unavoidable progressive derangement in mineral homeostasis due to renal dysfunction may trigger or accelerate cardiovascular disease\textsuperscript{12}. Already in early CKD, the plasma concentrations of the kidney-derived protein α-Klotho...
decrease while fibroblast growth factor-23 (FGF23) levels increase. The latter is probably responsible for decreased plasma 25 hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)\textsubscript{2}D) concentrations and all these factors, along with phosphate exposure, contribute to secondary hyperparathyroidism\textsuperscript{12}. The imbalance of each component worsens with advancing CKD and numerous studies established associations between these disturbances and cardiovascular calcification and heart disease. Experimental evidence, described here, also demonstrates that disturbed concentrations of the different components of the mineral homeostasis contribute to the development of a dysfunctional endothelium; however, this association is not well established in CKD patients.

- \textbf{α-Klotho}

Originally identified as an anti-aging protein, α-Klotho is now recognized as a major player in mineral homeostasis. α-Klotho-deficient mice (kl/kl mice) exhibit a phenotype that remarkably resembles human ageing, characterized by a short lifespan. Interestingly, human CKD shares many biochemical and histological features with the phenotype of kl/kl mice\textsuperscript{48, 49} including cardiovascular manifestations characterised by an increased propensity of left ventricular hypertrophy and fibrosis, vascular calcification, arterial stiffening, hypertension and, importantly, endothelial dysfunction\textsuperscript{48, 50}. Several abnormalities present in
α-Klotho mutant mice such as impaired angiogenesis, insufficient endothelium-derived NO formation and reduced levels of circulatory EPCs may account for the development of ECD\textsuperscript{50}. Membrane-bound α-Klotho is predominantly expressed in the distal tubule of the nephron. The mechanisms responsible for α-Klotho deficiency in CKD are not fully understood but are likely to be multifactorial\textsuperscript{51, 52}. In addition, membrane α-Klotho is cleaved from the cell surface by membrane-anchored proteases and released into the circulation where it is suggested to be continuously required to maintain vascular health\textsuperscript{53, 54}. In this regard, one of the first vasculo-protective activities described for α-Klotho was its role in the maintenance of endothelial homeostasis\textsuperscript{48, 55, 56}. Exposure of HUVECs to α-Klotho increased NO production and induced eNOS phosphorylation and iNOS expression\textsuperscript{57}. Along the same line, α-Klotho works through a cAMP-dependent pathway to upregulate angiotensin I-converting enzyme (ACE) in HUVECs\textsuperscript{58}. Moreover, α-Klotho has been suggested to also protect the vascular tissue by mediating anti-inflammatory actions. In this context, α-Klotho has been shown to suppress the expression of the adhesion molecules ICAM and VCAM by the attenuation of NF-kB signalling pathway upon TNF-α stimulation\textsuperscript{59}. Furthermore, induction of H\textsubscript{2}O\textsubscript{2}-mediated apoptosis was also prevented by α-Klotho protein in HUVECs by decreasing caspase 3 and 9 and limiting subsequent down-regulation of the p53 and p21 pathways\textsuperscript{60, 61}. Another mechanism by which α-Klotho protects the endothelium was observed by Kusaba et al. who showed that α-Klotho mediated the internalization of the Transient Receptor Potential Canonical 1 (TRPC1) and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) complex, thereby preventing hyperpermeability and apoptosis through an increase of calcium influx in EC incubated with VEGF\textsuperscript{62}. Although extensive experimental research has already provided much information on the beneficial effects of α-Klotho, protecting against endothelial damage, the relationship between α-Klotho and vascular dysfunction in patients with CKD remains poorly established. In CKD patients, lower α-Klotho levels were found to be an independent biomarker of arterial stiffness and defective FMD\textsuperscript{63}. However, while a deficiency in serum α-Klotho has been linked to cardiovascular complications in some studies\textsuperscript{63-65}, this issue is still debated as Seiler et al. found no relationship between soluble α-Klotho and cardiovascular outcomes in a cohort of CKD stages 2-4\textsuperscript{10}. Taken together, experimental studies suggest that α-Klotho preserves endothelial integrity in many different ways; however, there is no strong clinical evidence for the impact of α-Klotho deficiency in the CKD-mediated endothelial injury.

- **Vitamin D**

Vitamin D deficiency, defined as serum 25(OH)D concentrations below 20 ng/ml (50 nmo- l/L), is associated with an increased prevalence and incidence of cardiovascular comorbidity and mortality rates in CKD\textsuperscript{66, 67}. In the kidney, 25(OH)D is converted by 1α-hydroxylase to its
active form 1,25(OH)\(_2\)D to exert its effects on distant target tissue\(^{68}\). By binding the vitamin D receptor (VDR), 1,25(OH)\(_2\)D activates both genomic and non-genomic pathways related to cellular proliferation and differentiation, and also on the endocrine and immune system\(^{68, 69}\). As a consequence of CKD, there is a reduced production of active vitamin D, which limits the normal activity of VDR in target tissues, including the vascular endothelium\(^{68, 70}\). In this regard, in several studies, vitamin D deficiency was associated with an abnormal endothelial function in patients with CKD of different stages\(^{71-73}\). In experimental research, when compared to wild-type mice, the selective endothelial VDR knockout mice model exhibits impaired vessel relaxation with reduced eNOS expression and a state of endothelial cell activation promoting leukocyte recruitment \(^{68, 70, 74}\). This evidence suggests that VDR functions are essential for endothelial stability. In addition, plausible mechanisms of vitamin D protective effects on ECs \textit{in vitro} have been recently described, using different synthetic vitamin D analogs\(^{75-80}\). Vitamin D can promote both proliferation and migration in HUVECs, prevent inflammatory endothelial gene expression following exposure to lipopolysaccharide and TNF-\(\alpha\), and decrease the oxidative stress-mediated EMPs release\(^{75-79}\). In the cerebral endothelium, active vitamin D blocked the disruption of the endothelial barrier upon hypoxia preventing the MMP-9-mediated decrease of the tight junction proteins zonula occludens-1, claudin-5, and occludin\(^{80}\). In experimental models of CKD, vitamin D analogs also restored uremia-induced, abnormal expression of aortic genes and improved endothelial function in a 5/6 nephrectomy rat model\(^{81, 82}\). In contrast, however, in the majority of clinical trials among diverse populations, vitamin D administration has failed to show an improvement of endothelial function\(^{83-89}\). In patients with CKD stages 3 and 4, amelioration of FMD with vitamin D under low 25(OH)D circumstances has been reported\(^{90}\). Similar results were also observed in dialysis patients with vitamin D deficiency, where active vitamin D improved FMD of the brachial arteries\(^{91-93}\). However, no effect of active vitamin D on brachial artery FMD or biomarkers of inflammation and oxidative stress was found in patients with advanced CKD and type 2 diabetes\(^{94}\). Similarly, other cardioprotective effects of vitamin D therapy such as prevention of left ventricular hypertrophy or diastolic dysfunction are under debate since the benefits on cardiac function and structure, as observed in uremic animal models, could not be confirmed in clinical trials\(^{95, 96}\). These considerations urge the search for better positioning the potential role of vitamin D administration in patients with CKD, especially in relation to disturbances in vascular function and structure.

**Phosphate and FGF23**

CKD impairs phosphate balance, ultimately resulting in hyperphosphatemia\(^{97}\). In clinical studies, hyperphosphatemia and even high-normal serum phosphate concentrations represent one component of the increased risk of cardiovascular complications and mortality in both the general and CKD population\(^{98-100}\). Recently, a number of studies suggested that
phosphate may exert direct toxic effects on ECs\textsuperscript{101}. Specifically, \textit{in vitro} experiments with endothelial cells demonstrated that high phosphate concentration increases oxidative stress and decreases NO synthesis via inhibitory phosphorylation of eNOS\textsuperscript{102}. In addition, exposure of endothelial cells to high phosphate also promoted the formation of EMPs with impaired capacity of angiogenesis\textsuperscript{103,104}. Importantly, in healthy and CKD mice it was reported that a high-phosphate diet promoted endothelial inflammation and dysfunction and endothelial-cell detachment\textsuperscript{105}.

To compensate the decreased glomerular filtration of phosphate in the setting of CKD, FGF23 synthesized by osteocytes/osteoblasts, limits tubular reabsorption thereby restoring phosphate excretion. This effect of FGF23 is mediated by binding to α-Klotho-FGF receptor (FGFR) complex at distal segments of the nephron, and leads to internalization of the phosphate transporter (NaPi-2a)\textsuperscript{106}. Besides phosphate exposure, other factors such as hyperparathyroidism and exogenous 1,25(OH)\textsubscript{2}D, calcium loading and inflammation also contribute to the elevation of plasma FGF23 concentration in CKD\textsuperscript{107}. Other physiological effects of FGF23 include suppression of 1,25(OH)\textsubscript{2}D in the kidney and therefore, relatively higher FGF23 may be a surrogate of vitamin D deficiency\textsuperscript{108}. Although FGF23 may contribute to CVD by the disturbance of mineral metabolism, FGF23 is independently associated with cardiovascular complications in different stages of CKD\textsuperscript{109,110}. Furthermore, experimental data suggest that FGF23 can directly impair endothelium-dependent relaxation upon acetylcholine stimulation. This effect was mediated by a reduction of NO bioavailability due to an accumulation of superoxide levels\textsuperscript{111}. Recently, Lim K, \textit{et al.} demonstrated that FGF23 leads to a pro-calcific effect due to a CKD-mediated deficiency α-klotho and the specific receptor of FGF23 in vascular cells\textsuperscript{112}. The calcification was prevented by restoring vascular α-klotho and rendering vascular cells FGF23 responsive after the addition of active vitamin D\textsuperscript{112}. However, Mencke \textit{et al.} strongly refused the presence of functional membrane-bound α-Klotho in human arterial tissue and that was in line with other mice and human studies\textsuperscript{113-115}. Overall, further clinical studies are warranted to delineate the pathological mechanisms linking phosphate and FGF23 with vascular abnormalities in CKD patients.

**Uremic toxins:**

Progression of CKD leads to accumulation in blood and tissues of uremic retention solutes\textsuperscript{116}. As a result, the cardiovascular system is constantly exposed to the toxic effects of a range of uremic retention solutes inducing, among other complications, endothelial damage\textsuperscript{117,118}. One well-characterized uremic toxin is asymmetrical dimethyl arginine (ADMA), which is known to exert a negative impact on endothelial cell stability in both \textit{in vivo} and \textit{in vitro} experimental models\textsuperscript{118}. Indeed, ADMA is considered as a circulating endogenous inhibitor
of eNOS, and its accumulation has been associated with ECD in patients with CKD. In CKD mice, increased serum concentration of ADMA caused attenuated endothelium-dependent vasodilation of aortic rings by inhibiting eNOS phosphorylation. In cultured ECs, ADMA induces oxidative stress and enhanced pro-inflammatory pattern promoting the acceleration of senescence and endothelial monocyte chemotactic protein-1 (MCP-1) production. Furthermore, ADMA induces stress fibers and focal adhesion formation in a RhoA and Rho kinase-dependent pathway leading to a limited endothelial repair. Importantly, ADMA also impairs the regeneration of the injured endothelium by reducing the differentiation, mobilization and function of EPCs.

In patients with dialysis-dependent CKD, ADMA can be cleared by standard dialysis strategies such as hemodialysis and peritoneal dialysis. In contrast, other type uremic retention solutes, especially protein-bound uremic toxins, are difficult to remove by dialysis and are known to be pathogenic for the endothelial health. These toxins differ in their nature of protein binding: covalent for Advanced glycation end products (AGEs), and non-covalent for indoxyl sulfate (IS) and p-cresyl sulfate (PCS).

Formed by complex pathways, AGEs are the result of non-enzymatic glycation and oxidation of proteins, lipids and nuclear acids. Therefore, in CKD, accumulation of AGEs not only results from declined renal clearance, but also as a consequence of enhanced oxidative stress present during renal insufficiency, and leading to enhanced production of AGEs. In various cell types, AGEs exerts diverse cellular responses via the multiligand cell-surface receptor Advanced glycation end product-specific receptor (RAGE). The activation of RAGE in ECs in vitro, results in up-regulated expression of adhesion molecules, increased endothelial permeability, impaired NO production and increased ROS formation. In EPCs, AGEs induce apoptosis and limit migration capacity. Moreover, in patients with CKD, decreased endothelial reactivity has been correlated with increased circulating levels of AGEs. Using an in vitro approach, the same study demonstrated that AGEs isolated from serum of patients with CKD induced suppression of eNOS and this effect was attenuated after RAGE blockade.

Recently, several studies demonstrated that other protein-bound uremic toxins such as PCS and IS exert critical toxic effects on EC health during renal insufficiency. In patients with CKD, PCS is the main circulating form of p-cresol and is independently associated with cardiovascular complications. In addition, markers of endothelial damage such as EMPs have been directly related to free serum p-cresol concentrations in hemodialysis patients. The same study demonstrated in vitro that PCS induced a dose-dependent increase of shedding EMP whereas this effect was prevented by inhibition of Rho kinase. In addition, another in vitro study demonstrated the implication of the Rho kinase pathway on the p-cresol-me-
diated alterations of Vascular Endothelial (VE)-Cadherin distribution leading to an increased endothelial permeability and barrier disruption\textsuperscript{142}.

IS is another prototypical protein-bound uremic retention molecule and critical player in the development of vascular disease and elevated mortality rate observed in patients with CKD\textsuperscript{143}. IS is associated with worsened FMD and arterial stiffness in CKD patients\textsuperscript{144}. This study also demonstrated that IS impaired the chemotactic motility and colony-forming ability of EPCs, suggesting that IS contributes to the pathogenesis of ECD by limiting the vascular repair capacity\textsuperscript{144}. In addition, several \textit{in vitro} studies showed that IS can directly disrupt the stability of the ECs through other molecular pathways. Specifically, IS increases EMPs release and impairs wound healing capacity\textsuperscript{145, 146}. Moreover, it promotes endothelial activation by ROS-induced activation of NF-κB. Similarly to p-cresol, cell culture exposure of IS results in endothelial gap formation by VE-cadherin disassembly and stress-fiber formation\textsuperscript{147}.

Overall, uremic toxins may impact the vasculature by disrupting the integrity of the endothelial cell barrier, promoting endothelial activation and weakening its recovery capacity by impairing the EPCs function. Interestingly, the deleterious effects observed by uremic toxins in experimental research share many characteristics with the endothelial abnormalities present in CKD patients suggesting that they are important mediators in the development of uremia-induced ECD in patients.

**Oxidative stress and Inflammation:**

Numerous studies have demonstrated that CKD is associated with increased oxidative stress and inflammation\textsuperscript{148-151}. Oxidative stress can be considered as a high accumulation of reactive oxygen species (ROS) in parallel with impaired endogenous antioxidant mechanisms\textsuperscript{152}. ROS are classically defined as partially reduced metabolites of oxygen that possess strong oxidizing capabilities\textsuperscript{152}. The high production of ROS due to CKD may contribute directly or indirectly to the pathogenesis of the cardiovascular disease by promoting endothelial injury\textsuperscript{153, 154}. Findings in animal models of chronic renal failure confirmed that enhanced generation of ROS leads to decreased NO bioavailability and impairment of the normal function of the endothelium\textsuperscript{155, 156}. Furthermore, increased levels of oxidative stress markers have been related to impaired EC function in CKD patients\textsuperscript{157, 158}. Moreover, chronic or prolonged ROS production is considered critical to the progression of inflammatory processes\textsuperscript{159}, by activating transcription factors such as NF-κB, triggering a pro-inflammatory, pro-adhesion and pro-oxidant phenotype\textsuperscript{159}. In addition, the activation of NFκB pathway in ECs is also triggered by inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNFα)\textsuperscript{160}. These pro-inflammatory molecules are known to be elevated in patients with CKD and are linked to ECD\textsuperscript{161-163}. Taken together, the development of a pro-inflammatory
and pro-oxidative state during renal dysfunction is associated with oxidative stress, vascular NFκB activation, and inflammation, thus self-perpetuating a vicious cycle leading to ECD.

**THERAPEUTIC STRATEGIES TO PROTECT THE ENDOTHELium IN CKD**

Detailed knowledge of factors involved in ECD in CKD can pave the way to endothelial-protective therapeutic strategies, aiming to ameliorate cardiovascular disease in CKD. Based on the above, several options emerge. The overarching approach might be the restoration of mineral metabolism network by correcting hormonal disturbances. Circulatory levels of α-Klotho are affected in the early stages of CKD and its beneficial effects on mediating endothelial stability and its capacity to ameliorate the disrupted mineral balance could be a forthcoming treatment to CKD patients. One plausible strategy is the restoration of α-Klotho levels via gene delivery techniques although this therapeutic approach is not ready for clinical use\(^\text{164}\). Alternatively, exogenous soluble α-Klotho therapy could be a safer and effective way to overcome α-Klotho deficiency during CKD. Recently, exogenous α-Klotho therapy has been shown to be effective in attenuating high phosphate diet-induced renal and cardiac fibrosis and accelerated renal recovery after acute kidney injury in mice\(^\text{165, 166}\). Although the protective effects of exogenous α-Klotho administration in uremia-mediated ECD in animal models remain to be investigated, *in vitro* data suggests that the endothelial-protective properties of α-Klotho are worthy of being tested *in vivo*. In this context, α-Klotho protein exerts protective effects in cultured endothelial cells upon exposure of CKD serum from stage 5 patients\(^\text{167}\). Moreover, exogenous α-Klotho attenuates *in vitro* the endothelial damage induced by the uremic toxin IS and modulates the FGF23-mediated NO synthesis and oxidative stress\(^\text{168, 169}\).

An alternative option to increase circulating α-Klotho is the administration of active vitamin D. Recent data indicated that vitamin D plays a pivotal role by upregulating α-Klotho in cultured kidney-derived cell lines and by increasing serum α-Klotho in *in vivo* models of CKD\(^\text{115, 170-172}\). Although this close relationship could be a significant mechanism protecting against ECD and other cardiovascular complications, in a recent study in patients with CKD patients, active vitamin D supplementation induced no significant changes in circulatory α-klotho\(^\text{173}\). Consequently, further studies are needed to determine whether this connection is clinically relevant for the prevention of vascular abnormalities in CKD.

Importantly, vitamin D vasculoprotective effects are not only limited to the increase in circulating α-Klotho. In this context, vitamin D replacement has raised great expectations to treat cardiovascular complications in CKD patients. However, as mentioned previously, data regarding the beneficial effects of vitamin D supplementation on cardiovascular dis-
ease including endothelial function are conflicting. In CKD animal models, active vitamin D treatment has been shown to mitigate the impact of uremia on endothelial function. In randomized trials, several active vitamin D analogs lead to favourable changes on the vascular function in CKD patients of stages 3-4, haemodialysis with or without vitamin D deficiency, while other studies reported no improvement in FMD with patients of advanced CKD. Overall, active vitamin D may potentially play different roles in protecting the vascular endothelium during CKD but further studies are needed in this area.

Giving its potential role in ECD, direct neutralization of the effects of phosphate and FGF23 may be another therapeutic option to protect the development of ECD. Options to accomplish the reduction of serum phosphate concentrations include dietary phosphate restriction which has been demonstrated to improve endothelial function in CKD-induced in rats. Similarly, treatment of hyperphosphataemia with the phosphate-binder sevelamer hydrochloride was demonstrated to ameliorate the phosphate-mediated ECD in uremic mice. Thus, variation of serum phosphorus concentrations might translate to better endothelial function and cardiovascular health in CKD patients. Alternatively, strategies for targeting high FGF23 concentrations such as the application of monoclonal antibodies has been already tested and shown to be effective for reducing secondary hyperparathyroidism in CKD rats. Another study demonstrates that treatment with a FGF23 receptor-blocker attenuates the development of left ventricular hypertrophy in CKD mice. However, as demonstrated by Shalhoub et al., the beneficial effects achieved by the neutralization of FGF23 signalling can be countered by the increment of serum phosphate. Recently, FGF23-lowering treatment through calcimimetic and phosphate-binding therapy resulted in lower cardiovascular events and clinical benefits for CKD patients. Thus, limiting FGF23-mediated pathways and phosphate serum concentrations may be a potential therapeutic strategy for reducing endothelial damage in CKD.

Because of their harmful effects on ECs, reducing concentrations of uremic toxins, ROS and inflammatory cytokines in CKD patients by dialysis may promote endothelial cell health. Indeed, serum from CKD patients mediated ECD in vitro by remodelling the extracellular matrix and this effect was mitigated in cells treated with serum from the same patients after hemodialysis therapy. Unfortunately, most protein-bound uremic retention molecules cannot be removed by dialysis. Other alternatives require elements that may counterbalance the deleterious effects from oxidative stress, inflammation or toxicity by using anti-oxidants or inflammatory mediators. Finally, patients with dialysis-dependent CKD following renal transplantation, have improved endothelial function. These benefits also include the normalization of the functions of the EPCs, contributing to a better repair. Overall, future studies should focus on the effective removal of these retention solutes in uremic patients in order to attenuate ECD and promote endothelial repair.
CONCLUSIONS

In patients with CKD, the ongoing endothelial damage in the vascular system is thought to be a central process towards progressive cardiovascular complications. The pathogenesis of ECD in patients with renal dysfunction results from an imbalance between increased endothelial damage and impaired regeneration. These processes may result from the progressive loss of vasculoprotective factors vitamin D and α-Klotho together with an increment of ECD-mediators such as FGF23, uremic toxins, ROS and inflammatory cytokines. Therapeutic strategies toward a better endothelial health should be based on correcting the derangements of the mineral homeostasis and removing the retention solutes.
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Vitamin D attenuates endothelial dysfunction in uremic rats and maintains human endothelial stability

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ABSTRACT

Background: Dysfunctional endothelium may contribute to the development of cardiovascular complications in Chronic Kidney Disease (CKD). Supplementation with active vitamin D has been proposed to have vasoprotective potential in CKD, not only by direct effects on the endothelium but also by an increment of α-Klotho. Here, we explored the capacity of the active vitamin D analogue paricalcitol to protect against uremia-induced endothelial damage and the extent to which this was dependent on increased α-Klotho concentrations.

Methods and Results: In a combined rat model of CKD with vitamin D deficiency, renal failure induced vascular permeability and endothelial-gap formation in thoracic aorta irrespective of baseline vitamin D, and this was attenuated by paricalcitol. Downregulation of renal and serum α-Klotho was found in the CKD model which was not restored by paricalcitol. By measuring the real-time changes of human endothelial barrier function, we found that paricalcitol effectively improved the recovery of endothelial integrity following the addition of the pro-permeability factor thrombin and the induction of a wound. Furthermore, immunofluorescence staining revealed that paricalcitol promoted VE-cadherin-based cell-cell junctions and diminished F-actin stress fibers organization, preventing the formation of endothelial intracellular-gaps.

Conclusions: Our results demonstrate that paricalcitol attenuates the CKD-induced endothelial damage in thoracic aorta and directly mediates endothelial stability in vitro by enforcing cell-cell interactions.
INTRODUCTION

The risk of cardiovascular-related mortality is exceedingly high in individuals with chronic kidney disease (CKD). Impairment of endothelial function, which accompanies CKD, is recognized as a sentinel event in the development and progression of cardiovascular disease (CVD). As kidney function declines, there is a progressive decline of both plasma 25 hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)2D) concentrations, and of the kidney-derived protein α-Klotho, accompanied by secondary hyperparathyroidism and increased fibroblast growth factor-23 (FGF23) levels. These hormonal disturbances occur already in early-stage CKD and are proposed to make a notable contribution to the initiation and progression of CVD. Remarkably, there is limited evidence directly pointing to structural abnormalities and a mechanistic role for the endothelial layer in CKD.

Treatment with active vitamin D has been considered a key therapy for cardiovascular events in CKD, but controversy remains as beneficial effects on cardiac abnormalities as observed in animal models could not be confirmed in clinical trials. Moreover, while in vitro studies suggested a protective role for vitamin D in ameliorating damaged endothelium, experimental in vivo CKD studies have primarily focused on the prevention of vascular calcification of the medial layer and heart failure, but paid little attention to structural endothelial damage. A small number of prior studies have suggested benefits of the active vitamin D analogue paricalcitol (19-nor-1α,25(OH)2D2) on endothelial stability in animal models of CKD and even in patients. Interestingly, paricalcitol supplementation increased kidney and serum α-Klotho levels in in vivo models of CKD. α-Klotho is an anti-aging protein and its deficiency is associated with a cardiovascular phenotype including arteriosclerosis, vascular calcification and, importantly, endothelial cell dysfunction. In humans, deficiency in serum α-Klotho correlates with cardiovascular complications. However, a direct role of serum α-Klotho deficiency on endothelial dysfunction in patients with CKD has not been established.

In the present study, we evaluated the impact of vitamin D deficiency, α-Klotho concentrations and the effect of paricalcitol treatment on the development of endothelial cell dysfunction in a CKD rat model. Our data show that paricalcitol treatment attenuates endothelial damage in CKD and directly mediates endothelial stability in vitro. This beneficial effect was not mediated by an increment of α-Klotho concentrations, identifying paricalcitol as a key intervention stabilizing endothelial barrier function.
METHODS

Experimental models

Male Wistar rats (Charles River, Maastricht, The Netherlands) weighing 250 to 275 g were used. After one week of acclimatization, 3/4 nephrectomy was performed to induce uremia (CKD); healthy (non-CKD) controls were not surgically manipulated (experimental design on Figure 1A). The 3/4 nephrectomy involved the complete removal of the right kidney and ligation of 1 to 2 branches of the arteries supplying the left kidney in order to obtain residual kidney function of about one-fourth of the total capacity. Animals were anesthetized with inhaled isoflurane (2 %). For analgesia, tamgesic was administered (10 μl/g weight) intramuscularly pre and post-operatively. Thirty-two rats were made uremic by 3/4 nephrectomy. Four of the rats died during the surgery (n=28). Following one week of recovery from the 3/4 nephrectomy (week 1) animals were fed with a vitamin D deficient diet (TD.120503 Brown C.C. Vitamin D deficient diet; 20% lactose, 1% Ca, 0.65% P) and it was maintained until the end of the experiment as we described recently. To induce CYP24A1 expression, to accelerate the catabolism of endogenous 25(OH)D and calcitriol stores, the rats were subcutaneously injected with 32 ng of 19-nor-1,25-dihydroxyvitamin D2 (paricalcitol; Zemplar®, kindly provided by AbbVie) 3 times per week during three weeks in total. Non-vitamin D deficient groups received a standard diet (Teklad global 2016, Envigo). Oral administration of paricalcitol (0.1 μg/kg rat; kindly provided by AbbVie, Chicago, USA) dissolved in sugar water (1 %) was provided three times per week during seven weeks in total. In parallel, vehicle control rats received only sugar water (1 %). Food consumption was measured every day. All the animals were socially housed under standard conditions and were given food and water ad libitum. Health conditions were checked daily. The weight of the animals was checked daily after surgery during 7 days and weekly for the remainder of the experiment. Animals that lost more than 20 % of their maximum gained body weight at any point during the experiment or showed abnormal activity were excluded from the experiment. Animals were organized as indicated in Figure 1A: non-CKD group standard diet with vehicle control treatment (n=6), paricalcitol treatment (n=6), non-CKD group with vitamin D deficient diet with vehicle control treatment (n=6), paricalcitol treatment (n=6), CKD group standard diet with vehicle control treatment (n=7), paricalcitol treatment (n=7), CKD group vitamin D deficient diet with vehicle control treatment (n=8), paricalcitol treatment (n=6). At the end of the experiment, euthanasia was performed using CO₂/O₂ asphyxiation. The experimental protocol was in compliance with NIH Guideline for the Care and Use of Laboratory Animals and was approved by the Animal Welfare Committee at the VU University Medical Center, Amsterdam.
Measurement of blood parameters

Five hundred microliters of blood were drawn from tail vein in rats under inhaled isoflurane (2 %) anesthesia. At the time points (weeks: -1, 3, 6, 10) serum samples from CKD groups were analyzed for urea and creatinine levels. For determination of urea levels, a kinetic test with urease and glutamate dehydrogenase was used. Creatinine levels were detected by indirect immunofluorescence assay. Measurements were performed by using spectrophotometer Cobas8000 C702 (Roche Diagnostics, Risch-Rotkreuz, Switzerland). At the time point week 4 serum samples were analyzed for 25(OH)D by competitive binding protein assay (Diasorin, Stillwater, Minnesota, USA), 1,25(OH)₂D by radioimmunoassay after immunextraction (IDS, Tyne and Wear, UK). Parathyroid Hormone (PTH) serum levels from end-point (week 11) were analysed by ELISA (Scantibodies Laboratory, Santee, CA) as previously described²⁵.

Evans Blue extravasation in thoracic aorta

Vascular permeability was assessed by injecting 4 % of Evans Blue dye (Sigma) in 0.9 % of saline via the tail vein (1 ml/kg) under isofluorane (2 %) anesthesia. After 30 min of Evans Blue dye injection, animals were euthanized for tissue harvest. The thoracic aorta was removed, weighed after washing with phosphate-buffered saline (PBS) and the dye was extracted in formamide at 56 °C for 24 h. Dye concentration was quantified from the light absorbance at 620 nm, and its tissue content was calculated from a standard curve of dye concentration in the range of 0.5–10 μg/ml. The Evans Blue concentration in tissue was corrected by the weight of tissue and Evans Blue concentration in plasma (ng/ml dye in tissue /mg tissue/ ng/ml dye in plasma).

Histology and Immunohistochemistry

For immunohistochemical analysis, 4 μm sections were deparaffinized, rehydrated and incubated in methanol/H2O2 (0.3 %) for 30' to block endogenous peroxidases. Antigen retrieval was performed with boiling 0.01 M citrate buffer (pH 6.0). Tissue slides were then preincubated with normal rabbit serum (1:50, Dako, Eindhoven, The Netherlands) for 10' at room temperature (RT). Next, slides were incubated overnight (O/N) at 4°C with goat-antirat PECAM-1 (CD31, clone M-20; 1:50, Santa Cruz Biotechnology, CA) and then incubated with rabbit-anti-goat-horseradish peroxidase (HRP) (1:100, Dako) for 30’ at RT. Staining was visualized using 3,3’-diaminobenzidine (0.1 mg/mL, 0.02% H₂O₂). The slides were subsequently counterstained with haematoxylin, dehydrated and covered. Stainings of PBS controls were included. These controls yielded negative results (data not shown). The sections were scanned using the Panoramic Desk scanner (3DHistech, Budapest, Hungary). Analysis were assessed with Pannoramic Viewer (3DHistech, Budapest, Hungary), endothelial gaps
were determined by measuring μm negative surface CD31*100 / μm total CD31 from 3 random areas of the aortic tissue.

**Quantitative RT-PCR**

Kidney tissues were mechanically homogenized and total RNA was extracted using TRIzol Reagent (Invitrogen). Reverse transcription into complementary DNA (cDNA) was performed using Reverse Transcription System kit (Promega, Madison, WI). The synthesised cDNA was amplified with a standard quantitative Polymerase Chain reaction (qPCR) protocol including the use of SYBER GREEN (Applied Biosystems). Klotho forward: GAGCGGT-CACTAAGCGAATACG reverse: CGTGAATGAGGTCTGAAAGC; β-actin, forward: GACCAGAGG-CATAAGGGACAA, reverse: GGCCAACCGTGAAAAGATGA. The relative amount of mRNA was calculated using comparative Ct (ΔΔCt) methods. Amplification products were normalized against β-actin mRNA, which was amplified in the same reaction as an internal control. The x-fold change in mRNA expression was quantified relative to control (healthy non-treated) animals.

**α-Klotho immunoblot**

Kidney lysates were prepared by homogenizing of preserved tissue in lysis buffer containing Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). Protein concentrations were determined using the Pierce Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and 20 µg of protein was used for α-Klotho immunoblot with the following antibodies: rat anti-human Klotho monoclonal antibody (KM2076 at 1:1000 dilution, Transgenic Inc, Kobe, Japan), GAPDH (14C10 at 1:1000 Cell Signalling, Leiden, The Netherlands), followed by donkey anti-rat/rabbit conjugated with HRP (1:5000, Dako). Signal was visualized using enhanced chemiluminescence (Life Sciences) on LAS3000 (Fujifilm, Japan). Image J (NIH, Bethesda, Maryland) was used for analysis. For the IP-IB α-Klotho assay in serum from endpoint (week 11) was performed as previously described26.

**Human endothelial cell culture**

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords from healthy donors obtained from the Amstalland Ziekenhuis, Amstelveen. The use of human tissue for isolation of endothelial cells was approved by the Medical Ethical Committee of the VU University Medical Center. Patients gave informed consent for the use of tissue for research purposes. The study conforms with the declaration of Helsinki. After isolation, cells were resuspended in M199 medium (Biowhittaker/Lonza), supplemented with penicillin 100 U/mL and streptomycin 100 µg/mL (Biowhittaker/Lonza), heat-inactivated human serum 10 % (Sanquin Blood Supply, Amsterdam, The Netherlands), heat-inactivated new-born
calf serum 10 % (Gibco, Grand Island, NY), crude endothelial cell growth factor 150 µg/mL (prepared from bovine brains), L-glutamine 2 mmol/L (Biowhittaker/Lonza), and heparin 5 U/mL (Leo Pharmaceutical Products, Weesp, The Netherlands). Cells were cultured at 37 °C and 5 % CO₂, with a change of culture medium every two days. Cells were cultured up to passage 2, for experiments passage 1-2 cells were used.

**Endothelial barrier function assay**

For electric cell-substrate impedance sensing measurements (ECIS), cells were seeded in 1:1 density on L-cysteine (10 mM, Sigma) and gelatin-coated electric cell-substrate impedance sensing arrays, each containing 8 wells with 10 gold electrodes per well in a 0.49 mm² electrode area (Applied Biophysics, Troy, NY). ECIS software (v1.2.65.0 PC; Applied Biophysics) was used to calculate the level of overall resistance (at 4000 Hz). Culture medium was renewed 24 h after seeding, and experiments were performed 48 h after seeding. For stimulation pre-treatment medium was changed for 1 % Human Serum albumin (Sanquin Blood Supply) in M199 medium containing recombinant Human Klotho from Sigma (400 pM) and paricalcitol (10-100 nM; kindly provided by AbbVie, Chicago, USA). After 24 h of pre-treatment, thrombin (1 U/ml; Sigma Aldrich, Zwijndrecht, the Netherlands) was added directly to the wells for a final concentration of 1 U/mL or electrical wounding was applied (60 s; 100000 Hz), respectively. Thrombin was quantified by the % of drop in resistance after the addition of thrombin and the area under the curve was quantified from normalized baseline starting from time 0 h. Wound healing quantification was assessed by the slope of the wound healing (1 h to 3 h) and the area under the curve measured starting from 1 h.

**Immunofluorescence imaging**

HUVECs were seeded on glass coverslips coated with glutaraldehyde 0.5 % (Fluka, St. Gallen, Switzerland) -crosslinked gelatine. Cells were seeded at 1:1 density and grown to confluence in 24-48 h. Pre-treatment was performed in 1 % Human Serum albumin in M199, containing Klotho (400 pM) or Paricalcitol (10-100 nM). After 24 hours of pre-treatment, thrombin was added to the wells for a final concentration of 1 U/mL. At indicated time points (0'-60'-120'), the medium was replaced by 2 % paraformaldehyde (37 °C, Sigma Aldrich), followed by 15min incubation on ice. Cells were permeabilized with Triton X-100 0.05 % (Sigma Aldrich) in PBS, and incubated overnight with primary antibody against VE-cadherin (1:400; XP D87F2, Cell Signaling). Subsequently, cells were washed and incubated with FITC secondary antibodies (1:100; Invitrogen, Paisly, United Kingdom) and the F-actin cytoskeleton was visualized with rhodamine/phalloidin (1:140; PHDH1 Cytoskeleton Inc, Denver, CO, USA) for two h at room temperature. Cells were washed and nuclei were stained with DAPI (1:500; Sigma-Aldrich) for 15'. Next, cells were washed, and sealed with Mowiol mounting medium.
(Sigma-Aldrich). Imaging was performed with a Leica TCS SP8 STED using a 63x Zeiss oil objective. Image J (National Institutes of Health, Bethesda, Maryland) was used for analysis. For quantification of VE-cadherin/F-actin images were equally adjusted for contrast, mean fluorescence was quantified and divided by the amount of counted nuclei (DAPI).

**Statistical analysis**

Data were analysed using both GraphPad Prism 7 software (La Jolla, CA) as well as the statistical package R (version 3.4.3)\textsuperscript{28}. Two-group comparisons were made by paired or unpaired \(t\) tests. ANOVA (involving a single, two or three factors) was also used to perform comparisons, with Bonferroni multiple-testing correction applied where appropriate, as indicated in figure legends. Analysis of the different factors and their two-way and three-way interactions are reported in the Table 1. Correlation was assessed by using Pearson correlation. Differences were considered statistically significant for \(p<0.05\).

| Table 1 – Analysis of the different independent variables and their interactions for each dependent variable. Table summarizes the different \(p\) values obtained by two-way ANOVA in the 25(OH)D, creatinine and urea dependent variables and three-way ANOVA for the other dependent variables. Differences were considered statistically significant for \(p<0.05\) and were highlighted as green.

**RESULTS**

**Characterization of uremia and vitamin D deficiency on blood chemistry**

Figure 1A shows the experimental design of a combined model of 3/4 nephrectomy (CKD) with vitamin D deficiency\textsuperscript{25} and the corresponding controls (non-CKD). Serum concentrations of urea and creatinine increased two (5.5±0.8 to 11.9±2.4 mmol/l) to three-fold (15.4±1.7 to 44.3±8.9 µmol/l), respectively, three weeks following surgery, and this was stable for the remainder of the experiment (Figure 1B-C). No significant differences on the degree of renal failure were observed among the four CKD groups at the end-point (Supplementary Figure S1A-B). Three weeks after the induction of vitamin D deficiency, the average of 25(OH)D and 1,25(OH)\(_2\)D concentrations were below the detection levels (8 nmol/l and 20 pmol/l respectively) in most of the animals exposed to the vitamin D deficient diet (Figure 1D-E; \(p<0.0001\).
**Figure 1** - Uremia and vitamin D deficiency is established. (A) Representative experimental design and group distribution. CKD was induced by 3/4 nephrectomy (3/4 Nx) at week 0. Vitamin D deficiency was induced from week 1 to 4 and vitamin D deficient diet was maintained until the end of the experiment (week 11). Concurrently, animals received oral paricalcitol (0.1 µg/kg) or vehicle control treatment three times per week during seven weeks in total. To the bottom, an overview of the groups. (non-CKD= non-uremic animals; CKD= uremic animals; VD= vitamin D; PC= paricalcitol; vehicle control = VC). Scatter plot analyses of serum levels of urea (mmol/l) (B) and creatinine (µmol/l) (C) before (week -1) and after (week 3, 6, 11) surgery. Results are means ± SD (n=28). Differences were considered statistically significant for p<0.05, using one-way (repeated measures) ANOVA. (3/4 Nx= induction of 3/4 nephrectomy) ****p<0.0001. Serum levels of (D) inactive (25(OH)D; nmol/l) and (E) active (1,25(OH)2D; pmol/l) forms of vitamin D three weeks after vitamin D deficiency (week 4). Results are means ± SD (n=12-15). # indicates that most of the values were under the detection level and significance could not be calculated for that group. (non-CKD= non-uremic animals; CKD= uremic animals; VD= vitamin D). Differences were considered statistically significant for p<0.05, using two-way ANOVA in D (p=0.0064 for surgery, p<0.0001 for diet, p=0.0545 for surgery x diet) and unpaired t-test for E. **P < 0.001.
for diet by two-way ANOVA). Moreover, the influence of 3/4 nephrectomy was confirmed by two-way ANOVA (p<0.01 for surgery) where CKD animals with standard diet showed a higher concentration of 25(OH)D (92.93±24.8 nmol/l) and a lower 1,25(OH)₂D (187.8±33.1 pmol/l) when compared to non-CKD animals with standard diet (25(OH)D: 70.92±18.97 nmol/l and 1,25(OH)₂D: 244.9±46.78 pmol/l). Thus, serum measurements confirmed the induction of uremia and vitamin D deficiency.

In addition, we investigated whether paricalcitol treatment affected serum PTH levels. When analyzing the overall effect of 3/4 nephrectomy on PTH levels, a significant increase in uremic animals was found, when compared to non-CKD (Supplementary Figure S1C). When comparing individual groups, the significant influence of surgery was confirmed by three-way ANOVA (p<0.01; Supplementary Figure S1D). Furthermore, PTH concentrations where significantly affected by treatment and diet (p<0.01 in both cases) with effects differing between groups. Specifically, paricalcitol treatment reduced PTH concentrations in vitamin D-deficient CKD animals (261.5±80.1 untreated and 98±69.2 pg/ml treated) with no significant effect in other groups (p=0.024 for treatment x diet).

**Paricalcitol treatment attenuates uremia-induced endothelial damage in thoracic aorta**

To explore the effects of uremia and vitamin D deficiency on aortic endothelial permeability, animals were injected intravenously with Evans Blue dye 30’ before sacrifice. CKD was confirmed to have a significant effect on vascular permeability by a three-way ANOVA (p<0.01 for surgery). The permeability of the aortic endothelium was increased (53 %) in untreated CKD animals on standard diet when compared with non-CKD controls (Figure 2A). A similar trend (43 % increase in Evans Blue penetrance) was observed for non-treated uremic vitamin D deficient animals compared with non-CKD animals. Endothelial permeability was normalized in CKD animals treated with paricalcitol, irrespective of the diet (Figure 2A; p=0.021 for treatment, p=0.012 for surgery x treatment). No changes were observed in non-CKD animals irrespective of both diet and treatment. To further support the findings on Evans Blue extravasation, the surface perimeter of the aorta was examined for endothelial gaps, which are an anatomical correlate of the permeability of the aortic endothelium (Figure 2B; enlargements). CKD animals without paricalcitol treatment showed an increment (13.6-13.8 %) of the endothelial gaps (μm negative surface CD31*100 / μm total CD31) in good agreement with the data of the Evans Blue penetration (Figure 2C; p<2⁻¹⁶ for surgery). Paricalcitol treatment rescued these differences and protected against endothelial damage in CKD animals with both standard and vitamin D deficient diet (p=0.041 for treatment). Subsequently, three-way ANOVA analysis confirmed that the effect of surgery on endothelial damage is dependent of the type of treatment provided to the animals (p=0.006 for surgery x treatment)
Figure 2 - Paricalcitol ameliorates uremic-induced endothelial dysfunction. (A) Quantification of formamide-extracted Evans Blue (EB; EB tissue (µg/ml) / tissue weight (TW; mg)/ EB plasma µg/ml in thoracic aorta corrected for tissue weight and the Evans Blue plasma concentration (non-CKD= non-uremic animals; CKD= uremic animals; VD= vitamin D; PC= paricalcitol). p=0.003 for surgery, p=0.021 for treatment, p=0.911 for diet, p=0.012 for surgery x treatment. (B) Representative images of immunohistochemistry of thoracic aorta sections stained with CD31 antibody. Images represent one rat per group. Scale bars are 100 µm, magnification: 20x. Enlargements of the white boxes are included on the bottom of every figure. p=2x10^-16 for surgery, p=0.041 for treatment, p=0.958 for diet, p=0.006 for surgery x treatment. (C) Graph shows % of endothelial gaps measured on three sections of the aortic tissue (µm of CD31 negative surface*100 / µm total surface measured CD31). Data are represented as means ± SD (n=4-7). Differences were considered statistically significant for p<0.05 using three-way ANOVA. (D) Correlation between EB extravasation and % of endothelial gaps (Pearson regression; p<0.05; n=33).
while no significant two and three interactions were detected with the diet. In addition, a positive Pearson correlation ($r=0.475$, $p=0.0051$) was found between the amount of Evans Blue extravasation and the % of endothelial gaps suggesting a direct relationship between these two parameters (Figure 2D). Thus, we demonstrate that CKD drives aortic endothelial leakage and dysfunction independently from endogenous 25(OH)D status, whereas paricalcitol improved the integrity of the endothelium from uremia-induced barrier loss.

**Endothelial changes are partially associated with serum α-Klotho levels**

To determine if alterations in endothelial integrity are possibly mediated by α-Klotho, gene expression and protein abundance of α-Klotho were first examined in kidney tissue (Supplementary Figure S2). When testing the overall effect of uremia on α-Klotho, we observed that CKD leads to a significant downregulation (by 40 %) of both α-Klotho protein and mRNA levels when compared with non-CKD animals (Figure 3A, 3C). After comparing individual groups, three-way ANOVA analysis confirmed that surgery had a significant influence in α-Klotho protein ($p<2^{-16}$) and mRNA levels ($p=0.001$) while no differences were found when animals were challenged with standard or vitamin D deficient diet. Furthermore, no effect was detected after paricalcitol supplementation (Figure 3B, 3D). In accordance with the results in the kidney, we found a statistically significant effect of CKD on α-Klotho serum concentrations (50 % decrease) when comparing all CKD with non-CKD animals (Figure 3E). When evaluating the effect of paricalcitol on serum α-Klotho, there was a trend in both non-CKD (10.7±3.1 vs 23.8±7.1 pg) and CKD groups (4.8±2.0 vs 10.2±8.6 pg) with standard diet while no influence was detected in the vitamin D deficient groups. Three-way ANOVA analysis showed a significant independent effect from surgery on soluble α-klotho ($p=0.001$) while treatment displayed a borderline effect, both independently ($p=0.051$) and together with diet ($p=0.054$ for treatment x diet). Together, these findings indicate that CKD leads to α-klotho deficiency in kidney tissue while no effect was observed for vitamin D status. Although α-klotho levels were not restored by paricalcitol treatment, we cannot exclude the possibility that α-Klotho contributed to the vasoprotective effect observed in uremic animals.

**Paricalcitol stabilizes basal human endothelial barrier function**

To investigate the direct effects of paricalcitol and α-Klotho on endothelial integrity, in vitro studies focused on the real-time changes in barrier function measured by Electric Cell-substrate Impedance Sensing (ECIS). Paricalcitol (100 nM) effectively increased (8.4 %) the absolute basal endothelial resistance after 24 h exposure. No significant impact on the basal endothelial barrier function was detected 24 h after the addition of α-Klotho (400 pM; 4.3 %) nor for the lower dose of paricalcitol (10 nM; 5.3 %) when compared to control (Figure 4A).
Figure 3 - Effect of paricalcitol on α-Klotho levels in kidney tissue and in the circulation. Quantification of α-Klotho protein (α-Klotho /GAPDH) normalized to control group in kidney tissue, (respective immunoblot is shown in Supplementary Figure S2) of non-CKD and CKD animals pooled (A; n=23-27) and not pooled groups (B; n=6-8). p=2x10^-14 for surgery, p=0.935 for treatment, p=0.970 for diet. Relative mRNA expression of α-Klotho was assessed by qPCR in kidney tissue of pooled (C; n=23-28) and non-pooled (D; n=6-8) groups. B-Actin was used as housekeeping gene. p=0.001 for surgery, p=0.881 for treatment, p=0.855 for diet Serum α-Klotho (week=11) was measured by the IP-IB assay (E; pooled samples n=22-24) and (F; separate groups n=5-7). p=0.001 for surgery, p=0.051 for treatment, p=0.830 for diet (non-CKD= non-uremic animals; CKD= uremic animals; VD= vitamin D; PC= paricalcitol). Data are represented as means ± SD. Differences were considered statistically significant for p<0.05 using unpaired t-test for A, C, E and three-way ANOVA for B, D, F. **p<0.01, ***p<0.001, ****p<0.0001.
Based on our current findings, we proceeded by evaluating whether the distribution of the main adherens junction protein Vascular-Endothelial (VE)-cadherin, a determinant of endothelial junctional stability\textsuperscript{29}, and F-actin re-arrangement could explain the changes of endothelial barrier enhancements. Immunostaining for VE-cadherin revealed that paricalcitol significantly enhanced its peripheral localization in a dose-dependent manner (39 % for 10 nM and 48 % for 100 nM) compared to control conditions (Figure 4B-C). In addition, the F-actin content was found to be significantly strengthened and more densely arranged in the cortical area of paricalcitol-stimulated (100 and 10 nM) HUVECs while controls showed a normal F-actin distribution (Figure 4D). α-Klotho-treated HUVECs showed no changes for VE-cadherin and F-actin intensity. Thus, these findings suggest that paricalcitol modulates endothelial barrier enhancement by enforcement of junctional VE-cadherin and the cortical F-actin cytoskeleton.
Paricalcitol attenuates endothelial dysfunction following barrier-disruptive agents

To further test whether paricalcitol and α-Klotho contribute to the stabilization and restoration of reduced endothelial integrity induced by a challenge, we analysed HUVECs in an electric wounding assay. The restoration of endothelial resistance for paricalcitol 100 nM in this wound healing assay was significantly higher compared to control, as reflected by the slope of the recovery after wounding (140.7±7.5 vs 116.3±11.5 a.u.) and the area under the curve starting from 1h (5651±88.33 vs 5158±154.4 a.u.) (Figure 5A-C). Next, using thrombin as a pro-permeability factor, we evaluated the capacity of paricalcitol and α-Klotho in preventing the endothelial permeability and gap formation. In accordance with the wound assay, paricalcitol attenuated thrombin-induced endothelial dysfunction as shown by a 35% (100 nM) and 38% (10 nM) reduction of the resistance after one h of thrombin as compared to the control which showed a 45% reduction induced by thrombin (Figure 5D-E). The area under the curve of normalized data from baseline after the addition of thrombin was significantly higher for HUVECs pre-stimulated with paricalcitol 100 nM as compared to the control (3.35±0.14 vs 2.96±0.07 a.u.) (Figure 5F). There was a small, statistically non-significant, effect during the recovery phase for α-Klotho pre-stimulated HUVECs (43% drop; 3.20±0.18 a.u.). Finally, using immunofluorescent detection of VE-cadherin and F-actin fibers, we could confirm that paricalcitol attenuated the thrombin-induced intercellular gap formation after 60’ and 120’, which is in line with the real-time measurements of the barrier function (Figure 5G).

The induction of F-actin stress fibers upon 60’ thrombin stimulation was not prevented by paricalcitol although, after 120’, cells showed a more densely packed cortical F-actin ring (Figure 5G). Taken together these findings indicates that paricalcitol strengthened the endothelial barrier during disruptive conditions accompanied by enhanced endothelial migration and limited the inter-endothelial cell gap formation. We observed no statistically significant effect of α-Klotho-treated cells whereas paricalcitol confirmed distinct beneficial contributions to the maintenance of the endothelial barrier integrity.
Figure 5 - Paricalcitol protects against endothelial barrier dysfunction. (A) Representative time-course absolute endothelial electrical resistance measurements of HUVEC monolayers during migration after 24h stimulation in basal medium plus 1% Human Serum albumin. Arrow indicates the induction of an electrical wound. (B-C) Quantification of the wound healing capacities are represented as the slope of the curves (1 h to 3 h; a.u.) and the area under the curve (a.u.) measured from 1 h onwards. (D) Representative absolute endothelial electrical resistance
DISCUSSION

Our study demonstrates that CKD induces endothelial cell disruption in the thoracic aorta in vivo and that this can be attenuated by paricalcitol, an active vitamin D analogue. This conclusion is based on a striking parallel between Evans Blue penetration into the aortic wall and the detachment of endothelial cells from the aorta, both pathological features which normalized after administration of paricalcitol. Importantly, baseline vitamin D status, both active and nutritional, did not play a role in these effects. Originally, we hypothesized that presumed protection of paricalcitol was mediated by an increment of circulating α-Klotho. Although our data do suggest some increase in circulating α-Klotho following paricalcitol administration in vitamin D sufficient animals, this increase was not statistically significant. However, this does not exclude that α-Klotho did contribute to vasoprotection in our uremic model. Finally, we confirmed in vitro that paricalcitol enforces endothelial cell-cell adhesions and thereby the endothelial monolayer integrity. This feature most likely prevents the inter-endothelial gap formation seen in vivo by enhancing the restoration of the endothelial barrier following disruptive hits, suggesting that paricalcitol was the principal mediator of the endothelial stability following uremia.

Limited previous findings pointed to abnormal endothelial cell properties in CKD contributing to cardiovascular complications in patients. Although the underlying mechanistic pathway that might explain the loss of endothelial barrier function in CKD has been unclear so far, it was suggested that vitamin D deficiency might participate in this uremia-induced phenomenon. Here, we found that vitamin D deficiency induced in our recently developed rat model, did not aggravate the injury on the aortic endothelial layer caused by CKD. Despite these findings, supplementation with the active vitamin D analogue paricalcitol did result in amelioration of the aortic endothelial permeability and limited the gap formation observed in CKD rats. In line with our results, Wu-Wong et al. have reported that paricalcitol can improve endothelial-dependent relaxation in uremic rats independent from effects on PTH serum concentration. Recently, randomized clinical trials conducted in patients with CKD also demonstrated that treatment with active vitamin D leads to significantly favourable changes in endothelial function.
In accordance with our *in vivo* findings, we confirmed the potency of paricalcitol as endothelial barrier function stabilizer in HUVECs by ECIS. Further, paricalcitol was found to enhance recovery from endothelial disruptive hits such as an electric wound (simulating an injury in the endothelium) or thrombin (to induce permeability and gap formation). Interestingly, this effect was mediated by enhanced intercellular junctions resembling a protective mechanism previously reported in brain endothelial cells where active vitamin D preserved the barrier integrity following hypoxia. Here, we found that paricalcitol strengthened the adherens junction protein VE-cadherin in HUVECs, an endothelium-specific homotypic adhesion protein essential to control the integrity of the endothelial monolayer. In addition, VE-cadherin interacts, via other proteins, with the F-actin cytoskeleton to orchestrate the cell-cell contact and thus maintaining barrier function. Our data show that paricalcitol stabilizes the cortical F-actin ring formation, which is necessary for the maintenance of a quiescent endothelium. After thrombin addition, there is a re-organization of the cortical actin ring as a requirement for inter-endothelial gap formation. Interestingly, immunofluorescent imaging revealed that HUVECs, pre-stimulated with paricalcitol, displayed less gap formation upon thrombin, which is in line with the barrier function studies. These endothelial protective effects could be mediated by changes in Matrix Metalloproteinase 9 (MMP9) concentrations. As recently demonstrated, active vitamin D can reduce MMP9 expression in brain endothelial cells and prevent the hypoxia-mediated loss of cell-cell contact. Our observations are in line with previous studies where active vitamin D preserved the integrity of intestinal mucosal barrier and stimulated epithelial cell migration. Together, our findings suggest that paricalcitol limits uremia-induced endothelial damage by enhancing endothelial barrier integrity and recovery capacity following a disruptive hit. Furthermore, although adherens junctions were not examined in the *in vivo* study, we suggest that endothelial cell detachment and permeability observed in aortic tissue was attenuated by enforced VE-cadherin in paricalcitol treated rats.

Instead of direct effect on target tissue, as an alternative explanation for beneficial effects of active vitamin D, it has been postulated that it can upregulate renal α-Klotho as the actual effector of remote beneficial effects, after shedding its ectodomain. Indeed, α-Klotho deficiency in mice leads to a limited vasodilatory response and increased permeability in the endothelial cells among other vascular complications also observed in CKD patients. As reported previously in other uremic *in vivo* models and CKD patients, we confirmed that impaired kidney function induced downregulation of mRNA and α-Klotho protein levels in kidney tissue accompanied by lower serum concentrations. However, it was unexpected that neither vitamin D deficiency nor paricalcitol supplementation affected α-Klotho in the kidney tissue nor its concentration in serum. Only in paricalcitol-treated, vitamin D sufficient animals, a non-significant trend was noted. Consistent with this suggestion of increase, Lau...
et al. found, in intraperitoneally (IP) paricalcitol (100 ng/kg and 300 ng/kg) treated uremic mice on high phosphate diet, increased serum α-Klotho concentrations despite unchanged kidney tissue expression, leaving the origin of the increment elusive\textsuperscript{20}. In this experiment, we applied similar paricalcitol concentrations (100 ng/kg) as in that study, and mimicked the clinical setting by applying an oral, instead of IP, route of administration which might have limited the efficacy on serum α-Klotho concentrations in our animal model.

Although our experiments do not support a protective effect of α-Klotho on the aortic endothelium, this cannot be completely ruled out either. In previous experimental in vitro studies, the α-Klotho protein was found to suppress oxidative stress in the endothelium, to increase Nitric Oxide production and to limit endothelial permeability by the internalization of both VEGF receptor-2 and TRPC-1\textsuperscript{31, 44}. Moreover, other experimental studies found an endothelium-protective effect by overexpression of α-Klotho in endothelial cells\textsuperscript{45} or application of culture media from α-Klotho-expressing cells\textsuperscript{44}. Here, when we applied an effective exogenous α-Klotho concentration (400 pM) in the in vitro parts of our study as previously reported\textsuperscript{46, 47}, we observed a statistically non-significant trend of the basal endothelial barrier function. However, this effect was limited when compared to paricalcitol effects suggesting that the active vitamin D analog was the more important mediator.

Our study has some limitations. While we applied established experimental models of uremia and vitamin D deficiency, the experimental design did not include sham surgery for non-CKD animals, so we cannot exclude that some differences are the consequences of the surgery itself and not of kidney failure. Based on the study duration however and our experience when developing the model, we consider it highly unlikely that differences between the groups are based on anything else than differences in kidney function, and decided to limit animal discomfort for the control group. Another possible limitation is that we administered paricalcitol orally, making bioavailability less predictable. However, based on the PTH suppressive effect and the effect on our primary read-outs we think this was of limited relevance and may only lead to underestimation of the true effect. Similarly, it might have been of interest to determine whether changes in the renin-angiotensin system and blood pressure could partially explain the results observed in the uremic model, as active vitamin D suppresses renin, which in turn may have an effect on the endothelium. Despite these limitations, the beneficial effects of paricalcitol in the in vitro setup are unlikely explained by effect-mediation of renin suppression by paricalcitol. In addition, we cannot exclude the possibility that the beneficial effects of paricalcitol on the endothelium are related to a local increment of α-Klotho. More research is required to determine the influence of α-klotho during active vitamin D-mediated endothelial protection. Finally, electric wound and thrombin were used to simulate endothelial injury and permeability detected in rat aortic endothelium and evaluate paricalcitol and α-Klotho protective capacities. However, these two
approaches provide a close, but incomplete, estimation of the damage exerted by uremia in the endothelium. Moreover, the results were similar for these two different forms of endothelial injury.

Due to recent negative results from clinical trials in patients with CKD that aimed to improve cardiac function (PRIMO and OPERA), enthusiasm to use active vitamin D was dampened, because of the obviously slightly higher risk for hypercalcemia. However, nihilism about potential benefits of the clinical use of active vitamin D seems inappropriate. Collectively, we demonstrated a consistent, protective effect of the oral use of paricalcitol on uremia-induced endothelial disruption. Although α-Klotho has been suggested to be an important mediator of endothelial cell stability, we could not demonstrate its role in the paricalcitol-mediated vasoprotective effects. Alternatively, we provide evidence that paricalcitol directly maintains endothelial barrier integrity by enhancing the cell to cell contacts. Clinically, this benefit may translate into improved vascular health in patients with CKD.

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**SUPPLEMENTARY FIGURES**

### Supplementary Figure 1 - Kidney function and PTH serum levels in male Wistar rats (end-point).
Comparison of urea (μmol/l) (A) and creatinine (mmol/l) (B) serum measurements in CKD animals at week 11 (n=7-8). p=0.0098 for diet in creatinine. PTH (pg/ml) serum analysis at week 11 (C) (pooled groups n=17-19) and (D) (not pooled groups n=4-5). p=0.003 for surgery, p=0.002 for treatment, p=0.007 for diet and p=0.024 for treatment x diet. Data are represented as means ± SD. (non-CKD= non-uremic animals; CKD= uremic animals; VD= vitamin D; PC= paricalcitol). Differences were considered statistically significant for p<0.05 using unpaired t-test (C) two-way (A,B) and three-way ANOVA (D)**p<0.01.

### Supplementary Figure 2 - α-Klotho protein in kidney tissue.
Representative Immunoblotting analysis of α-Klotho with KM2076 antibody in total protein lysates of Kidney (n=6-8). GAPDH was used as loading control. (non-CKD= non-uremic animals; CKD= uremic animals; VD= vitamin D; PC= paricalcitol).
Stabilization of cell-cell junctions by active vitamin D ameliorates uremia-induced loss of human endothelial barrier function

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ABSTRACT

BACKGROUND

Uremia induces endothelial cell (EC) injury and impaired repair capacity, of which the underlying mechanism remains unclear. Active vitamin D (VD) may promote endothelial repair, however, the mechanism that mediates the effects of VD in chronic kidney disease are poorly understood. Thus, we investigated uremia-induced endothelial damage and the protection against such damage by active VD.

METHODS

We applied Electric Cell-substrate Impedance Sensing (ECIS) to study real-time responses of human ECs exposed to pooled uremic and non-uremic plasma with or without the addition of active VD. The effects of Indoxyl Sulfate and p-cresol were tested in non-uremic plasma. Structural changes for VE-cadherin and F-actin were assessed by immunostaining and quantified.

RESULTS

The exposure of ECs to uremic media significantly decreased endothelial barrier function after 24h. Cell migration after electrical wounding and recovery of the barrier after thrombin-induced loss of integrity were significantly impaired in uremic-medium stimulated cells and cells exposed to Indoxyl Sulfate and p-cresol. This effect on ECIS was dependent on loss of cell-cell. Mechanistically, we found that EC, exposed to uremic media, displayed disrupted VE-cadherin interactions and F-actin reorganization. VD supplementation rescued both endothelial barrier function and cell-cell interactions in endothelial cells exposed to uremic media. These events were associated with an increment of VE-cadherin at intercellular junctions.

CONCLUSIONS

Our data demonstrate a potentially clinically relevant mechanism for uremia-induced endothelial damage. Furthermore, active VD rescued the uremic medium-induced loss of cell-cell adhesion revealing a novel role of active VD in preservation of endothelial integrity during uremia.
INTRODUCTION

Increased mortality risk in patients with Chronic Kidney Disease (CKD) is frequently linked to cardiovascular disease. CKD impacts vascular health, and especially vascular calcification of the medial layer of the vessel wall or secondary calcifications of atheromatous plaques has received much attention. The integrity of the endothelium is a somewhat neglected aspect, yet it plays a pivotal role in different aspects of the vascular function. Endothelial dysfunction is suggested to be an instigator of the development and progression of cardiovascular events in CKD. Signs of endothelial cell (EC) injury such as impaired endothelium-dependent vasodilatation, suppression of nitric oxide production and inflammation are frequently observed in CKD patients. However, while it is conceivable that uremic toxins may interfere with vascular endothelial integrity, there is only limited evidence for a direct effect of uremia on EC.

Several kidney disease-related risk factors, including the progressive decline of circulating active vitamin D (VD) levels, are suggested to cause adverse changes in vascular function. In addition, 25 Hydroxivitamin D (25(OH)D) deficiency is suggested to have a crucial contribution to the development and progression of EC dysfunction in CKD patients. Moreover, active VD supplementation was suggested to reduce cardiovascular events such as vascular calcification or cardiac abnormalities in CKD animal models. However, these results contrast with clinical trials using active VD therapy in CKD patients and controversy remains regarding its beneficial potential, especially with regard to the attenuation of cardiac disease in CKD. In EC dysfunction, it was shown that uremia impaired vasodilatory response in animals, and disturbed artery flow-mediated dilatation in selected patients was prevented by active VD supplementation. However, while it is recognized that active VD analogs modulate endothelial inflammation, cell-cell junction stability and oxidative stress in in vitro studies, the mechanisms that modulate these effects of active VD in CKD, are poorly understood.

This lack of knowledge of molecular mechanisms that explain the effects of uremia on endothelial barrier function and the beneficial effect of active VD, formed the rationale to use innovative in vitro measurements to study the EC behaviour in CKD. For this, we applied Electric Cell-substrate Impedance sensing (ECIS) to measure the real-time changes of the barrier function of human EC under the influence of uremic plasma (eGFR<18). We found impaired barrier function and disrupted cell-cell contact of EC exposed to human uremic plasma. These results were in agreement with the effects of the uremic toxins indoxyl sulfate and p-cresol. Altered levels of the adherens junction protein Vascular-Endothelial (VE)-cadherin were identified as potentially underlying this EC dysfunction. Finally, the active VD analogue paricalcitol attenuated the disrupted barrier function induced by uremia and restored
VE-cadherin at junctions. We suggest that targeting impaired stability of adherens junctions might form a therapeutic goal for EC dysfunction in CKD.

**MATERIALS AND METHODS**

**Patients and sample collection**

CKD plasma samples were obtained from 6 patients (3 male and 3 female) non-dialyzed with CKD (stage IV-V), age 75.8±7.9, (mean±SD), glomerular filtration rate (eGFR) 18.1±6.7ml/min/1.73 m². Non-CKD plasma samples were from 6 healthy donors (4 male and 2 female), age 35.9±14 with >90eGFR. Plasma samples were obtained from each patient by centrifugation at 2500xg during 10 min at 4°C. Supernatant was centrifuged for 5 min at 13500xg to achieve platelet-free plasma. Plasma samples were analyzed for 25(OH)D by liquid-liquid extraction and liquid chromatography-tandem mass spectrometry detection (The precision of the assay is 4% mean intra-assay coefficient of variation, CV and 7.5% mean inter-assay CV) and 1,25-dihydroxyvitamin D (1,25(OH)₂D) by solid-phase immunoextraction and liquid chromatography-tandem mass spectrometry detection (3.5% mean intra-assay CV and 7.5% mean inter-assay CV). Details of human plasma donors are provided in Table 1.

**Endothelial cell culture**

Human umbilical vein endothelial cells (HUVECs), were isolated as described previously, from umbilical cords from healthy donors, obtained from the Amstelland Ziekenhuis, Amstelveen. Cells were taken up in M199 medium, supplemented with penicilline 100U/mL and streptomycin 100µg/mL (Biowhittaker/Lonza), human serum 10% (Sanquin Blood Supply, Amsterdam, The Netherlands), new-born calf serum 10% (Gibco, Grand Island, NY), crude EC growth factor 150µg/mL (prepared from bovine brains), L-glutamine 2mmol/L (Biowhittaker/Lonza), and heparin 5U/mL (Leo Pharmaceutical Products, Weesp, The Netherlands). HUVECs were cultured at 37°C and 5% CO₂, with a change of culture medium every two days. Cells were cultured up to passage 2; for experiments. For experiments, HUVECs were washed with bare medium (bM199) consisting of M199 supplemented with penicillin 100 IU/ml and streptomycin 100 µg/ml, and exposed to bM199 with 20% of pooled non-CKD/CKD plasma during 24h, combined with or without paricalcitol (100 nM; kindly provided by AbbVie, Chicago, USA). Based on measurements of concentrations in CKD patients with <30 eGFR, uremic toxins Indoxyl Sulfate and p-cresol (Sigma Aldrich) were tested at 100 µM in bM199 with 20% (v/v) of pooled non-CKD plasma.

**Electric cell-substrate impedance sensing (ECIS)**

Transendothelial electrical resistance of HUVEC monolayers was measured using ECIS (Applied BioPhysics, Troy, NY, USA) as previously described. Using 8-well ECIS arrays (10 electrodes per well, 0.49mm² electrode area) HUVECs were seeded in a 1:1 density on a gel-
tin-coated array. ECIS software (v1.2.65.0 PC; Applied BioPhysics) was used to calculate the level of overall resistance (4000Hz), cell–cell contact (Rb) and cell–matrix contact (α) as depicted in Supplementary Figure 1A. Cells were grown to confluency (48h) and medium was renewed 24h after seeding. After 24h of pre-treatment, electrical wounding was applied (60s; 100000Hz). For quantification of the wounding response, the slope (assessed by linear regression) and the area under the curve (AUC) of the recovery phase from each replicate (measured after electrical wound induction) was calculated. The effect of thrombin (1U/ml; Sigma Aldrich, Zwijndrecht, the Netherlands) was quantified after 60 minutes pre-treatment with bM199 containing 1% human serum albumin (HSA; Sanquin Blood Supply). Resistance was normalized to the initial value before the addition of thrombin. The % of drop after the addition of thrombin and the AUC from each replicate were quantified to assess the extend of the thrombin response.

**Immunofluorescence imaging**

HUVECs were seeded in 1:1 density on glass coverslips coated with glutaraldehyde 0.5% (Fluka, St. Gallen, Switzerland)-crosslinked gelatine and grown to confluency in 24-48h. After 24 hours of treatment, medium was replaced by 2% paraformaldehyde (37°C, Sigma Aldrich), followed by 15 min incubation on ice. Fixed cells were permeabilized with Triton X-100 0.05% (Sigma Aldrich) in PBS, washed, and incubated overnight with primary antibody against VE-Cadherin (1:400; XP D87F2, Cell Signaling). Afterwards, HUVECs were washed and incubated with FITC secondary antibodies (1:100; Invitrogen, Paisly, UK) and the F-actin cytoskeleton was stained with rhodamine/phalloidin (1:140; PHDH1 Cytoskeleton Inc) for 2h at room temperature. Subsequently, cells were washed and nuclei were stained with DAPI (1:500; Sigma-Aldrich) for 15 minutes. Cells were washed, and sealed with Mowiol mounting medium (Sigma-Aldrich). Imaging was performed with a Leica TCS SP8 STED using a 63x Zeiss oil objective. ImageJ (NIH, Bethesda, Maryland) was used for analysis. For quantification of VE-cadherin/F-actin, images were equally adjusted for contrast, mean fluorescence was quantified and divided by the amount of counted nuclei (DAPI).

**Fluorescence-activated cell sorting (FACS)**

VE-cadherin membrane expression was quantified in HUVECs using a specific antibody (Allexa Fluor® 647 anti-mouse CD144) for 30 minutes. HUVEC were rinsed with PBS twice and then detached using Accutase (GE Healthcare, Eindhoven, The Netherlands). Detached cells were resuspended in ice-cold PBS supplemented with 0.5% (w/v) BSA, centrifuged and resuspended in PBS + 0.5% BSA. FACS analyses were performed with Calibur flow cytometer (Becton & Dickinson, San Jose, CA, USA) and FlowJo (Oregon, USA) software.
**Statistical analysis**

Data were analysed using GraphPad Prism software (La Jolla, CA). Statistical analysis were performed using unpaired t-test for two groups and three or more groups using One-way ANOVA with Bonferroni correction. Data were shown as means ± SD. Differences were considered statistically significant for \( p<0.05 \) (*\( p < 0.05 \), **\( p<0.01 \), ***\( p<0.001 \)).

<table>
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<tr>
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<td>Age (years)</td>
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<td>eGFR (ml/min/1.73 m²)</td>
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<tr>
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<td>56</td>
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<tr>
<td>1,25(OH)2D (pmol/L)</td>
<td>96</td>
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Table 1. Clinical and Biochemical characteristics of the non-CKD and CKD plasma. Gender is indicated as (m: male; f:female). Age, and eGFR are expressed as mean ± SD (n=6). Biochemical measurements of 25(OH)D and 1,25(OH)2D are measured in plasma pools.

**RESULTS**

**Uremic plasma induces endothelial barrier disruption by reduced cell-cell interaction**

To test whether a uremic environment affects endothelial barrier integrity, confluent HUVEC monolayers were exposed to plasma from patients with CKD (eGFR<18ml/min/1.73 m²) or healthy subjects (eGFR>90ml/min/1.73 m²) (v/v; 20%). Baseline characteristics of the plasma donors are reported in Table 1. Initially, endothelial resistance was reduced (1536±21.8 vs 1477±27.4Ω; \( p=0.0426 \)) in uremic plasma-treated HUVECs after 3h when compared to non-CKD plasma (Figure 1A). Although the endothelial resistance normalized to the control values, a second decay of the resistance was observed with statistically significant differences at 24h (1530±10.1 vs 1432±24.5 Ω; \( p=0.0031 \)). Subsequently, we tested recovery after thrombin-induced (1U/ml) contraction of HUVECs inducing a loss of barrier function, 24h after plasma exposure (Figure 1B). Increased loss of barrier function was observed for CKD-plasma exposed EC after thrombin stimulation. This was reflected by the % of drop in resistance and accompanied by a significantly reduced AUC measured after data normalization: CKD (49.5%; 3.24±0.01a.u.), vs. non-CKD controls (41.5%; 3.55±0.01a.u.) (Figure 1B). Similarly, HUVECs challenged with uremic media displayed a reduced recovery capacity in the wound healing assay (Figure 1C). Quantitative analysis showed a significant reduction for both slope (1h to 5h) and AUC of the recovery phase (1h to 8h) of CKD-plasma exposed cells.
Figure 1. Uremic plasma induces endothelial barrier disruption in HUVECs (A) Representative time-course of absolute endothelial electrical resistance measurements (Ω) of confluent HUVECs stimulated for 24h with 20% (v/v) of non-CKD plasma (dark) or CKD plasma (grey). Insert: quantification of the absolute endothelial resistance (Ω) after 3h and 24h of stimulation as indicated by arrowheads. (B) Normalized resistance of thrombin (1U/ml)-stimulated HUVECs. Upon 24h incubation with indicated media, HUVECs were exposed to a basal medium plus 1% human serum albumin during 1h and challenged with thrombin (arrow). Bar graphs: (left) quantification of the thrombin response represented by the area under the curve (starting at timepoint 0) and (right) to maximum drop of resistance (%) following stimulation. (C) Resistance measurements (Ω) after electrical wounding (arrowhead) of 24h 20% plasma pre-stimulated cells. Bar graphs: (left) quantification of the wound healing as the area under the curve (from 1h to 8h) and (right) the slope of the recovery (from 1h to 5h). a.u., arbitrary units. Data shows means ± SD (n=3, from pool of 3 donors). Representative data of at least 3 experiments. Differences were considered statistically significant for p<0.05 using unpaired student’s t-test where *P < 0.05, **p<0.01, ***p<0.001.
(170±1a.u.; 7636±43.1a.u.) compared to non-CKD controls (217±13.6a.u.; 8544±159.3a.u.). Furthermore, measurements 48h after the induction of a wound confirmed that differences in AUC between CKD and non-CKD plasma sustained without normalization of CKD condition (11.34% less of AUC in CKD after 24h and 14% after 48h when compared to non-CKD) (Supplementary Figure 2).

Figure 2. Impaired endothelial barrier function in CKD is caused by reduced cell-cell interaction. Absolute endothelial electrical resistance attributable to cell-matrix interaction (α; Ω⁰.⁵cm⁻¹) (A) and cell-cell interaction (Rb; Ωcm²) (B) of confluent HUVECs stimulated for 24h with 20% (v/v) non-CKD plasma (dark) or CKD plasma (grey). Normalized values of α (C) and Rb (D) upon thrombin (1U/ml; arrow) stimulation. Time course of endothelial α (E) and Rb (F) values after electrical wounding (arrow). Data shows means ± SD (n=3, from a pool of 3 donors). Representative data of at least 3 experiments. *, p<0.05 using unpaired student’s t-test.

To elucidate whether changes in basal EC barrier function were related to effects on the cell-cell contact or cell-matrix contact, ECIS modelling of the parameters Rb (resistance
between cells) and α (resistance between cells and extracellular matrix) was performed\cite{31} (Supplementary Figure 1A). As shown in Figure 2A, no significant changes were observed for cell-matrix interactions after 24h exposure of EC to different media. In contrast, impaired cell-cell contact (9% reduction; p=0.039) was found in HUVECs challenged with uremic media (Figure 2B). No differences in cell-matrix interactions were detected during the recovery phase in both assays (Figure 2C, 2E). The changes observed in cell-cell interactions after thrombin treatment or electrical wounding mimic the EC basal alterations (Figure 2D, 2F). Together, these findings indicate that uremic media decrease endothelial barrier function and limit the recovery capacity after an electrical wound healing and thrombin exposure. Abnormal cell-cell- but not cell-matrix contact mediates these uremic plasma-induced changes in resistance.

**Uremic toxins indoxyl-sulfate and p-cresol aggravate non-CKD plasma wound recovery**

To investigate whether the uremic retention solutes indoxyl sulfate and p-cresol may be involved in the uremic media-mediated loss of the endothelial barrier, we tested how the addition of these toxins interfere with the barrier function of a non-CKD condition. The direct addition of these toxins in isolation (100 µM), resulted with no changes on resistance during measurements of basal endothelial barrier function or wound healing (data not shown). When a combination of indoxyl-sulfate and p-cresol (100 µM) was tested, no differences in basal EC resistance were detected after 24h (Supplementary Figure 3A). Alternatively, uremic toxins significantly delayed the recovery of an electric wound as reflected by measurements of the slope of resistance recovery (2-6h after wounding; 0.30±0.01a.u. for non-CKD and 0.25±0.02a.u. for indoxyl sulfate + p-cresol) (Supplementary Figure 3B-C). Quantitative analysis of the AUC confirmed that the combined uremic toxin-exposure results in a declined recovery of barrier integrity at 12h after wounding (12.58±0.64a.u. vs 10.82±0.79a.u.) and the differences were further increased after 40h (87.67±3.98a.u. vs 71.44±5.85a.u.) (Supplementary Figure 3D). Similar to CKD media, the joint effects of indoxyl sulfate and p-cresol on wound recovery were mediated by reduced cell-cell interactions (Supplementary Figure 4B) while cell-matrix interactions remained unaffected (Supplementary Figure 4B).

However, no significant differences were found when the AUC was measured (Supplementary Figure 4C) although the effect of the uremic toxins in cell-cell interactions was more pronounced after 40h mirroring the AUC measurements assessed after wound (Supplementary Figure 3C). Thus, the addition of uremic toxins at 100µM to healthy plasma showed impaired recovery after a wound with disturbed cell-cell contact.

**VE-cadherin localization is impaired in uremic conditions**

Next, we explored the structural changes within the EC using VE-cadherin and F-actin (im-
VE-cadherin, which is essential to modulate and maintain the inter-EC contacts, showed a markedly disrupted peripheral localization and discontinuous organization after uremic media exposure (Figure 3A). Consistently, after uremic plasma stimulation, there was a significant reduction of the VE-cadherin fluorescence intensity when compared not only to non-CKD plasma (45% less) but also to a control media (42% less). Similarly, quantitative analysis using flow cytometry confirmed a decreased membrane expression of VE-cadherin in HUVECs exposed to CKD plasma (41% vs non-CKD) (Figure 3D-E). Furthermore, non-uremic plasma-exposed EC showed a peripheral distribution of the F-actin organized as a cortical actin rim which promotes the assembly of endothelial cell-cell and cell-matrix adhesions. In contrast, exposure to uremic plasma resulted in prominent F-actin stress fibers, characteristic of cell contraction albeit without significant changes when compared to non-CKD (0.82±0.03 vs 0.64±0.09a.u.; p=0.061) (Figure 3C). No changes in VE-cadherin expression where found in cells exposed to control media when compared to non-CKD media although HUVECs incubated with control media displayed a significant increment of F-actin stress fiber formation. These findings suggest that disrupted adherens junction stability underlies the effect on EC dysfunction observed in uremic-plasma exposed cells. These alterations in adherens junctions were accompanied by a reorganization of F-actin in uremic plasma-exposed HUVECs.

Active VD attenuates uremia-mediated endothelial dysfunction

Measurements of vitamin D levels confirmed that, in CKD plasma, 1,25(OH)₂D concentrations were 42% lower of those in non-CKD plasma (96 vs 55 pmol/l) while 25(OH)D concentrations were similar between groups (50 vs 56 nmol/l) (Table 1). We therefore further tested the potentially beneficial effects of the active VD form paricalcitol (PC) in the uremic media-mediated endothelial dysfunction. Quantifying basal endothelial resistance, we found that addition of 100nM PC rescued the significant reduction in resistance induced by uremic plasma after 24h when compared to non-CKD conditions (Figure 4A, insert). However, no significant differences between CKD groups were detected. No significant improvement of the barrier function was detected for healthy plasma combined with PC. Furthermore, the addition of PC to uremic medium improved the impaired recovery after electrical wounding (Figure 4B). This was reflected by a pronounced increase of the slope (from 1-6h) in CKD + PC (140±9.5a.u.) rescuing the significant differences induced by uremic plasma when compared to non-CKD conditions (121±13a.u.) although there was no significant difference between CKD groups (Figure 4C). In addition, the AUC (from 1-24h) was significantly higher (7.5%) for PC-treated cells in CKD plasma when compared to CKD alone. Moreover, the addition of PC to non-uremic media increased the differences of the AUC and slope against CKD media when compared to non-CKD alone as shown in Figure 4C. These data show amelioration by PC of the uremia-mediated changes on basal endothelial resistance.
In addition, PC ameliorated the recovery capacity of endothelial barrier following electrical wounding in uremic plasma.

Figure 3. Membrane VE-cadherin localization is disturbed in CKD plasma exposed HUVECs. (A) Immunostaining of VE-cadherin (green), F-actin (red) and nuclei (blue) of HUVECs after 24h of incubation in medium (bM199) plus 1% HSA (Control; white) or 20% (v/v) non-CKD (dark) or CKD plasma (grey). Scale bar represents 50 µm. Zoomed images correspond to the white boxes. Normalized intensity quantifications are in the corresponding graphs for VE-cadherin (B) and F-actin (C). Surface expression of VE-cadherin was assessed by flow cytometry (D) in 24h-stimulated cells. (E) Normalized mean fluorescence intensity (MFI) measured by flow cytometry of membrane VE-cadherin is shown for the different conditions. Data are mean ± SD of 3 independent experiments. Differences were considered statistically significant for p<0.05 using one-way ANOVA where *P < 0.05, **p<0.01, ***p<0.001.
Disrupted VE-cadherin-mediated cell-cell junctions in uremic plasma are restored by active vitamin D

After determining the potency of PC to improve the barrier function during uremia, we explored whether this effect is the consequence of the restoration of cell-cell contact. In line with the previous experiment, PC significantly improved (9.6%) the strength of the cell-cell interaction after 24h as reflected by the quantification of the resistance between cells (Rb) (Figure 5A,B). PC supplementation rescued the significant differences encountered in uremic-plasma treated EC after quantification of the AUC (24h; 12.6%) of the cell-cell quantification in the wound healing process when compared to non-CKD conditions (Figure 5C).

Figure 4. PC stabilizes uremic plasma-induced endothelial dysfunction

(A) Representative measurements of absolute endothelial electrical resistance (Ω) of HUVECs, stimulated for 24h as indicated (PC=paricalcitol), (non-CKD=dark; non-CKD+PC=striped dark; CKD=grey; CKD+PC= striped grey). Insert: absolute endothelial resistance (Ω) after 24h. (B) Electrical wound healing (arrow) of cells pre-stimulated during 24h as indicated. (C) The quantifications of the wound healing capacity are shown as the area under the curve (from 1h to 24h) and slope of the recovery phase (a.u.; from 1h to 6h). a.u., arbitrary units. Representative data of at least 3 experiments. Data shows means ± SD (n=3, pool of 3 donors). Differences were considered statistically significant for p<0.05 using one-way ANOVA where *P < 0.05, **p<0.01, ***p<0.001.
However, no significant differences were found between uremic conditions with or without PC treatment. No significant changes were found regarding the slope of the wound healing phase although uremic medium alone showed a negative trend (-15 to -10%) when compared to other conditions. Further, PC induced enhanced VE-cadherin peripheral intensity under uremic conditions as shown in Figure 6A, which is supported by significant differences of intensity measurements (38% higher; p=0.015) when compared to uremia alone (Figure 6B). Evaluating adherens junctions morphology, the jagged distribution of VE-cadherin was improved to a more linear distribution when HUVECs in uremic conditions where supplemented with PC (Figure 6A, zoom).

PC slightly, but not significantly, increased VE-cadherin intensity (14%) in non-uremic medium. Finally, although cytoplasmic F-actin filaments were dominant in uremic conditions as observed in Figure 6A, the fluorescence intensity of F-actin did not differ significantly when compared to other conditions (Figure 6C). In addition, immunostaining for F-actin showed that PC reduced the arrangement of stress fibers upon uremic plasma while in non-uremic conditions PC slightly enhanced F-actin cortical distribution although no significant changes were observed after quantification. Thus, we confirmed that PC strengthens the endothelial barrier integrity under uremic conditions by mediating the stabilization of junctional VE-cadherin.

Figure 5. Cell-Cell interactions are stabilized by PC in uremic media (A) Representative cell-cell interaction (Rb; Ωcm²) measurements of HUVECs incubated for 24h as indicated (PC=paricalcitol), (non-CKD=dark; non-CKD+PC=striped dark; CKD=grey; CKD+PC= striped grey). (B) Rb of an electric wounding response (arrow) of cells previously stimulated for 24h (C) Quantification of the wound healing capacities are represented as the area under the curve (from 3h to 24h) and the slope (a.u.; from 3h to 7h). a.u., arbitrary units. Data shows means ± SD (n=3). Representative data of at least 3 experiments. Differences were considered statistically significant for p<0.05 using one-way ANOVA where *P < 0.05, **p<0.01.
DISCUSSION

This study demonstrates that uremic media negatively affect the integrity of the EC barrier function and also impair the recovery following exposure to barrier-disruptive mediators. Specifically, cell-cell interactions reduced in uremic plasma-exposed EC were driving the overall barrier dysfunction which was corroborated by reduced peripheral VE-cadherin and cortical F-actin. The active VD compound paricalcitol attenuated the endothelial barrier-disruptive effects of uremia in basal and electrical wounding assays by improving the cell-cell contact and restoring endothelial integrity.

Prolonged exposure of uremic toxins present in the plasma of CKD patients can affect the integrity and repair capacity of EC. In our study, ECIS has shown suitable to characterize in real time, the effects of uremic media on confluent endothelial barrier function. Adverse changes on EC by uremic plasma such as limited resistance to flow, activation of NFkB including increased permeability, have been previously demonstrated in vitro and in vivo. Our data show that EC exposed to uremic plasma (derived from patients with eGFR<18ml/min/1.73 m²) indeed displayed a reduced resistance when compared to healthy plasma (eGFR>90). Further, when the barrier integrity was compromised by thrombin (inducing cell contraction and intercellular gap formation) or electrical wounding, mimicking an injury, HUVECs exhibit a reduced recovery capacity after exposure for 24h to CKD plasma. Based on this data, we suggest that EC that have been exposed to uremia are more sensitive to barrier-disruptive conditions. Interestingly, this is supported by data showing altered proliferation and wound repair on HUVECs exposed to the uremic toxins p-cresol and indoxyl sulfate. These uremic retention toxins, poorly removed by hemodialysis therapies, are kidney-disease related cardiovascular risk factors and are suggested to contribute to endothelial toxicity in CKD. In this study, the addition of indoxyl sulfate and p-cresol in a concentration detected in serum from CKD patients with eGFR<30 (100 µM), mimicked the deleterious effects of CKD plasma in the recovery of the EC barrier function after a wound. Alternatively, the differences found in basal EC resistance after CKD plasma stimulation were not detected in media with uremic toxins indicating that the effects from CKD in EC dysfunction are not exclusive from indoxyl sulfate and p-cresol toxicity.

There are several cellular structures which are essential to the maintenance of the EC barrier integrity. By mathematically modelling our data obtained with ECIS, we concluded that the changes in transendothelial electrical resistance which we previously observed can be attributed to impaired cell-cell contact in uremic media. This was confirmed after measuring of the resistance between cells (Rb), which was reduced, while the current between the cell-matrix interactions (α) remained unchanged. In good agreement with this data, our imaging analysis showed that the adherens junction protein VE-cadherin, essential for
Figure 6. Reinforced VE-cadherin contact mediates the effect of PC in uremic media (A) Immunofluorescence staining of VE-cadherin (green), F-actin (red) and the nuclei (blue) of HUVEC following 24h of incubation (scale bar, 50 µm). Zoomed images correspond to the white boxes. Normalized mean VE-cadherin (B) and F-actin (C) intensity of the immunostainings are shown. (PC=paricalcitol), (non-CKD=dark; non-CKD+PC=striped dark; CKD=grey; CKD+PC= striped grey). Data shows means ± SD of 3 independent experiments. Differences were considered statistically significant for p<0.05 using one-way ANOVA where *P < 0.05, **p<0.01, ***p<0.001.
endothelial barrier integrity, showed disrupted localization and was less concentrated at cell boundaries upon uremic conditions. The decrease of VE-cadherin in cells was not attributable to redistribution to the cell surface as confirmed by FACS. Furthermore, VE-cadherin is linked to the actin cytoskeleton and its association is essential to barrier function. On exposure to uremic media, EC displayed a reduced cortical ring-like F-actin distribution. A prominent cortical actin cytoskeleton structure is characteristic for quiescent endothelium, and therefore, stable barrier integrity. In addition, immunostaining suggested increased presence of stress fibers indicating a reorganization of the F-actin protein leading to a different cell shape and cell contact destabilization albeit not statistically significant. Again, this is in line with in vitro studies performed with uremic toxins. HUVECs exposed to p-cresol displayed limited VE-cadherin and F-actin colocalization while indoxyl sulfate addition resulted in adherens junction disassembly and cytoskeleton reorganization. This mechanism is suggested to be mediated by an enhanced dissociation of VE-cadherin and actin cytoskeleton induced by the Rho/Rho kinase pathway. Upon activation of Rho, there is an increase of myosin light chain (MLC) phosphorylation leading to the formation of stress fibers, myosin-based contraction and opening of cell junctions which increases permeability. Indeed, by measuring Rb during wound healing we confirmed that indoxyl sulfate and p-cresol combination impact cell-cell- but not cell-matrix interactions, similarly as CKD plasma.

Further evidence for an uremia-induced disturbed actin cytoskeleton comes from a proteomic analysis in endothelial cells, showing an increment of the actin depolymerizing protein dextrin upon uremic serum stimulation. Interestingly, this was accompanied by a downregulation of annexin A2 which plays a key role in the establishment of adherens junctions. Our data extend these observations by comparing the effects of uremic plasma with those of a control (bare medium). Deficiencies in kidney-specific factors such as 25(OH)D, 1,25(OH)2D or α-Klotho are suggested to also contribute to cardiovascular pathology. In our study, VD measurements confirmed that 1,25(OH)2D in CKD plasma was lower than in plasma from healthy donors. However, when compared to CKD condition, control media showed no changes in VE-cadherin intensity suggesting that the effects observed in CKD were not related to some unidentified deficiency in the plasma, but a direct effect of uremic toxins. Yet, our experiment does not rule out that 1,25(OH)2D deficiency aggravates uremia-mediated endothelial damage.

In CKD animal models and patients, active VD supplementation has been shown to mitigate endothelial dysfunction. In addition, dietary VD and 1,25(OH)2D or PC can modulate endothelial stability by modifying inflammation, thrombosis, and vasodilation. We found that adding PC partially prevented the changes in electrical resistance induced by uremia and improved the recovery following electrical wounding. Interestingly, Won et
al. showed that $1,25(\text{OH})_2\text{D}$ attenuated the decrease of electrical resistance upon hypoxia in brain EC and restored tight junction expression which has a prominent contribution to blood-brain barrier function\textsuperscript{25}. This protective effect was mediated by a decrease of the Matrixmetalloproteinase-9 (MMP-9) which mediates disruption of cell-cell interaction\textsuperscript{25}. In line with this, we found a restoration of junctional VE-cadherin in cells incubated with uremic media by PC. Interestingly, increased MMP-9 has also been examined \textit{in vitro} in EC after the application of uremic media\textsuperscript{45}. Although more detailed studies are necessary to elucidate the vasculo-protective mechanism of active VD upon uremia, altered MMP9 expression appears to be one possible mechanism of endothelial protection. This possible mode of action is illustrated in the Supplementary Figure 1B. In addition, it remains to be studied whether uremia-disrupted cell-cell interaction also includes the impairment of the endothelial tight junction. In our study, however, we focused on the effects on VE-cadherin since non-brain EC exhibit less developed tight junctions. Importantly, besides being the major determinant of endothelial cell-cell interaction\textsuperscript{32}, VE-cadherin plays a key role controlling the level of expression and localization of other junctional molecules\textsuperscript{46}. As an alternative mechanism of action, it has been proposed that targeting the uremia-induced oxidative stress in the endothelium could be a potential strategy against endothelial dysfunction in CKD\textsuperscript{47}. In this regard, $1,25(\text{OH})_2\text{D}$ has been shown to be protective against oxidative stress in EC\textsuperscript{48}, and therefore, this mechanism could also be related to the endothelial-protective proprieties of active VD against uremia. Finally, it is also important to consider that EC has the ability to transform 25(OH)D to the active metabolite $1,25(\text{OH})_2\text{D}$\textsuperscript{49}, and comparable endothelial protective effects could be achieved by 25(OH)D supplementation. This alternative therapeutic approach could be of importance in a setting with a 25(OH)D deficiency, a feature that, however, was not applicable in our study.

Our study bears some limitations as the exact signaling mechanism involved in the VE-cadherin-disrupted contact and F-actin reorganization during uremia remains to be established. Moreover, it would be interesting to determine whether the uremia-mediated endothelial damage could be prevented by the previous addition of PC. Nevertheless, this setting would not be representative of a clinical situation. Likewise, additional EC-types need to be tested to confirm the effects observed during uremia. Despite those disadvantages, we provide valuable \textit{in vitro} data by the combination of human uremic plasma with real-time measurements of endothelial barrier functions.

In conclusion, we have extended our insight of the effects of uremia on EC dysfunction. Although several kidney-disease related risk factors were suggested to contribute to endothelial dysfunction in CKD, our \textit{in vitro} findings show similarities with the effects of the uremic toxins indoxyl sulfate and p-cresol. We propose that limited cell-cell interaction caused by reduced VE-cadherin and F-actin reorganization affects the integrity of the EC barrier
during uremia. These changes may carry important clinical implications since they restrict the capacity of the EC to resist and recover from barrier-disruptive conditions aggravating vascular complications during CKD. Additionally, as a therapeutic approach, we describe a novel mechanism how the active VD analogue paricalcitol modulates the uremia-damaged endothelium by restoring cell-cell interactions. Overall, stabilizing the intercellular endothelial contact might be a crucial step to prevent the damage in the endothelium and ameliorate future vascular complications in CKD.

ACKNOWLEDGMENTS

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Supplementary Figure 1. Schematic representation of the ECIS measurement (A) Endothelial monolayer on a collagen-coated ECIS electrode (ECM= extracellular matrix). The electrical current can flow underneath and between the cells (black arrows). Moreover, electrical current can also flow through the cells (dashed black arrows). Resistance of the endothelial cells against the electrical current is measured at 4000 - 32000 Hz. The resistance at 4000 Hz is representative of the barrier function of the endothelial cells. ECIS software is able to measure the resistance between cells (Rb; cell-cell interaction) or between the cells and electrode (α; cell-matrix interaction, measure at 32000 Hz). (B) Representative model of the mechanisms underlying the results obtained in ECIS. Left: non-CKD media results in stable endothelial barrier function with enforced cell-cell interactions and normal F-actin cytoskeleton distribution. Middle: CKD or uremic toxins media mediates cell-cell disruption and stress fibers formation leading to a cell contraction and the increment of flux of current representative of the loss of barrier function. This mechanism could be mediated, although not confirmed, by the increment of MMPs. Right: active VD stimulation strengthened the cell-cell interaction and reduced the formation of stress fibers maintaining the integrity of the barrier function and reducing the flux of current.
Supplementary Figure 2. Long-term measurements of the effects of uremic media in HUVECs. Representative time-course of absolute endothelial electrical resistance measurements (Ω) of confluent HUVECs stimulated for 24h and after electrical wounding measured for 48h with 20% (v/v) of non-CKD plasma (dark) or CKD plasma (grey). Bar graphs: (left and right) quantification of the wound healing as the area under the curve (24h and 48h). a.u., arbitrary units. Data shows means ± SD (n=4, from pool of 3 donors). Representative data of at least 3 experiments. Differences were considered statistically significant for p<0.05 using unpaired student’s t-test where *P < 0.05, **p<0.01.
Supplementary Figure 3. Uremic toxins impair endothelial wound recovery with no changes in basal resistance
(A) Representative time-course of normalized endothelial electrical resistance measurements of confluent HUVECs stimulated for 24h with 20% (v/v) of non-CKD plasma (non-CKD; dark) or non-CKD plasma with 100 µM each of Indoxyl sulfate and p-cresol (IS + PS; stripped-grey). (B) 40h normalized resistance measurements (Ω) after electrical wounding of 24h 20% plasma pre-stimulated cells. (C) Quantification of the slope of the recovery (from 1h to 6h). (D) Quantification of the wound healing as the area under the curve (left:12h and right:40h). a.u., arbitrary units. Data shows means ± SD (n=3, from pool of 3 donors). Representative data of at least 3 experiments. Differences were considered statistically significant for p<0.05 using unpaired student’s t-test where *P < 0.05.
Supplementary Figure 4. Uremic toxins mediates impaired recovery of the barrier by low cell-cell interactions

Absolute endothelial electrical resistance attributable to cell-matrix interaction ($\alpha; \Omega \cdot \text{cm}^2$) (A) and cell-cell interaction ($R_b; \Omega \cdot \text{cm}$) (B) of wound healing measurements from Supplementary Figure 3B of confluent HUVECs stimulated for 24h with 20% (v/v) non-CKD plasma (dark) or non-CKD plasma with 100µM Indoxyl sulfate and p-cresol (IS + PS; stripped-grey). (C) Quantification of the wound healing of the cell-cell interactions as the area under the curve (left:12h and right:40h). a.u., arbitrary units. Data shows means ± SD (n=3, from pool of 3 donors). Representative data of at least 3 experiments.
CHAPTER 4

Effect of uremia and active vitamin D therapy in Nε-(carboximethyl) lysine depositions in intramyocardial blood vessels

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ABSTRACT

Myocardial microvascular dysfunction might impact the normal function of the cardiovascular system in Chronic Kidney Disease (CKD) patients, but this has been insufficiently studied. As an underlying mechanism, local oxidative stress in vascular tissue might play a role in the pathogenesis of cardiovascular disease during CKD. Previous studies reported that N(ε)-Carboxymethyllysine (CML), as a product of oxidative stress mechanisms, represents an integrative biomarker for oxidative stress. In addition, vitamin D has been demonstrated to be protective against oxidative stress and CKD-mediated cardiovascular damage. Here, we investigated the presence of CML depositions in the intramyocardial blood vessels of the heart of uremic rats treated or untreated with active vitamin D and relate this feature with oxidative stress processes. We found enhanced levels of CML in the myocardial blood vessels of uremic animals. Active vitamin D treatment did not prevent CML depositions in uremic animals. Furthermore, CML depositions were not accompanied by the increased expression of the reactive oxygen species-producing enzyme NOX2 in the myocardial blood vessel. In conclusion, we demonstrate for the first time the presence of uremia-mediated accumulation of CML in the heart microvasculature. This pathological feature was not prevented by active vitamin D treatment. We could not link the presence of CML with increased local oxidative stress leaving the source of local CML accumulation undetermined.
INTRODUCTION

Chronic kidney disease (CKD) is a risk factor for the occurrence and progression of cardiac and vascular complications\(^1\). Cardiac changes observed in CKD patients are of multifactorial origin. Traditional risk factors fail to fully account for the increased incidence of cardiovascular death in CKD, thereby suggesting the implication of specific CKD-related causes\(^1\). Myocardial microvascular dysfunction occurs in CKD, and is thought to contribute to myocardial disease and the development of cardiovascular alterations in these patients\(^2\). However, how abnormalities in myocardial microvascular function originate and contribute to the development of cardiovascular complications in CKD remains poorly understood.

\(N(\epsilon)\)-Carboxymethyllysine (CML) is one of the major advanced glycation end products (AGEs), and its production and accumulation in the vascular tissue may play a role in the pathogenesis of cardiovascular disease in CKD\(^3,5\). CML formation is enhanced during uremia and mediated by inflammatory factors or reactive oxygen species (ROS)\(^6,7\). Subsequently, CML can stimulate different pathways via interaction with the receptor for AGEs (RAGE) on the endothelial surface resulting in the activation and translocation of nuclear transcription factors, including NF-\(\kappa\)B\(^8\). As a consequence, the activation of this pathway causes a feed-forward loop of the inflammatory state and oxidative stress that may contribute to the aggravation of cardiovascular abnormalities in patients with CKD\(^8-12\). Besides increased production, decreased clearance of AGEs in kidney failure contributes to elevated CML levels\(^6,7\), thus aggravating the development of lesions in cardiovascular tissue of the CKD population.

Among other cardiovascular localizations, it has been demonstrated that CML depositions can be found in intramyocardial blood vessels of both rat and human after acute myocardial infarction and in an arteriosclerotic mouse model\(^13-16\). However, whether CML accumulates in intramyocardial blood vessels in CKD and aggravates or generates cardiac complications remains to be determined.

Active vitamin D supplementation has been demonstrated to reduce oxidative stress and prevent ROS-mediated vascular toxicity\(^17-20\). Importantly, active vitamin D has been shown to attenuate endothelial dysfunction upon AGEs stimulation and limit CML depositions and oxidative stress in the medial layer of the aortic wall in diabetic rats\(^21,22\). Therefore, in the present work, we studied the presence of CML depositions and the ROS-generating enzyme NADPH Oxidase 2 (NOX2) expression in the intramyocardial blood vessels of a rat uremic model. In addition, we studied the potential protective effect of the active vitamin D analogue paricalcitol. We found that uremia induces accumulation of CML but without changes of NOX2 expression in the intramyocardial blood vessels. However, this was not prevented by paricalcitol.
MATERIALS AND METHODS

Experimental models

Male Wistar rats (Charles River, Maastricht, The Netherlands) weighing 250 to 275 g were used. After 1 week of acclimatization and handling, a 3/4 nephrectomy was performed to induce uremia; non-CKD (healthy) controls were not surgically manipulated. The nephrectomy involved the complete removal of the right kidney and ligation of 1 to 2 branches of the arteries supplying the left kidney in order to obtain residual kidney function of about one-fourth of the total capacity. In sixteen rats, uremia was induced (CKD) by 3/4 nephrectomy. One rat died during the surgery leaving 15 animals for analysis. Four weeks after 3/4 nephrectomy was established, oral administration of paricalcitol (0.1 ug/kg rat; kindly provided by AbbVie, Chicago, USA) dissolved in sugar water was provided three times per week during seven weeks in total. In parallel, vehicle control rats received only sugar water. Animals groups were as follows: non-CKD with vehicle control treatment (n=6), CKD with vehicle control treatment (n=8), CKD group with paricalcitol treatment (n=7).

All the animals were socially housed under standard conditions and were given food and water ad libitum. Health conditions were checked daily. The weight of the animals was checked daily after surgery during a period of 7 days and weekly for the remainder of the experiment. Animals that lost more than 20% of their maximum gained body weight at any point during the experiment or showed abnormal activity were excluded from the experiment. The experimental protocol was in compliance with NIH Guideline for the Care and Use of Laboratory Animals and was approved by the Animal Welfare Committee at the VU University Medical Center, Amsterdam.

Measurement of blood parameters

Five hundred microliter of blood were drawn through tail vein in rats under inhaled isoflu-rane (2%) anesthesia. At the time points (weeks: -1, 10) serum samples from CKD groups were analyzed for urea and creatinine levels. Urea levels were determined by measuring the substrate levels by ultraviolet enzymatic method of a urease reaction coupled to glutamate dehydrogenase reaction. Creatinine levels were detected by indirect immunofluorescence assay. Measurements were performed by using spectrophotometer Cobas8000 C702 (Roche Diagnostics, Risch-Rotkreuz, Switzerland).

Immunohistochemical stainings

For immunohistochemical analysis, formalin-fixed, paraffin embedded cardiac tissue was cut into 4-µm sections, deparaffinized, rehydrated and incubated in methanol/H2O2 (0.3%) for 30 minutes to block endogenous peroxidases. For the NOX2 staining, antigen retrieval was
performed by boiling the slides in a 10 mM Tris/1mM EDTA-buffer, pH 9.0. The slides were incubated with normal rabbit serum (1:50, Dako) prior to the incubation of mouse-antihuman NOX2 (1:25, obtained from Sanquin Research at CLB, Amsterdam, The Netherlands), for 1h at room temperature. The sections were then washed with phosphate-buffered saline and incubated with a rabbit-anti-mouse-horseradish peroxidase (Dako) for 30 min at room temperature. For the CML staining, antigen retrieval was performed by incubating the sections with 0.1% pepsin/0.02M HCl for 30 min at 37°C. After an avidin-biotin blocking step (incubation with a 0.1% avidin solution and 0.01% biotin solution, both for 10 min at room temperature (Biotin Blocking System, Dako), and a serum step (normal rabbit serum for 10 min at room temperature), sections were incubated with a mouse-antihuman CML antibody (1:2000, a gift from C.G. Schalkwijk) for 60 min at room temperature. After washing, sections were subsequently incubated with rabbit-antimouse biotin for 30 min at room temperature (1:500, Dako) and streptavidin–horseradish peroxidase (1:200; Dako) for 60 min. Both stainings were visualized using 3,3′-diaminobenzidine (DAB; 0.1 mg/ml, 0.02% H2O2) for 10 min. The slides were subsequently counterstained with haematoxylin, dehydrated and covered. With each staining, a phosphate-buffered saline control was included. Both controls yielded negative results (not shown).

Immuno-scoring

For CML intensity scoring, each positive vessel in the heart was given a score according to the intensity of staining: 1 = weak staining; 2 = moderate staining; or 3 = strong staining. To obtain the CML immuno-histochemical score, the respective intensity scores (1, 2 and 3) were first multiplied by the amount of blood vessels positive for this score as previously described. These three numbers were then added and subsequently divided by the surface area of the tissue, resulting in an immunohistochemical score per millimeter square. The number of NOX2-positive vessels were scored with a microscope and divided by the surface area of the tissue.

Statistical analysis

Data were analysed using GraphPad Prism software (La Jolla, CA), different analysis were used. Statistical analyses were performed using two-way ANOVA for Figure 1 (treatment x time) and Figure 2B (score x group) measurements and One-way ANOVA with Bonferroni correction for the rest of the analysis. Data were shown as means ± SD. Differences were considered statistically significant for p<0.05.
RESULTS

CML accumulates in the intramyocardial blood vessels of uremic rats

At the end of the experiment (week 10), the nephrectomized animals had a three to four-fold increase in both creatinine (15.09±1.94 to 55.25±12.27 µmol/l for CKD; 15.63±1.86 to 69±24.22 µmol/l for CKD + PC) and urea (5.61±0.82 to 14.25±3.84 mmol/l for CKD; 5.57±1 to 17.29±7.27 mmol/l for CKD + PC) serum levels when compared to the measurements performed before surgery (week -1) indicating the establishment of substantial CKD (Figure 1A-B).

Figure 1: Uremia is established by 3/4 nephrectomy. Serum levels of creatinine (mmol/l) (A) and urea (µmol/l) (B) before (week -1) and at the end of the experiment (week 10) (PC=paricalcitol). Results are means ± SD (n=6-8). Differences were considered statistically significant for p<0.05 using two-way ANOVA (A: treatment p=0.2205, time p<0.0001, treatment x time p=0.1454; B: treatment p=0.3544, time p<0.0001, treatment x time p=0.2954). *p<0.001 ** p<0.0001.

Next, we investigated the presence of CML in the intramyocardial blood vessels from the heart. The immunohistochemical score per cm² revealed that CML depositions were significantly increased in uremic rats when compared to non-CKD group (1.14±0.38 vs 0.53±0.28 score/mm²; p=0.021). However, the uremia-mediated increase of CML depositions was not prevented in paricalcitol-treated rats (1.28±0.42 score/mm²) (Figure 2A). The majority of CML positive vessels in non-CKD rats displayed a weak intensity of the immunohistochemical score per cm² (scored as 1; Figure 2B). In contrast, uremic animals displayed more increased (2-score; moderate intensity) CML positive vessels than non-CKD animals, with statistically significant changes for the paricalcitol-treated group (10.38±4.71 non-CKD; 19.44±5.70 p=0.072 CKD; 24.29±7.01 p=0.0032 CKD+PC; positive vessels/100mm²). Regarding the maximum intensity, all CKD animals showed a significant increment CML-positivity when compared to non-CKD (3-score; strong intensity) (3.88±2.92 non-CKD; 15.78±9.58 p=0.0108 CKD; 19.19±11.12 p=0.001 CKD+PC; positive vessels/100mm²). Thus, enhanced presence of CML in the intramyocardial blood vessels was found in CKD animals without attenuation of this deposition by paricalcitol treatment.
**Figure 2:** Quantification of CML in blood vessels. Quantitative evaluation of CML blood vessels in heart tissue (PC=paricalcitol). (A) Immunohistochemical score per cm² for CML (B) Intensity score of CML. Three different intensity scores for CML (1=minor; 2=moderate; 3=strong; defined in detail in Methods). Bars represent mean ± SD of scores (n=6-8). Differences were considered statistically significant for p<0.05 using one-way ANOVA for (A) and two-way ANOVA for (B; score p=0.0068, group p<0.0001, score x group 0.384). *P < 0.05, **p<0.01.

**CML accumulation in intramyocardial vessels is not related to oxidative stress**

Based on these findings, we tested whether the accumulation of intramyocardial CML was the result of enhanced oxidative stress. Therefore, the amount of NOX2, a key generator of ROS, was measured in intramyocardial vessels. NOX2 positive vessels/mm² were detectable in CKD animals although without significant differences when compared to control (0.45±0.13 vs 0.31±0.16 positive vessels/mm², p=0.612) (Figure 3). No statistically significant changes were found in paricalcitol treated CKD animals (0.57±0.27 positive vessels/mm²). In contrast to CML depositions, no differences were found in the NOX2 positive vessels among uremic and non-uremic animals.

**Figure 3:** Immunohistochemical analysis of NOX2 in myocardial microvascular tissue. Quantitative evaluation of NOX2 in intramyocardial blood vessels (PC=paricalcitol). Bars represent mean ± SD of NOX2 positive vessels/mm² (n=6-8). Differences were considered statistically significant for p<0.05 using one-way ANOVA.
DISCUSSION

Our study demonstrates that CKD leads to the accumulation of CML in the blood vessels of intramyocardial tissue. The deposition of CML in tissue was not prevented by active vitamin D supplementation. Finally, we could not demonstrate that CML accumulation was associated with increased local oxidative stress processes as reflected by unchanged NOX2 positive vessels/mm².

Abnormalities in the myocardial microvascular tissue may underlie pathological consequences for the normal function of the cardiovascular system in CKD patients. However, little is known about how uremia mediates microvascular dysfunction in the heart or its relationship with increased risk of cardiovascular events in patients with CKD. Oxidative stress is frequently increased in CKD, and it is conceivable that this can make a notable contribution to the structural vascular damage and microvascular dysfunction. Under high oxidative conditions, there is an increment of the production of AGEs, specifically CML, the accumulation of which may aggravate vascular damage. In our uremic model, we found increased CML depositions in the intramyocardial blood vessels which might represent myocardial microvascular dysfunction. However, when we analyzed the levels of the ROS-generating enzyme NOX2, we did not find significant changes in the blood vessels of myocardial tissue. Therefore, alternative mechanisms that might promote the generation of AGEs, such as inflammation, need to be considered.

Alternatively, it is important to take into account that in chronic renal failure, increased levels of circulating AGEs result from decreased renal clearance and increased inflammation and oxidative stress in the kidney. Indeed, several studies have shown an association between high levels of AGEs in serum and cardiovascular complications and mortality in CKD patients. Furthermore, soluble CML correlated with markers of endothelial activation such as VCAM-1 and limited microcirculatory blood flow in the skin of non-diabetic CKD patients. Therefore the increased amount of CML in observed the myocardial microcirculation might either be the consequence of local CML production or deposition from increased circulating CML. In this study, we did not measure circulating CML levels but our animal model of renal failure showed a three to four-fold increase of serum urea and creatinine. Thus, it is possible that serum CML levels in our animal models were also increased due to the limited renal clearance.

Experimental studies have shown that active vitamin D therapy plays an important role in the prevention of cardiovascular alterations in models of CKD. Specifically, vitamin D therapy potentially can modulate inflammation, improve endothelial function, attenuate myocardial hypertrophy and prevent heart failure. In a diabetic rat model, induced by streptozotocin
administration, active vitamin D was able to reduce CML deposition in the aortic wall\textsuperscript{22}. The same model resulted in liver oxidative stress which was also prevented by vitamin D\textsuperscript{22}. Thus, the reduced CML accumulation in vascular tissue could be related to a protection against oxidative stress. In contrast, in the present study, the active vitamin D analog paricalcitol did not prevent uremia-induced CML accumulation in the blood vessels of myocardial tissue. In addition, oxidative stress did not play a major role in CML depositions as demonstrated by NOX2 staining. As mentioned previously, the CML depositions observed in our study could have been related to a reduced renal clearance. However, the increment of urea and creatinine observed in uremic animals was not reduced by paricalcitol treatment suggesting that renal function, and therefore, CML soluble levels were similar in all CKD animals.

Clinical trials gave rise to controversy of the protective effects of active vitamin D in CKD-induced heart disease\textsuperscript{32,33}. In this regard, Tadhani \textit{et al.} showed in the PRIMO trail no improvement of diastolic dysfunction or no alteration in left ventricular mass-index after forty-eight weeks of paricalcitol therapy in CKD patients\textsuperscript{32}. Consistent with this, fifty-two weeks of treatment with paricalcitol did not regress left ventricular hypertrophy or improve left ventricular systolic or diastolic function in patients with CKD stages III-V in the OPERA trail\textsuperscript{33}. Taken together, there is still a need to expand the knowledge and the possible optimal timing of the effects of active vitamin D therapy in CKD-induced cardiovascular complications.

Our study has several limitations. First, we could not determine the origin of CML depositions in the heart microvascular tissue of the uremic animals, being either locally formed or deposited from the circulation. Second, the overall impact of the CML presence in the heart microvascular function and implication in the development of heart dysfunction remains to be determined. Nevertheless, we demonstrate, for the first time, the \textit{in vivo} accumulation of CML, a kidney-related cardiovascular non-traditional risk factor, in the blood vessels of myocardial tissue of uremic animals.

In conclusion, microvascular dysfunction in the heart might impact the health of the cardiovascular system during CKD. Here, we suggest that CML might be a contributing player for the development of lesions in the myocardial blood vessels. We could not relate the increment of CML to oxidative stress leaving the origin elusive. In addition, we extend the knowledge of active vitamin D therapy in the uremic-related cardiovascular field.
REFERENCES


CHAPTER 5

Prevention and treatment of peritoneal remodeling in Peritoneal dialysis: targeting chronic inflammation

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ABSTRACT

The peritoneal membrane undergoes to progressive deterioration by the exposure to bio-incompatible dialysis fluids in patients with Peritoneal dialysis (PD). The structural alterations in the peritoneal membrane inflicted by long-term treatment with PD solutions include the development of peritoneal fibrosis and angiogenesis which may lead to limited ultrafiltration capacity and technique failure. Depending on the cytokine microenvironment, macrophages can adopt a proinflammatory (M1) or a profibrotic (M2) phenotype where the latter has been reported to contribute to the excessive peritoneal fibrotic tissue formation in PD. Similarly, the interleukin 17 (IL-17)-mediated immune response, produced by the T helper 17 (Th17) cell subset, has been suggested to be a key player in the development of peritoneal damage. As a potential therapeutic strategy, diminishing the impact of these inflammatory mediators may lead to the preservation of the peritoneal membrane integrity during PD. In this regard, the addition of active vitamin D and Alanyl-Glutamine dipeptide in conventional PD fluid may carry important beneficial effects by modulating not only the inflammatory milieu but also protecting the mesothelial integrity. In addition, biocompatible PD fluids, which are reported to better preserve the peritoneal membrane structure, might display different inflammatory cell infiltration compared to conventional PD solutions.
INTRODUCTION

Peritoneal dialysis (PD) is an established renal replacement treatment modality for end-stage renal disease patients. Based on the ability of the peritoneum to function as a dialyzing membrane, the primary goal of dialysis is to exchange waste products between the blood compartment and the PD fluid instilled through a permanent catheter. The effectiveness of PD on the removal of metabolic waste products depends on the accurate functioning of the peritoneum. Unfortunately, long-term PD is associated with the development of pathological changes in the peritoneal membrane leading to a dysfunctional peritoneum and limited ultrafiltration capacity. Due to the increased number of worldwide patients depending on renal replacement therapies including PD, research has focused its attention on understanding factors that may limit its long-term application. Specifically, the continuous exposure to conventional PD fluids which usually contain glucose as an osmotic agent, the glucose degradation products (GDPs) formed during heat sterilization of the solutions and the low pH may drive inflammatory processes that play a central role in the PD-mediated peritoneal remodelling. Furthermore, peritonitis and mechanical injury as a result of a constant instillation of PD fluid also contribute to peritoneal deterioration. Taken together, PD therapy, although performing excellent dialysis and removal of solutes, gives a set of specific challenges to the peritoneal membrane. Additional scientific understanding of the pathophysiological mechanisms of peritoneal injury is required to develop therapeutic strategies that help to preserve the integrity of the peritoneal membrane and improve the long-term effectiveness of PD treatment.

PD-RELATED REMODELLING

- Peritoneal fibrosis

Prolonged exposure of the peritoneal membrane to PD fluids is associated with morphological (fibrosis, angiogenesis) and functional (increased peritoneal solute transfer rate, ultrafiltration failure) alterations. One of the most serious complications of long-term PD treatment is encapsulating peritoneal sclerosis, a condition characterized by an exaggerated fibrogenic response. The peritoneal membrane is lined by a monolayer of mesothelial cells, a specialized cell type that has some characteristics of epithelial cells which cover a thin layer of submesothelial connective tissue, acts as a permeability barrier and secretes various substances involved in the regulation of peritoneal permeability and local host defence. As reported by in vitro and in vivo studies, upon PD fluid exposure, mesothelial cells lose their polarity and differentiate into fibroblasts/myofibroblasts through epitheli-
al-to-mesenchymal transition (EMT) gaining migratory and invasive properties. This progressive fibrotic process is induced by several pathogenic factors, such as inflammatory mediators, high glucose content, the presence of GDP, and low pH, resulting in the accumulation of smooth muscle actin-positive myofibroblasts in the submesothelial connective tissue. As an additional mediator, uremia itself contributes to the fibrotic response and the release of mesothelial-derived inflammatory mediators. Among a number of different underlying mechanisms of progressive membrane injury, overactivation of Transforming Growth Factor (TGF)-β1 signalling has been proposed as a pathogenic mechanism for the PD-mediated mesenchymal phenotype. TGF-β1 enhances the production of extracellular matrix macromolecules like collagen, laminin and fibronectin altering the structure and function of the peritoneal membrane. In PD patients, increased TGF-β1 protein levels have been reported in PD effluents during peritonitis indicating that, indeed, TGF-β1 is a critical mediator of peritoneal fibrosis. This event was also reproduced in an animal model, where it was demonstrated that the transduction of TGF-β1 into rat peritoneum causes peritoneal epithelial-to-mesenchymal transition and impairs membrane function. Similarly, it has been shown that conventional dialysis fluids can induce IL-6 synthesis by peritoneal membrane cells. In vitro, exposure of human mesothelial cells to IL-6 promotes a switch to a mesenchymal-like phenotype through JAK2/STAT3 signalling pathway. Interestingly, IL-6 has been found to upregulate the expression of the main pro-angiogenic factor Vascular Endothelial Growth Factor (VEGF) from mesothelial cells in the presence of soluble IL-6 receptor. This event suggests a close link between inflammatory cytokines and the development of both peritoneal fibrosis and angiogenesis.

Angiogenesis

Peritoneal microvessels also play a crucial role in PD. Angiogenesis increases the effective surface area for exchange between the peritoneal membrane and PD fluid present in the peritoneal cavity. Although this promotes clearance of uremic toxins, it also enhances increased dissipation of the glucose-gradient across the blood compartment and the PD fluid. As a result, this structural change decreases the glucose-driven osmotic pressure of the PD fluid, leading to a decline of ultrafiltration capacity. Furthermore, the enhanced vascular network contributes to the peritoneal thickening and increased permeability. Local VEGF production during PD seems to play a central role in the processes leading to peritoneal neo-angiogenesis. Interestingly, it has been shown that VEGF is widely expressed in peritoneal mesothelial cells undergoing EMT while it has much lower expression in other cells. This event suggests that angiogenesis and fibrosis may be interconnected processes. Besides VEGF, other factors are involved in the formation of new blood vessels such as the Macrophage produced Monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor...
(TNF)-α and interleukin (IL)-1β suggesting that chronic inflammation plays an important role in the new vessel formation during PD treatment.

**Peritoneal inflammation**

Systemic inflammation has been shown to predict outcome in PD, just as it does in chronic kidney disease in general. This inflammation is characterized by the release of chemokines and cytokines and enhanced migratory properties of various cells of the immune system. Some studies suggested that chronic inflammatory reactions during PD treatment can lead to increased peritoneal fibrosis and angiogenesis together with increased peritoneal permeability. In the dynamic process of wound healing, inflammation and epithelial healing are the primary response against damage. In contrast, fibrosis is rather a second-line danger response program that only becomes relevant when epithelial healing is insufficient, incomplete, or persistently suppressed by ongoing injury and inflammation. The differentiation of T cells is crucial for immune and inflammatory responses and its regulation may be a therapeutic target to control peritoneal damage, as deranged T cell responses may contribute to pathological peritoneal membrane changes. It has been found that there are different rates between CD4+ and CD8+ cells in the peritoneum during PD treatment with respect to healthy individuals. In an experimental model, Chung et al. have reported an increase of activated T cells homing to the peritoneal cavity shortly after peritoneal injury and proposed a central role for CD4 positive (CD4+) T cells. Moreover, Habib et al. confirmed the hypothesis that recruitment of CD4+ T cells in the peritoneal membrane may drive the progression of excessive fibrotic tissue formation in PD patients. Regarding the distinct CD4+ T cells, peritoneal T cells can be polarized towards two classical subsets, the T helper (Th)1 and Th2. In general terms, Th1 cells are defined by the secretion of interferon (IFN)-γ, while Th2 cells produce predominantly IL-4. In chronic PD patients, a deviation toward Th2 pattern has been described while a Th1 response has been observed during episodes of acute peritonitis.

Aside from the Th1/Th2 paradigm, additional populations of CD4+ T cells - designated as regulatory T (Treg) and Th17 cells - were identified and consequently related to many autoimmune and chronic inflammatory diseases. While Treg cells limit inflammatory responses and are associated with immune-tolerance, Th17 cells are characterized by the production of IL-17, one of the major pro-inflammatory cytokines. The reciprocal relationship between Treg cells and Th17 cells represents a delicate balance between tolerance and elicitation of immune responses. The enhanced production of TGF-β1 and IL-6 mediated by the continuous exposure of the mesothelial cells by PD fluids, may promote Th17 differentiation. As a consequence, the induced Th17 activity will result in increased IL-17 production, ag-
gravating the peritoneal damage during PD treatment as demonstrated in nondiabetic PD patients\textsuperscript{44}. Furthermore, in peritoneal biopsies from dialyzed patients, IL-17 immunostaining was found mainly in inflammatory areas and was absent in the healthy peritoneum\textsuperscript{45}. This result was accompanied by enhanced IL-17 concentrations in PD effluent from long-term PD patients\textsuperscript{45}. Furthermore, the critical implication of IL-17 in peritoneal inflammation and fibrosis has been confirmed in a mice PD model pointing to IL17 as a key player of peritoneal damage and a candidate to consider for therapeutic interventions\textsuperscript{16, 45}.

Besides Th17/Treg axis, several studies make a statement that also alternatively activated macrophages (M2) play an important role in tissue remodeling and fibrosis\textsuperscript{35}. Macrophages are the most plastic immune cells distributed widely in organs and tissues, which polarize into distinct populations determined by the microenvironment\textsuperscript{46, 47}. These cells have been classified either into ‘pro-inflammatory’ M1 or ‘immunoregulatory’ M2 macrophages, although this simple nomenclature does not fully reflect the broad spectrum of macrophage function and phenotypes\textsuperscript{48}. IFN-γ, the main cytokine produced by Th1 cells, is associated with the macrophage polarization towards classically activated macrophages (M1), whereas M2 macrophages reciprocally engage with Th2 cells by the effects of IL-4\textsuperscript{46, 47}. M1 macrophages release large amounts of pro-inflammatory mediators such as TNF-α with autocrine/paracrine effect on macrophage activation and with pro-apoptotic effects in order to limit the survival of activated immune cells. This causes tissue inflammation and local cell damage. In contrast, M2 macrophages release anti-inflammatory mediators such as IL-10 which suppresses the local inflammation. Such macrophages, however, predominantly secrete cytokines including TGF-β1 promoting fibrosis in the local tissue\textsuperscript{46, 47}. In terms of PD, loss of peritoneal function linked to peritoneal membrane fibrosis development has been related to the profibrotic CC chemokine ligand 18 (CCL18) characteristic of M2 macrophages\textsuperscript{49}. Here, Bellón et al. proved that the capacity of M2 macrophages to stimulate fibroblast proliferation was proportional to the mRNA level of CCL18, and they believed that M2 macrophages may participate in human peritoneal fibrosis through the CCL18 production\textsuperscript{49}. Additionally, they showed that an increased concentration of CCL18 was found in the peritoneal effluent of patients who later developed encapsulating peritoneal sclerosis. In support of this finding, together with CD4+ T cells, pro-fibrotic M2 macrophages have been pointed as important contributors to the excessive fibrosis during encapsulating peritoneal sclerosis\textsuperscript{35}. Overall, the intraperitoneal presence of pro-inflammatory molecules mainly secreted by Th17 cells and M2 macrophages may orchestrate the development of a chronic inflammatory condition in the peritoneal cavity triggering the peritoneal remodeling (Figure 1). This concept suggests that the immune imbalance is the fundamental key for the loss of peritoneal membrane integrity in PD.
Figure 1: Representative figure of a normal peritoneal structure (left) and its changes during PD treatment (right). The direct contact of conventional PD fluid impairs the preservation of the mesothelial barrier function leading to several structural changes in the peritoneum. After PD fluid exposure, mesothelial cells release inflammatory cytokines such as TGF-b and IL-6 mediating epithelial-to-mesenchymal transition and increasing the appearance of fibroblasts together with an increment of the extracellular matrix. Enhanced new vessel formation, as a result of the increment of the pro-angiogenic factor VEGF, rapidly decreases the glucose-driven osmotic pressure of the PD fluid leading to a functional decline of ultrafiltration. In parallel, those changes are accompanied by the infiltration of immune cells resulting in a chronic inflammatory environment in the peritoneum that aggravate the PD-mediated peritoneal fibrosis and angiogenesis. Among other immune cell types, M2 macrophages together with the IL-17 cytokine released by the Th17 cell subset are suggested to dominate the peritoneal cell infiltrate and relate its presence with both peritoneal structural and functional changes during PD.

PRESERVING THE PERITONEAL MEMBRANE INTEGRITY DURING PD

The addition of anti-inflammatory compounds to the conventional PD fluids to diminish the impact of both Th17/IL-17 and M2 macrophages mediated peritoneal damage may carry important benefits for the preservation of the peritoneal membrane integrity. Neutralization of IL-17 in a mice model of PD has been shown to diminish peritoneal fibrosis and inflamma-
tion, pointing IL-17 as a potential candidate to target\textsuperscript{45}. Recently, active vitamin D treatment has been demonstrated to mitigate the peritoneal membrane deterioration by lowering the amount of IL-17 in the effluent in an experimental model of PD\textsuperscript{50}. Interestingly, other studies identified vitamin D as an effective treatment for preserving the renal epithelial barrier in uremic conditions or modulating other immune cells such as the monocytes and macrophages\textsuperscript{51, 52}. These additional beneficial effects suggest that the protective properties of vitamin D in the peritoneum may not be limited to attenuating the effects mediated by the IL-17 pathway. As a novel therapeutic intervention, Alanyl-Glutamine (Ala-Glu) dipeptide raised as an interesting therapeutic strategy against PD fluid-mediated stress proteome of mesothelial cells exposed with PD fluids\textsuperscript{53}. Interestingly, Ala-Glu dipeptide also has been reported to have immunomodulatory proprieties\textsuperscript{54}. In a mice model of lung injury, Ala-Glu administration reduced local inflammation and injury mediated by LPS instillation by reducing the Th17 subset while Treg cell population was increased\textsuperscript{54}. This study points Ala-Glu treatment as an interesting therapeutic strategy against chronic inflammation developed upon PD exposure.

Improving the unphysiological composition of standard PD fluids towards a more biocompatible one is another logical approach to better preserve the peritoneal membrane. Standard PD fluids contain glucose as osmotic agent, high content of GDPs, low pH and the presence of lactate which have been described to be the main responsible factors driving the structural changes in the peritoneal membrane during PD treatment\textsuperscript{4, 5}. In addition, peritoneal accumulation of advanced glycosylation end-products (AGEs), as a result of the continuous exposure of the mesothelial cells with the high glucose concentrations, are suggested to contribute to the impairment of the peritoneal membrane integrity\textsuperscript{55}. As a result of the several disadvantages of conventional PD fluids, different solutions with different combinations of buffers and pH have been developed and brought into clinical practice. These more biocompatible PD solutions contain alternative osmotic agents, lower GDPs, more physiological pH with biocarbonate/lactate-buffered solutions which appear to offer advantages in terms of peritoneal membrane preservation, peritoneal homeostasis and AGEs formation\textsuperscript{56-58}. Although clinical and experimental data indicate that bicarbonate/lactate solution results in a better preservation of the peritoneal integrity, the underlying mechanisms still remain unexplained. The differences observed between these two types of PD fluids could be related to changes in the inflammatory milieu. In this regard, more biocompatible PD fluid was associated with a more stable Th1/Th2 balance and reduced peritonitis rate and with lower IL-6 concentrations in effluent from patients\textsuperscript{59, 60}. Overall, data suggest that the inflammatory environment during PD treatment may be different in a more biocompatible setting. However, additional clinical and experimental studies are necessary to fully explore the connection between these inflammatory changes and the preservation from biocompatible PD fluids.
CONCLUSIONS

Despite being a lifesaving treatment, PD is characterized by chronic peritoneal dysfunction mainly due to peritoneal fibrosis and neoangiogenesis. Increasing evidence points to chronic peritoneal inflammation as a primary cause of structural and functional changes of the peritoneal membrane, and in turn with PD clinical outcomes. In particular, M2 macrophages and Th17 cells have gained interest as main regulators of peritoneal fibrosis through secretion of pro-fibrotic mediators. Modulation of the inflammatory mediators could reduce the negative effects of these cell types and their production of inflammatory cytokines to prevent peritoneal damage in PD. Alternatively, the addition of more biocompatible fluids may result in an alternative inflammatory microenvironment leading to more preserved peritoneal membrane integrity.
REFERENCES


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CHAPTER 6

Protective effects of paricalcitol on peritoneal remodeling during peritoneal dialysis

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ABSTRACT

Peritoneal dialysis (PD) is associated with structural and functional alterations of the peritoneal membrane, consisting of fibrosis, angiogenesis and loss of ultrafiltration capacity. Vitamin D receptor activation (VDRA) plays an important role in mineral metabolism and inflammation, but also anti-angiogenic and anti-fibrotic properties have been reported. Therefore, the effects of active vitamin D treatment on peritoneal function and remodeling were investigated. Rats were either kept naïve to PDF exposure or daily exposed to 10 ml PDF and were treated for five or seven weeks with oral paricalcitol or vehicle control. Non PDF-exposed rats showed no peritoneal changes upon paricalcitol treatment. Paricalcitol reduced endogenous calcitriol but did not affect mineral homeostasis. However, upon PDF exposure, loss of ultrafiltration capacity ensued which was fully rescued by paricalcitol treatment. Furthermore, PD-induced ECM thickening was prevented and omental PD-induced angiogenesis was less pronounced upon paricalcitol treatment. No effect of paricalcitrol treatment on total amount of peritoneal cells, peritoneal leukocyte composition and epithelial to mesenchymal transition (EMT) was observed. Our data indicates that oral VDRA reduces tissue remodeling during chronic experimental PD and prevents loss of ultrafiltration capacity. Therefore, VDRA is potentially relevant in the prevention of treatment technique failure in PD patients.
INTRODUCTION

Peritoneal dialysis (PD) is a renal replacement therapy for patients with end stage renal disease (ESRD). During long-term PD, morphological changes occur in the peritoneum including interstitial fibrosis leading to thickening of the membrane and neovascularisation. Together with the induction of inflammatory processes this can lead to loss of peritoneal membrane function, technique failure and premature discontinuation of PD therapy. The mechanisms involved in these pathological changes are incompletely understood.

Vitamin D was originally identified as a key regulator for bone metabolism and calcium homeostasis. Novel insights revealed that its biological actions go beyond this and also include regulation of inflammation, angiogenesis, as well as cell growth, differentiation and apoptosis of many cell types. Chronic kidney disease (CKD) patients have low levels of active vitamin D as conversion 25-hydroxy-vitamin D₃ (25D) into the bioactive form 1,25-dihydroxy-vitamin D₃ (1,25D), occurs mainly in the kidney.

Based on these previous findings we hypothesized that active vitamin D can attenuate or prevent the changes observed after long term PD. To address this question we applied an experimental model of peritoneal dialysis.

METHODS

Animals and experimental design

Male Wistar rats (Harlan CPB, Horst, The Netherlands), weighing 280-330 grams were used in all experiments. After arrival, the rats were allowed one week of acclimatization. Animals were maintained under conventional laboratory conditions and were given food and water ad libitum. The experimental design (figure 1) was approved by the Animal Care Committee of the VU university medial center, Amsterdam.

Animals were randomly assigned into one of four groups and treated for five weeks: I: controls receiving 3 times weekly sugar water as vehicle control (n=9), II: control rats receiving 3 times weekly orally paricalcitol, dissolved in sugar water, (40 ng/kg rat, Zemplar®, kindly provided by AbbVie, Chicago, USA; n=9), III: animals receiving daily instillation of 10 ml conventional PDF and 3 times per week sugar water according to the same regime as group I (Dianeal, 3.86% glucose, pH5.2, Baxter R&D Utrecht, The Netherlands; n=13), and IV: rats receiving daily instillation of 10 ml conventional PDF and paricalcitol treatment according to the same regime as group II (n=13). PDF was instilled via a subcutaneously implanted access port as described previously. Oral administration of paricalcitol was achieved in the
following way: the rats were taught to drink sugar water via a syringe when offered. In this way we could limit the discomfort for the animals caused by oral gavage. Since multiple animals were housed per cage dissolving paricalcitol in drinking water would lead to unknown amounts of paricalcitol administered per animal.

To corroborate our results, additional animals were also treated for seven weeks as described above (I: n= 8; II: n= 8; III: n=15; IV: n= 15, respectively) with a small dose adjustment of the oral treatment of paricalcitol to 30 ng/kg rat paricalcitol, three times weekly, in order to maintain a stable cumulative dose of paricalcitol among treated groups.

**Readouts:**

Table 1 summarizes the analysis performed after five or seven weeks of treatment.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Effluent</th>
<th>Time period treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 weeks</td>
</tr>
<tr>
<td><strong>Effluent</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Transport parameters</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cell count</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cell differentiation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TGFβ</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VEGF</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MCP-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL10</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL12p70</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GRO/KC</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Morphological</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECM thickness</td>
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<td>+</td>
</tr>
<tr>
<td>Liver imprints</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Omentum</strong></td>
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<td></td>
</tr>
<tr>
<td>Vasculature</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Mesentery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vasculature</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 1: Analyses performed after 5 and/or 7 weeks of treatment. + indicates the analysis has been performed; - indicates the analysis has not been performed

<table>
<thead>
<tr>
<th>Serum</th>
<th>Transport parameters</th>
<th>25D</th>
<th>1,25D</th>
<th>Ca</th>
<th>P</th>
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<tr>
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<td>-</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25D</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,25D</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>+</td>
<td>+</td>
<td></td>
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</tr>
</tbody>
</table>

Analysis of effluent: At the end of the experiment, a 90-minute peritoneal equilibrium test (PET) was performed under fentanyl-citrate-fluanisone (0.05 ml/100 gram bodyweight; VetaPharma, Leeds, UK) and midazolam (0.08 ml/100 gram bodyweight; Actavis B.V., Baarn, The Netherlands) anesthesia. 30 ml Conventional PDF was instilled into the peritoneal cavity via a direct intraperitoneal catheter (Venflon Pro, BD Medical Systems, Franklin Lakes, NJ, USA). After drainage, the ultrafiltration capacity was calculated (effluent volume minus 30 ml) and the cell pellet was collected. Cell number and viability was determined in a haemocytometer by trypan blue exclusion. Cytocentrifuge preparations were stained with May-Grünwald-Giesma and cells were differentiated. Cell-free effluent was stored at -20°C or -80°C for determination of biomarkers for both five and seven weeks PETs.

In the animals treated for five weeks, glucose, creatinine, urea (GLUC3, CREA and UREAL respectively, COBAS 8000, Roche Diagnostics, Basel, Switzerland) and sodium (ABL 800 FLEX, Radiometer, Zoetermeer, The Netherlands) concentrations were analysed in serum samples, collected via a heart puncture after sacrificing the animals by CO₂/O₂ induction, and in cell-free effluents and dialysis/serum (D/P) transport ratios were calculated. Based on a glucose determination of a pure Dianeal sample, and glucose detection in the cell free effluent of the PETs, the percentage of glucose absorption was calculated, whereby was corrected for the PET volume.

Hyaluronic acid (HA) was determined in the effluent using an ELISA-based assay according to Fosang et al. The concentrations TGFβ (TGFβ1, Promega GmbH, Manheim, Germany), VEGF (R&D systems, Abingdon, UK or Milliplex™ MAP rat cytokine kits, Millipore, Billerica, MA, USA), and MCP-1 (Merck MILLIPORE, Darmstadt, Germany or Milliplex™ MAP rat cytokine kits) were also analyzed in the PET effluents of both experiments. In addition, IL4, IL10, IL12p70, IL5 and GRO/KC (IL8 related protein in rodents) were analyzed only for the PET effluents collected after 7 weeks of PDF exposure using multiplex bead arrays (Milliplex™ MAP rat cytokine kit). Milliplex™ MAP is based on Luminex® xMAP™ technology, and used as recommended by the manufacturers.
**Morphological analysis:** Parietal peritoneum samples were taken at the contra-lateral side of the tip of the catheter. Cryostat sections were cut and stained with Van Gieson (Merck, Darmstadt, Germany) to quantify fibrosis. To determine the submesothelial thickness, images were analyzed by measuring, on average, 10 independent points per animal (Leica LAS AF version 2.6.0, Leica Microsystems, CMS GmbH, Mannheim, Germany). A part of the omentum, of both five and seven week treated rats, and mesenteric tissue, only of five week treated rats, was dissected and spread on a glass slide. To visualize vasculature and macrophages the tissues where stained with CD31 (PECAM; Serotec, Oxford, UK) and ED2 (Serotec, Oxford, UK). Images were analyzed by digital image analysis (AnalySIS Soft Imaging System, Olympus, Hamburg, Germany or Cellprofiler: image analysis software for identifying and quantifying cell phenotypes).

Liver imprints of the mesothelial monolayer were made, after seven weeks of treatment, by pressing 6% gelatine coated glass slides on the slightly dried liver after sacrificing, and stained for vimentin (Serotec, Oxford, UK), cytokeratin (DakoCytomation, Glostrup, Denmark) and DAPI (Invitrogen, Breda, the Netherlands) to determine epithelial to mesenchymal transition. On average seven images per rat were analyzed manually (Leica LAS AF version 2.6.0, Leica Microsystems, CMS GmbH, Mannheim, Germany), whereby increased vimentin expression and change in morphology from cobbled stone like cells towards spindle like cells where counted as cells that underwent EMT.

**Serum analysis:** Serum samples were analyzed for 25-hydroxyvitamin D3 by competitive binding protein assay (Diasorin, Stillwater, Minnesota, USA), 1,25-dihydroxyvitamin D3 by radioimmunoassay after immunoextraction (IDS, Tyne and Wear, UK), PTH by ELISA (Scantibodies Laboratory, Santee, CA, USA) and calcium (Ca) and phosphate (P) by colorimetric assays (Roche diagnostics, Mannheim, Germany). With exception of 25D, only measured in serum samples of seven weeks treated animals, the analysis were performed after both five and seven weeks of treatment.

**In vitro macrophage migration assay:** Macrophage migration was examined in Boyden transwell cell culture chambers using gelatine-treated polycarbonate membranes with 10 μm pore size (Neuro Probe, Inc., Gaithersburg, MD, USA). Briefly, rat bone marrow cells were isolated and macrophages were allowed to adhere for 7 days in the presence of DMEM (Gibco, BRL, Gaithersburg, MD, USA) enriched with 15% v/v L-cell conditioned medium (LCM) and supplemented with 2% v/v penicillin-streptomycin-glutamine (PSG; invitrogen, Breda, The Netherlands) and 10% fetal calf serum (Biowest, Nuaillé, France). Other cells were washed away and macrophages were harvested by lidocaine treatment. Cells were resuspended in serum free DMEM to a concentration of 2*10⁵ cells/ml. Aliquots of 50μl were added to the upper chamber, while the lower chamber was filled with 25μl of
DMEM containing MCP-1 (10 ng/ml), paricalcitol (1*10^{-6} M) or a combination of both, with or without the addition of Dianeal (1:4 with DMEM). After 6 hours of incubation at 37°C, cells were removed from the upper chamber side of the membrane. The membrane was washed and stained with Coomassie. The cells on the bottom side of the filter were counted and expressed as percentage of migrated cells compared to control DMEM medium without chemo attractant. The experiment was performed in triplicate using different cell isolations.

**Statistical analysis**

Data presented as median and inter-quartile range are analysed by using the Kruskall-Wallis test followed by Dunn’s Multiple Comparison test to compare the following groups: I vs II, I vs III, I vs IV and III vs IV. The detection limit was used for statistical analysis for VEGF, IL4, IL10, IL12p70, IL5 and GRO/KC levels in the effluent, when the concentrations were below detection limit.

**RESULTS**

The well being of all rats was monitored daily and no unexpected abnormalities were observed. Twelve of the total 56 animals exposed to PDF were taken out the experiment due to abdominal fat or omental tissue wrapping around the tip of the catheter, which was consistent with previous experience. All control animals (groups I and II), 25 out of 30 in group III, and 19 out of 30 in group IV remained for analysis after five and seven weeks of PDF exposure.

**Vitamin D mineral homeostasis**

1,25D Levels showed a declining trend in paricalcitol treated animals after five weeks and a significant decrease after seven weeks of treatment compared to the control animals (Table 2). In addition, the same effect of paricalcitol on 25D levels was detected in the serum samples measured after seven weeks of treatment. Ca and P levels were not affected by
paricalcitol treatment, except for animals in the control group receiving 40 ng/kg paricalcitol which had significantly higher P level compared to the control animals.

### Table 2: Effect of paricalcitol on mineral homeostasis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Five weeks</th>
<th>Seven weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>1.25 vitamin D3 (pmol/l)</td>
<td></td>
</tr>
<tr>
<td>IQR</td>
<td>392.6–519.3</td>
<td>342.0</td>
</tr>
<tr>
<td>Group II</td>
<td>25 vitamin D3 (pmol/l)</td>
<td></td>
</tr>
<tr>
<td>IQR</td>
<td>no data</td>
<td>no data</td>
</tr>
<tr>
<td>Phosphate (mmol/l)</td>
<td>3.4</td>
<td>4.6</td>
</tr>
<tr>
<td>IQR</td>
<td>[3.4–3.8]</td>
<td>[4.0–4.7]</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>IQR</td>
<td>[3.6–3.8]</td>
<td>[3.4–3.8]</td>
</tr>
</tbody>
</table>

*p*<0.05 compared to group I, *p*<0.05 compared to group III.

**Peritoneal transport**

The PET resulted in ~10 ml net ultrafiltration (UF) in control animals (group I and II). Chronic PD treatment significantly reduced ultrafiltration capacity towards a median of 7.3 ml after five weeks and, even worse, 5.8 ml net UF after seven weeks (*p*<0.05 group II vs I; figure 2). Paricalcitol treatment in the PDF exposed group prevented these significant changes in UF (*p*>0.05 vs group I) and resulted in a 10-15% increase in UF capacity compared to PDF exposure alone.

![Figure 2: Net ultrafiltration after 90 minute PET. Net ultrafiltration after a 90 minute PET with 30 ml conventional PD fluid after five (A) and seven weeks of treatment (B). All data presented as Median and Inter Quartiles. Whiskers indicate the extremes. *p*<0.05 compared.](image)

To further analyze the effect of PDF exposure and vitamin D receptor activation on peritoneal functional decline, transport parameters were measured in serum and PET effluents after five weeks of treatment (Table 3). Exposure to PDF changed D/P creatinine from 0.2 to 0.4 (*p*<0.01 vs group I). Paricalcitol did not affect the D/P creatinine in both control and PD-treated rats. Although compared to control levels (group I) D/P urea (0.5) and D/P sodium (0.8) were slightly higher in group III (0.6, *p* > 0.05, and 0.9, *p* < 0.05, resp.) and group...
IV (0.6, $p < 0.01$, and $0.8, p > 0.05$, resp.), absolute changes in ratios were minimal. Glucose absorption increased, although not significantly, from 33.3% to 42.8% upon PDF exposure. Paricalcitol treatment lowered, but not significantly, glucose absorption in both non-PDF exposed and PDF exposed animals (33.3 % in group I vs 22.3 % in group II) and PDF exposed animals (46.5% in group III vs 42.8 % in group IV).

<table>
<thead>
<tr>
<th>Group</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose absorption (%)</td>
<td>[28.5-38.5]</td>
<td>[21.5-26.2]</td>
<td>[35.8-53.7]</td>
<td>[35.9-46.8]</td>
</tr>
<tr>
<td>D/S Creatinine</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4*</td>
<td>0.4*</td>
</tr>
<tr>
<td>[0.1-0.2]</td>
<td>[0.2-0.3]</td>
<td>[0.3-0.5]</td>
<td>[0.3-0.5]</td>
<td></td>
</tr>
<tr>
<td>D/S Urea</td>
<td>0.5</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>[0.4-0.6]</td>
<td>[0.4-0.6]</td>
<td>[0.5-0.7]</td>
<td>[0.5-0.7]</td>
<td></td>
</tr>
<tr>
<td>D/S Sodium</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>[0.6-0.8]</td>
<td>[0.7-0.8]</td>
<td>[0.8-0.9]</td>
<td>[0.8-0.9]</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Peritoneal transport parameters determined by PET effluent D /serum P after 5 weeks of treatment. All data presented as Median and Inter Quartiles. $^a p<0.05$ compared to group I, $^b p<0.01$ compared to group I.

**Cell numbers and macrophage migration**

Total cell numbers increased significantly upon PD treatment and were approximately five times higher compared to control animals ($p< 0.01$ and $p<0.001$ for group III and IV vs group I, respectively; fig 3A and B). However, there was no significant difference between the paricalcitol and vehicle control treated groups. Cell differentiation of peritoneal cells in the effluents revealed a reduction of eosinophil and mast cell count and an increase in neutrophils after PD treatment. In all groups macrophages remained the dominant cell type (+/- 80%; Table 4). Paricalcitol treatment did not induce significant differences in leukocyte composition in control nor PD treated rats neither after five or seven weeks of PDF exposure.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Five weeks</th>
<th>Seven weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Cell number</td>
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<td>22.2</td>
</tr>
<tr>
<td>IQR</td>
<td>[19.2-24.2]</td>
<td>[16.2-24.2]</td>
</tr>
<tr>
<td>Macrophages</td>
<td>77.0</td>
<td>80.0</td>
</tr>
<tr>
<td>IQR</td>
<td>[74.8-81.0]</td>
<td>[76.6-82.1]</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>IQR</td>
<td>[0.0-0.0]</td>
<td>[0.0-0.0]</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>IQR</td>
<td>[0.0-0.0]</td>
<td>[0.0-0.0]</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.3</td>
<td>1.9</td>
</tr>
<tr>
<td>IQR</td>
<td>[1.1-13.9]</td>
<td>[11.3-14.6]</td>
</tr>
<tr>
<td>Mast cells (%)</td>
<td>9.8</td>
<td>7.4</td>
</tr>
<tr>
<td>IQR</td>
<td>[8.6-13.6]</td>
<td>[4.5-10.4]</td>
</tr>
</tbody>
</table>

Table 4: Composition of peritoneal leukocytes. All data presented as Median and Inter Quartiles. $^a p<0.05$ compared to C group, $^b p<0.01$ compared to C group, $^c p<0.001$ compared to C group.
**Analysis of peritoneal effluents and in vitro macrophage migration**

MCP-1 was measured in the PET effluents and showed to be unaffected by paricalcitol treatment in the control situation (group II, fig 3C). After five weeks of PD treatment, MCP-1 levels were ~5 fold increased in PD rats compared to control rats (p=0.05). Paricalcitol treatment in combination with PD tended to increase MCP-1 levels even further, although not significantly, to a median of 2 ng/ml compared to 1.2 ng/ml in group III and 0.3 ng/ml in both control groups. No difference was found in MCP-1 levels between the different groups in the animals treated for seven weeks.

**Figure 3:** Cell numbers and macrophage migration. Total peritoneal cell number in effluent after 90-minute PET with 30ml of conventional PD fluid after five (A) and seven weeks of treatment (B) ** p<0.01, *** p<0.001. Effluent concentration of MCP-1 after five weeks of treatment (C) * p<0.05. Rat macrophage migration towards paricalcitol, MCP-1 in standard medium (white bars) or medium containing Dianeal (1:4) (grey bars; D * p<0.05 vs medium, # p<0.05 vs Dianeal+medium).
To examine the effect of paricalcitol and peritoneal dialysis fluid on macrophage migration, in vitro migration assays with primary rat macrophages were performed. As expected, MCP-1 induced macrophage migration. Paricalcitol did not change migration under these (control) conditions (fig 3D, white bars). However, when Dianeal was added to the culture medium (1:4), which is known to result in macrophage activation, paricalcitol enhanced migration, similar to the levels of MCP-1 induced migration (p<0.05) (fig 3D, grey bars). Simultaneous addition of paricalcitol and MCP-1 did not further increase macrophage migration when Dianeal was present.

VEGF levels in peritoneal effluents were below detection limits in most control animals but were statistically significantly higher in the PDF exposed groups. Seven weeks of paricalcitol treatment reduced VEGF levels to 40 pg/ml (median) compared to 72 pg/ml in the PDF exposed group (p= 0.01; fig 4A). However, after five weeks no effect of paricalcitol treatment on effluent VEGF concentration was found (data not shown).

As shown previously, PD treatment induced a significant increase in HA production, indicating an inflammatory state in the peritoneum (p=0.001) (fig 4B). Paricalcitol treatment mitigated the increase in HA concentrations in the rats exposed to PDF as well as after five as seven weeks of treatment.

In PD treated animals, TGFβ concentrations were on average 2.7 times higher compared to control animals (p=0.05) after as well five (data not shown) or seven (fig 4C) weeks of treatment. Paricalcitol treatment didn’t affect the TGFβ levels in the effluent.

IL4 levels were below detection limit in almost all animals in the control groups (18 pg/ml; groups I and II). IL4 levels were significantly increased in group III compared to group I (p= 0.001) with an average of 186 pg/ml. Although there was no significant difference between group III and IV, IL4 levels were less pronounced upon paricalcitol treatment with an average concentration of 92pg/ml (fig 4D).

IL12p70 concentrations in the effluent significantly increased upon PD treatment (groups III and IV) compared to group I, in which all levels were found to be below detection limit (24 pg/ml), p=0.001 and p=0.05 respectively. A non significant trend was observed whereby, upon PDF exposure, paricalcitol mitigated the rise of IL12p70 with a median concentration of 68 pg/ml compared to 207 pg/ml in group III (fig 4 E).

Also IL5 concentrations were below detection limit (24 pg/ml) in all control animals (groups I and II). PDF exposure alone led to an increase of IL5 concentrations in the effluent (74 pg/ml), whereas paricalcitol treatment completely prevented this significant elevation (24 pg/ml; p=0.01 vs group III; fig 4F).
Figure 4: Peritoneal effluent concentrations. VEGF (A), HA (B), TGFbeta (C), IL4 (D), IL12p70 (E), IL5 (F), IL10 (G), GRO/KC (F) levels in the peritoneal effluent after seven weeks of treatment. Data presented as Median and Inter Quartiles. Whiskers indicate the extremes.  p<0.05, *p<0.05, ** p< 0.01.
Even though a few animals in the PDF exposed groups had IL10 levels above the detection limit, the medians of all groups were 98 pg/ml IL10, so no difference was determined (fig 4G). GRO/KC concentrations were not significantly increased upon PDF exposure and no affect of paricalcitol treatments was observed. Median GRO/KC levels were 23, 27, 37 and 48 pg/ml for group I, II, III and IV, respectively (fig 4H).

**Peritoneal tissue remodeling**

Histological analysis showed that five-week PDF exposure resulted in increased submesothelial matrix thickness (median 22 µm) compared to control rats (median 15 µm; p=0.05 vs group I; fig 5). Additional paricalcitol treatment prevented thickening of the parietal mesothelial matrix layer (16 µm; p>0.05 vs group I). After seven weeks of PDF exposure no difference in submesothelial matrix thickness was found between all groups, thus also not between PDF and non-PDF treated groups (data not shown).

![Figure 5: Extra cellular matrix thickness. ECM thickness of parietal peritoneum (A) and representative examples of peritoneal sections of group I, III and IV, respectively, after 5 weeks of treatment. All data presented as Median and Inter Quartiles. Whiskers indicate the extremes. *p<0.05.](image)

Chronic PD treatment resulted in increased recruitment of activated M2 tissue macrophages and in new vessel formation in omentum and mesentery, determined by respectively ED2 and CD31 staining (fig 6). In the mesentery, paricalcitol treatment could not prevent PD induced macrophage accumulation nor angiogenesis (fig 6B and D). However, a declining trend in median omental ED2 positive macrophage accumulation was observed during paricalcitol treatment for both the control (group II 1 % vs group I 3% positive area) and the PDF exposed groups (group IV 5% vs group III 10% positive area; fig 6C+E). Moreover, PD-induced angiogenesis in the omentum (14% positive area; p=0.01 compared to group I) was less pronounced for paricalcitol treated animals (4% positive area; p>0.05 compared to group I) after five weeks of treatment (fig 6D+E). Although no significant differences in omental CD31 positive area were observed after seven weeks between group I and III, a declining trend in the CD31 positive area upon paricalcitol treatment was observed (33% vs 14% vs and 32 % vs 27% for groups I vs II and III vs IV, respectively; data not shown).
Figure 6: Angiogenesis and macrophage accumulation in visceral peritoneum. Macrophage accumulation (Ed2 positive staining) in omentum (A) and mesentry (B), and angiogenesis (CD31 positive staining) in omentum (C) and mesentry (D) after 5 weeks of PDF exposure and paricalcitol treatment. Representative examples of the omentum with ED2 in green and CD31 in red of control (group I), PDF exposed (group III) and PDF exposed treated with paricalcitol (group IV) treated rats (E). All data presented as Median and Inter Quartiles. Whiskers indicate the extremes. *p< 0.05, **p<0.01.

Liver imprints

Liver imprints were taken after seven weeks of PDF exposure. Mesothelial cell density and the number of vimentin positive- cytokeratin negative cells increased in the PDF exposed animals (figure 7A group III and IV compared to group I) indicating mesothelial cell regeneration and epithelial to mesenchymal transition. In addition, the cobbled-stone appearance
of the mesothelial cells (figure 7B group I) is partly lost in groups III and IV, in which the cells are more stretched. The ratio vimentin positive- cytokeratin negative cells/mesothelial cells approximately doubled in the PDF exposed groups (III and IV) compared to the control (group I). Paricalcitol treatment did not influence this process (figure 7A).

**Figure 7:** Epithelial to mesenchymal transition on liver imprints. Ratio vimentin positive/mesothelial cells on liver imprints (A). Representative examples of liver imprints with nuclei in blue, vimentin in green and cytokeratin in red of control (group I), PDF exposed (group III) and PDF exposed treated with paricalcitol (group IV) treated rats (B). All data presented as Median and Inter Quartiles. Whiskers indicate the extremes. **p<0.01.

**DISCUSSION**

In the present study the role of VDR activation on peritoneal remodeling in a chronic rat PD-model was investigated, while validating our model by comparing the control and PDF exposure only groups as well (group I vs III). In the animals exposed to PDF, compared to the control situation, we observed worsening of ultrafiltration capacity, elevation in inflammation markers, partly increased vascular surface area, and a higher number of cells undergoing epithelial to mesenchymal transition. This is in line with previous observations. Paricalcitol treatment influenced several of the examined parameters. Loss of ultrafiltration capacity, increase in ECM thickness, angiogenesis and IL5 levels due to PDF exposure were significantly attenuated by paricalcitol treatment. In addition, a trend towards decreased glucose absorption, less ED2 positive macrophage accumulation in the omentum and mes-
entry, lower HA, VEGF, IL12p70 and IL4 levels was observed upon paricalcitol treatment in PDF exposed animals. However, not all factors involved in peritoneal remodeling upon PD were affected by paricalcitol treatment, such as total cell number and epithelial to mesenchymal transition.

Importantly, paricalcitol treatment decreased endogenous 1,25D levels, which has also been found by others in both animal and human studies\textsuperscript{11,12}, and is the consequence of up-regulation of the catabolic enzyme 25(OH)D-24-hydroxylase. This indicates successfulness of applying oral paricalcitol treatment in our rat model.

The main finding of this paper is the demonstration that paricalcitol can attenuate the loss in ultrafiltration capacity upon PDF exposure. Driving forces for ultrafiltration in peritoneal dialysis are the maintenance of an osmotic gradient and the existence of low barrier resistance for water transport. Glucose absorption showed a slight increase after five weeks of PDF exposure, although not significant, indicating a small loss of osmotic gradient. In the control situation, and to lesser extent in the PDF exposed group, addition of paricalcitol resulted in a trend towards positively affecting the osmotic gradient. Despite, the fact that the trend was small and not significant this could have contributed to the partial preservation of the ultrafiltration capacity in animals receiving oral paricalcitol in a PD environment. Consistent with this is the observed attenuated neovascularization by paricalcitol. Neovascularization results in increased perfusion of the peritoneal membrane, which is considered to be one mechanism leading to enhanced dissipation of the osmotic gradient, by early enhanced glucose uptake form the peritoneal dialysis fluid.

In line with the observed partial prevention of ultrafiltration failure by paricalcitol treatment, we showed prevention of increasing ECM thickness, which may indicate a lower barrier resistance for water transport, upon PDF exposure after five weeks of treatment with paricalcitol. This latter finding is in accordance with the compelling evidence that vitamin D treatment can reduce fibrosis\textsuperscript{13,14}. Possible explanations for reduced fibrosis after paricalcitol treatment may be found in proteins involved in the thickening of the ECM-layer such as collagen type-1 and the renin-angiotensin-aldosterone (RAAS) system, which are down regulated upon VDR activation\textsuperscript{15,16}.

The immunomodulatory effects of paricalcitol can have beneficially contributed to the development of ECM thickness. In our experiments paricalcitol modulated the concentrations of IL5, IL4 and possibly also affected HA and VEGF, factors derived from cells of the immune system and/or mesothelial cells. Although we cannot prove the direct effect of these cytokines on peritoneal membrane remodeling, our data are in line with a recent study showing the importance of cytokines by correlating reduction in IL-17 and activation of regulatory T-cells with reduced fibrosis\textsuperscript{17}.
In our experiments paricalcitol treatment led to a ~50% decreased population of ED2 positive ‘M2’ macrophages in the omentum in both control and PDF exposed rats, which might have led to lesser thickening of the submesothelial matrix we observed after five weeks of treatment. In other studies it has also been shown that macrophages play an important role in fibrosis whereby M2 macrophages correlate with fibrosis in sclerotic skin, pulmonary- and kidney fibrosis. Moreover, there is compelling evidence that M2 macrophages are involved in peritoneal fibrosis in PD. In addition, paricalcitol treatment mitigated IL12p70 concentrations, which production is related to M1 macrophages, upon PDF exposure.

Vitamin D receptor activation has acknowledged anti-angiogenic properties. In this study it is also shown that paricalcitol tended to reduce angiogenesis, especially in the omentum. This observation is in line with previous in vitro studies. These studies demonstrate reduced proliferation of human umbilical vein endothelial cells upon paricalcitol treatment. In addition, in a mouse model for PD it has been shown that paricalcitol can prevent angiogenesis. Several humoral factors can be involved in this reduced angiogenesis following paricalcitol. IL12p70, however, which is described to have anti-angiogenic properties declined in the paricalcitol group. This suggests that the effects of paricalcitol are not mediated by this factor. VEGF is another prominent pro-angiogenic factor. Here, we show indeed that the PDF exposure induced increase in VEGF, after seven weeks of treatment, is attenuated by paricalcitol treatment. Finally MCP-1, which has been shown also to have pro-angiogenic capacities, was not reduced by paricalcitol.

In line with previous studies we found an increase in peritoneal cells observed after PD treatment. The higher cell numbers could be due to the enhanced levels of chemo attractants such as MCP-1 and IL5. Although paricalcitol didn’t influence total cell numbers, as described above, it tended to influence the type of cells including the decrease of ED2 positive cells.

The ratio vimentin positive/mesothelial cells, as indicator of EMT, increased upon PDF exposure compared to the control situation, which makes our model suitable to study the effects of paricalcitol on EMT. However, contrary to other studies, we were not able to find a decrease in EMT which might be due to the different models or concentrations of paricalcitol used.

To summarize, although we didn’t observe an effect of paricalcitol on EMT, a part of the effects of paricalcitol are found to be consistent. Paricalcitol partly preserved the ultrafiltration capacity upon PDF exposure. We observed partial prevention of angiogenesis, and thus a smaller vascular surface area, which could contribute to the observed trend in preservation of the glucose driven osmotic gradient. In addition, prevention of increase in ECM thickness was found, which indicates less resistance of the peritoneal membrane and thus
a better ultrafiltration capacity.

Our study has several limitations. Firstly, differences between the control and PDF exposed groups, such as ultrafiltration capacity and ECM thickness, were less pronounced after seven weeks of PDF exposure. This was likely caused by the apparently less mesothelial toxic effects in this particular control group of animals. We know from earlier experiments that differences in separate experiments may occur. Besides sampling error and unequal distribution of toxicity could be of importance too. Therefore, we could have missed potential protective effects of paricalcitol treatment in this group due to a lack of pathological changes in the PD control animals. However, we did use a wide range of additional parameters in which morphological, functional and biochemical components were included, and did observe all well-described changes in the PD treated groups for five weeks.

A second limitation is that we did not use a uremic model and vitamin D deficient model. However, we hypothesize that under those conditions the affect of VDR activation is likely even more pronounced.

Taken together, we have shown that VDR activation can partly restore ultrafiltration failure due to limiting ECM thickening and angiogenesis even in a calcitriol sufficient environment. Future studies should be carried out to address the clinical benefit of improved PD efficacy on ultrafiltration in particular.

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The dipeptide alanyl-glutamine ameliorates peritoneal fibrosis and attenuates IL-17 dependent pathways during peritoneal dialysis

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ABSTRACT

Peritoneal dialysis (PD) can result in chronic inflammation and progressive peritoneal membrane damage. Alanyl-Glutamine (Ala-Gln), a dipeptide with immunomodulatory effects, improved resistance of mesothelial cells to PD fluids. Recently, interleukin-17 (IL-17) was found to be associated with PD-induced peritoneal damage. Here, we studied the capacity of intraperitoneal Ala-Gln administration to protect against peritoneal damage by modulating IL-17 expression in uremic rat and mouse PD exposure models. Supplementation of PD fluid with Ala-Gln resulted in reduced peritoneal thickness, αSMA expression and angiogenesis. Addition of Ala-Gln also attenuated the IL-17 pathway expression induced by PD, reflected by substantial reduction or normalization of peritoneal levels of IL-17, transforming growth factor β, IL-6, and the transcription factor retinoic acid receptor-related orphan receptor gamma T. Moreover, increased levels of IL-17 were associated with PD-induced peritoneal thickening. Conversely, Ala-Gln treatment prevented peritoneal extracellular matrix deposition, an effect seen with IL-17 blockade. Thus, intraperitoneal administration of Ala-Gln, a stable dipeptide commonly used in parenteral nutrition, ameliorates PD-induced peritoneal damage in animal models, in part by modulating IL-17 expression. Hence, Ala-Gln supplementation of dialysate may be a potential strategy to ameliorate peritoneal deterioration during PD.
INTRODUCTION

Dialysis is a life-saving renal replacement therapy for about 2,000,000 ESRD (End Stage Renal Disease) patients worldwide, from which more than 200,000 patients are treated with peritoneal dialysis (PD). PD is based on the ability of the peritoneum to function as a dialysing membrane, allowing the exchange of solutes and waste products between the blood stream and the Peritoneal Dialysis Fluid (PDF) instilled via a permanent catheter. This home-based treatment preserves the patient’s residual renal function and provides better quality of life while ensuring equivalent patient survival when compared to haemodialysis. Unfortunately, long-term PD is associated with the development of functional and structural alterations to the peritoneal membrane. Peritoneal fibrosis, thickening of the submesothelial extracellular matrix (ECM) and vascular changes, together with epithelial to mesenchymal transition (EMT), are major causative factors in the development of ultrafiltration failure, necessitating a withdrawal from PD. These pathological changes are the consequences of inflammatory processes generated in part by the continuous exposure to conventional glucose-based dialysis solutions. Glucose is used as an osmotic agent to allow the exchange of solutes and is itself implicated in the alterations that occur in peritoneal cells, together with glucose degradation products (GDPs), formed during heat sterilization of the solutions. Several other factors can contribute to peritoneal deterioration in PD patients, including peritonitis and mechanical injury during instillation of PDF. In the last decade ‘biocompatible’ PD solutions have entered the market, many of which have been tested in various experimental PD models and clinical trials and are currently the first choice in the most developed countries. However, many of these solutions still feature high osmolarity, high glucose concentration and low pH, features that are potentially harmful to the peritoneal membrane and its integrity.

Glutamine is the most abundant nonessential free amino acid in the body. It is involved in nitrogen transport and hence represents an important fuel source during stress conditions. It is commonly used as a nutritional supplement in critically ill patients where its use associated with reduction in hospital mortality and is thought to have a protective immunomodulatory role by reducing Tumor necrosis factor α (TNFα) and Interleukin 6 (IL-6) expression. Alanyl-Glutamine (Ala-Gln) is a dipeptide featuring a glutamine amino group joined to an alanyl residue. This more stable and soluble Ala-Gln dipeptide form is able to restore the stress proteome of mesothelial cells when exposed to PDF. In lung injury, the dipeptide is able to reduce inflammation by modulating the T helper 17 (Th17) / Regulatory T cell (T reg) balance.

Th17 cells are a subset of T helper cells related to many autoimmune and inflammatory diseases. Naive T cells differentiate to Th17 cells under the combined effects of Transforming
Growth Factor Beta (TGFβ) and Interleukin 6 (IL-6). The main effector cytokine secreted by Th17 cells is Interleukin 17 (IL-17), expressed in TH17 cells under the control of the transcription factor Retinoic-acid-receptor-related orphan receptor gamma T (ROR(γ)t)\textsuperscript{20}. Elevated IL-17 levels have been found in experimental animal models of multiple sclerosis\textsuperscript{21}, psoriasis\textsuperscript{22}, collagen-induced arthritis\textsuperscript{23} and other autoimmune diseases. A key factor in the development of fibrosis is the interplay between IL-17 and TGFβ\textsuperscript{24}. Notably, a role for IL-17 in inducing peritoneal fibrosis has recently been shown in an experimental peritoneal dialysis model\textsuperscript{25}.

In this study we used uremic rat and mice PD exposure models to examine the effects of supplementing PDF with pharmacological doses of Ala-Gln dipeptide on IL-17 expression, Th17 balance, and peritoneal fibrosis.

**METHODS**

**Experimental models of PD exposure**

**Rat PD model.** Male Wistar rats (Charles River, Maastricht, The Netherlands) weighing 250-275 g were used. 15 rats were made uremic by 5/6 nephrectomy. These animals were then split into 3 groups. A control group (n=5) which did not have a catheter implanted, a PD exposed group that was injected daily for 5 weeks with standard PDF (Dianeal\textsuperscript{®} 4 3.86% glucose, pH 5.2, Baxter R&D, Utrecht, The Netherlands) as previously described\textsuperscript{26, 27} and a PD group that was also injected daily for 5 weeks but with Dianeal\textsuperscript{®} enriched with 8 mM Alanyl-Glutamine (Ala-Gln) Dipeptide (Dipeptiven\textsuperscript{®}, Fresenius Kabi Austria, Graz). Following one week of acclimatization and handling a 5/6 nephrectomy was performed in order to induce uremia. The nephrectomy involved the complete removal of the right kidney and ligation of 1 to 3 branches of the arteries supplying the left kidney in order to obtain a residual kidney function of around 1/6 of the total capacity. In order to verify uremia induction urea and creatinine serum levels were measured at day 0 (before 5/6 nephrectomy) and 21 (data shown in Supplementary figure 2a-b). After two weeks of recovery a peritoneal catheter connected to a subcutaneous access port (Venflon Pro, BD Medical, New Jersey, USA) was implanted as previously described\textsuperscript{27}. For 1 week following catheter implantation 2 ml of saline with 1 IU/ml heparin was administered each day (n=10). Animals were randomised to two treatment groups. For the following 5 weeks rats were instilled daily with 10ml of standard PDF (n=5) or (n=5) with PDF enriched with Ala-Gln.

**Mouse PD model.** C57BL/6J female mice (Charles River, Maastricht, The Netherlands) aged 12-14 weeks and weighing approximately 20 g at the start of the study were used. Animals were organized as follow: 1 healthy control group (n=5), 3 PD groups (n=5 per group) daily
exposed to 2ml standard Dianeal® during a period of 8 weeks and 3 PD groups (n=5 per group) daily exposed for 8 weeks to Dianeal® enriched with Dipeptiven® (Ala-Gln). PD groups were weekly injected i.p. respectively with IL-17 recombinant (human recombinant protein, R&D Minneapolis, MN at the dose of 10ng/g of body weight), αIL-17 antibody (eBioMM17F3, eBioscience, 100 μl /mouse) or received no additional injection during the whole experiment. Mice in all the PD groups underwent 5/6 nephrectomy and catheter implantation (Customized mouse catheter MMP-4S-061108A, Access Technologies, Ridgeway, USA). 5/6 nephrectomy consisted in the complete removal of the right kidney and the removal of the anterior and posterior 1/3 part of the left kidney by using a monopolar electric blade as described previously described28. In order to verify uremia induction urea and creatinine serum levels were measured at day 0 (before 5/6 nephrectomy), 15 and 70 (end point) (Supplementary figure 2c-d).

All the animals were housed under standard conditions and were given food and water ad libitum. Health conditions were checked daily. The weight of the animals was checked daily after surgery during a period of 10 days and weekly for the remaining of the experiment. Animals that lost more than 20% of their initial body weight or showed abnormal activity were excluded from the experiment. The experimental protocols were approved by the Animal Welfare Committee at the VU University Medical Center, Amsterdam.

**Serum analysis**

500 and 200μl of blood were drawn via tail and facial vein puncture in rats and mice respectively at the time points previously indicated. At all the time points, serum samples were analysed for urea and creatinine levels. For determination of urea levels a kinetic test with urease and glutamate dehydrogenase was used. Creatinine levels were detected by indirect immunofluorescence assay. Measurements were performed by using spectrophotometer Cobas8000 (c702), Roche diagnostics.

**Cell counting**

At the end point, following the injection of 2ml standard PDF via catheter in mice or 30 ml via an extra placed catheter (Venflon Pro, BD Medical, New Jersey, USA) in rat, peritoneal effluents were collected (after 30 and 90 min respectively), cells were isolated by centrifugation and counted. Cytocentrifuge preparations were made and cells number determined by May Grünwald staining for rat cells. Cell suspensions obtained from peritoneal lavage in mouse were stained with fluorochrome-conjugated mouse-specific antibodies against CD3, CD4, CD8α, B220, CD11b, Ly6C, F480 and IL-17 purchased from eBiosciences. Before intracellular staining, cells were re-stimulated for 4 h with 50ng/ml PMA and 500ng/ml ionomycin in the presence of 1μg/ml BD Golgi Plug (eBiosciences). Samples were analysed in a BD FACS Fortessa (BD Biosciences) flow cytometer and further analyses were performed with FloJo.
Histology and Immunohistochemistry

Parietal peritoneal biopsies were collected from the opposite side from the catheter installation. The biopsies were fixed in Bouin’s solution, embedded in paraffin, cut into 5 μm sections and stained with Masson’s Trichrome. Peritoneal membrane thickness was determined using light microscopy (Leica CTR6000, with a Leica Microsystems LAS-AF6000). Microscope photographs were obtained using an Olympus BX41® clinical microscope and an Olympus DP20® digital camera using cell Acquisition software. Peritoneal thickness of each animal was calculated by the median of measurement taken every 50 μm from one side to the other of the tissue sample.

Biopsies were frozen in Tissue-Teck® (O.C.T.® Sakura) and cut into 5 μm sections. To identify myofibroblasts and vessels samples were stained for anti-rat Alpha Smooth Muscle Actin (αSMA 1A4, DAKO, 1:500) combined with anti-mouse-IgG (H+L) AF (Invitrogen) and Cluster of Differentiation 31 (αCD31, PECAM, Serotec, Oxford, UK, 1:1000) coupled to anti-mouse-IgG-555 according to the manufacturer’s instructions. Nuclei were stained with DAPI. Florescence microscopy was performed with a Zeiss (Zeiss LM). The areas positive for CD31 were calculated by CellProfiler software (2.1.1, Broad Institute, UK).

To identify peritoneal infiltrated CD4+ IL-17+ T cells double immunofluorescence was performed by using an anti-mouse / rat-IL-17 (APC) (eBio17B7, ref: 17-7177-81, eBioscience) and an anti-mouse / rat-CD4 (PE) (Lot: 3130616, ref: 553730, BD eBiosciences). Nuclei were stained with DAPI. Microscopy was performed with a confocal microscope (Leica TCS SPE with LAS0AF software, version 2.0.1 build 2043).

Real-time quantitative (q) PCR

Parietal peritoneum biopsies were mechanically homogenized and total RNA was extracted using TRIzol Reagent® (Invitrogen). Reverse transcription into complementary DNA (cDNA) was performed using Reverse Transcription System kit (Promega). The synthesised cDNA was amplified with a standard quantitative Polymerase Chain Reaction (qPCR) protocol including the use of SYBR GREEN® (Applied Biosystems®) and rat or mouse specific primers were used (Supplementary table 3). The relative amount of mRNA was calculated using comparative Ct (ΔΔ Ct) methods. All samples were analysed in triplicate and averages compared. Amplification products were normalized against GADPH mRNA, which was amplified in the same reaction as internal control for each analysed gene.

Quantification of cytokines

Supernatants were made cell free by centrifugation (300G, 5mins, RT), stored at -20°C. Protein levels of rat IL-17A, TGFβ and HA were quantified by ELISA-based assay (Rat Platinum software.
ELISA kit eBioscience). Protein levels of mouse IL-17A, IL-6, INFγ and IL-4 were quantified by ProcartaPlex™ Multiplex Immunoassays (affymetrix eBioscience).

**Statistical analysis**

Data were analysed using GraphPad Prism software (La Jolla, CA). Statistical analysis was performed using One-way ANOVA test to compare the groups. Bonferroni test was used to correct for multiple analysis. Data were shown as means ± SEM. Confidence intervals used: 95%. Correlations were assessed using Spearman’s correlation tests (GraphPad Prism 5.03). A P-value <0.05 was considered statistically significant (‘*’ = P<.05, ‘**’ = P<0.01, ‘***’ = P<.001).

Figure 1. Effect of Alanyl-Glutamine (Ala-Gln) dipeptide on the parietal peritoneal thickness of PD-exposed rats. Panel (a) shows representative peritoneal membrane thickness for each group in the rat PD model. Masson’s trichrome staining shows a drastic increase of extracellular matrix deposition in rats exposed to standard PD fluid (PDF) and a significant reduction by Ala-Gln dipeptide treatment (PDF ALA-GLN). Magnification x20. (b). Column bars represent peritoneal thickness (μm) measurements of control group (grey) compared with PDF group (striped) and PDF ALA-GLN group (white). (c-d). Induction of Hyaluronic Acid protein level and Fibronectin gene expression in the PD group (striped) and subsequent reduction in the PDF ALA-GLN group (white)* P<.05, ** P<.01, *** P<.001.)
RESULTS

Supplementation of PDF with Ala-Gln prevented peritoneal thickness and decreased the expression of fibrotic markers in both uremic rat and mouse PD model

Histological analysis of peritoneal sections showed that fibrosis took place in our uremic models. Masson’s Trichrome staining of peritoneal sections, obtained in rats after 5 weeks of daily exposure to the PD fluid, clearly showed a significant increase of peritoneal thickness compared to the control group as well as an induced expression of other fibrosis markers, including Fibronectin (FBN) and Hyaluronic Acid (HA) (figure 1).
Ala-Gln dipeptide plays a role in immunomodulation and is associated with improved resistance of mesothelial cells to PDF exposure\textsuperscript{16}, thus we examined its effect in our uremic rat PD model. Parietal peritoneal sections from the Ala-Gln PDF group showed reduced peritoneal thickness compared to the standard PDF group. Masson’s Trichrome staining revealed that the peritoneal thickening in the Ala-Gln PDF group and control group is comparable, indicating a protective effect of the dipeptide (figures 1a-b). Decreased gene expression of FNC and protein expression of HA were found in the Ala-Gln group when compared to the PDF group (figures 1c-d). Immunofluorescence analysis of peritoneal biopsies showed that exposure to Ala-Gln PDF resulted in a significant reduction of αSMA positive cells accumulating in the parietal membrane upon daily instillation with conventional PDF (figures 2a-b). A positive linear correlation was also observed between the number of αSMA positive cells and the peritoneal thickness (figure 2c). Additionally, the number of CD31 positive vessels was lowered in the Ala-Gln enriched group (figures 2d-e).

Regarding ultrafiltration (UF), there was a non-significant minor decline with PDF exposure that was reversed with Ala-Gln (Supplementary figure 1a). Total number of peritoneal cells increased in the PDF groups with and without addition of Ala-Gln (Supplementary table 1). White cell differential demonstrated that these changes were mainly due to an increase in lymphocytes and, macrophages and a decrease in eosinophils, in accordance with previous observations after PDF exposure\textsuperscript{14, 29}.

**Ala-Gln modulated IL-17 expression induced by PDF**

Recent findings have shown activation of Th17 immune response in PD patients and preservation of peritoneal membrane integrity by blockade of IL-17 in an experimental mouse PD model\textsuperscript{25}. Therefore, we quantified IL-17 in the current study. IL-17 was elevated by PDF exposure in peritoneal membrane and effluent and was reduced by addition of Ala-Gln (figures 3 a-b). Examination at the mRNA level of IL-17 regulatory transcription factor ROR\textgreek{t} revealed expression in PDF Ala-Gln comparable to control, in contrast with the elevated levels of the PDF group (figure 3c). Moreover, a positive Spearman’s correlation between IL-17 protein and peritoneal thickness was shown in rat (figure 3d).
Figure 3: Alanyl-Glutamine (Ala-Gln) dipeptide reduces IL-17 expression. IL-17 is markedly reduced upon treatment with Ala-Gln dipeptide. Analysis of IL-17 levels in peritoneal effluents by ELISA (a) and in peritoneal membrane biopsies by qPCR (b) demonstrated that Ala-Gln (white bar) reduces significantly the levels of IL-17 when compared with the group exposed to conventional PD fluid (striped bar). (c) qPCR analysis of ROR(γ)t in peritoneal membrane biopsies showed a downregulation of this transcription factor in the Ala-Gln treated group (white bar) when compared with the PDF group (striped bar). (d) Correlation between IL-17 cytokine levels (pg/ml) in the peritoneal cavity detected by ELISA and thickness of the peritoneum compact zone of rats treated with PD fluid with and without Ala-Gln (Spearman regression, p < 0.05, r²=0.568, n=15). Data shows in figures (a-d) are obtained from analysis performed in rat tissues/samples. (e-f) Masson’s trichrome staining of extracellular matrix deposition in mouse peritoneal membranes. Pictures in (e) represent one mouse per group. Graph (f) represents measurements of peritoneal thickness (μm) in mouse. Groups are indicated as follow: healthy controls (small squares), PDF group (big squares), PDF ALA-GLN (horizontal stripes), PDF group with IL-17 blockade (vertical stripes), PDF group injected with recombinant IL-17 (right oblique stripes), PDF ALA-GLN group with IL-17 blockade (left oblique stripes), PDF ALA-GLN group injected with recombinant IL-17 (empty squares). Data are represented as means ± SEM of 5 animals per group. Graphs (g-h) represent IL-17 expression levels in mice respectively measured in peritoneal effluents by ELISA for protein and peritoneal biopsies by qPCR for mRNA. Groups are indicated as follow: healthy controls (small squares), PDF group (big squares), PDF ALA-GLN (horizontal stripes), PDF group with IL-17 blockade (vertical stripes), PDF group injected with recombinant IL-17 (right oblique stripes), PDF ALA-GLN group with IL-17 blockade (left oblique stripes), PDF ALA-GLN group injected with recombinant IL-17 (left oblique stripes). IL-17 Data in the column bars are represented as means ± SEM of 5 animals per group. (* P<.05, ** P<.01, *** P<.001).
To elucidate molecular mechanisms underlying the protective role of Ala-Gln on the peritoneal membrane, we examined the effect of IL-17 antagonism in a mouse model of peritoneal fibrosis.

As previously shown in the rat model, also in mice Ala-Gln was associated with reduced peritoneal thickness (drastically increased after 8 weeks of daily exposure to PDF). Moreover, increased levels of IL-17 were related to higher PD-induced peritoneal thickening, since the addition of recombinant IL-17 significantly increased ECM thickness in PDF-treated mice. Conversely, IL-17 blockade prevented formation of peritoneal ECM in this group (figures 3 e-f).

Thus, IL-17-dependent thickening of the peritoneal membrane was directly demonstrated in the mouse model by i.p. injections of recombinant IL-17 or IL-17 blocking antibody. The same interventional model was used to demonstrate effects of Ala-Gln on IL-17-dependent thickening of the peritoneal membrane. Addition of Ala-Gln reduced peritoneal thickness (figures 3e-f) and both protein and mRNA IL-17 levels (figures 3g-h) with combined exposure to recombinant IL-17 and PDF whereas blockade of IL-17 reduced peritoneal ECM in the PDF group and further decreased it in the Ala-Gln PDF exposed group (figures 3e-f). Ala-Gln thus appeared to act in a way comparable with blockade of IL-17 expression.

In order to further investigate whether the protective role played by Ala-Gln addition to PD fluid is caused by the modulation of IL-17 expression, key factors involved in peritoneal Th17 cell differentiation were studied. In the rat model, a significant up-regulation of TGFβ (figures 4a-b) and IL-6 (figures 4c-d) in the PDF-only group and down-regulation in the PDF-Ala-Gln group when compared with the control group was noted. In the mouse model, no significant differences were found between groups regarding both mRNA and protein levels of IL-6 (figure 4e-f), Interferon γ (INF γ) (figures 4 g-h) and Interleukin 4 (IL-4) (figures 4i-j) measured respectively in peritoneal effluents and parietal peritoneal biopsies. Nevertheless IL-6 protein expression was reduced in the Ala-Gln group similarly as previously observed in rat.

Similarly to the rat model, also in mice not significant differences regarding UF were found and total number of peritoneal cells resulted increased in the PDF-exposed groups (without reaching significance) (Supplementary table 2). No significant variation was found in the number of macrophages and lymphocytes but further subtyping suggested that differences in lymphocytes were restricted to CD4+ IL-17+ lymphocytes.

Analysis of infiltrating cells by confocal microscopy indeed showed a clear reduction in CD4+ IL-17+ parietal peritoneum infiltrating T cells when Ala-Gln enriched group was compared with the PD treated group (figure 5a). Moreover analysis of the effluent showed that Ala-Gln was able to quantitatively reduce PD fluid enhanced recruitment of CD4+ IL-17+ T
lymphocytes (figure 5b).

**DISCUSSION**

In this study it is shown that the addition of Ala-Gln to PD fluid is protective against peritoneal fibrosis in both uremic rat and mouse models. This is substantiated by decreases in peritoneal thickening, expression of EMT markers, pathological neovascularisation, and

![Figure 4](image-url)
a reduction in inflammatory/immune cell numbers in both the effluent and the parietal peritoneal tissue. Finally, these data provide compelling evidence that Ala-Gln plays its protective role, at least partly, by modulating the expression of IL-17, a key factor involved in peritoneal membrane integrity.

Although PD represents a life-saving treatment for many CKD (Chronic Kidney Diseases) patients, long term exposure to conventional PDFs causes a progressive increase of the peritoneal compact zone thickness over the time of exposure, vascular changes, fibrosis and inflammatory events that ultimately result in treatment failure. The peritoneal dialysis procedure is a well recognized stimulus for inflammation, but uremia itself also activates inflammatory pathways, resulting in a state of micro-inflammation. In this study we wanted to mimic as closely as possible patient situation by combining uremia with long term exposure to PDF in both rat and mouse model.

In congruence with patient studies this investigation showed peritoneal membrane thickening and up-regulated expression of fibrotic markers after extended PDF exposure. Interestingly, thickness of the peritoneal membrane and fibrosis (indicated by HA, FNC and TGFβ up-regulation) have been successfully restored by the addition of Ala-Gln dipeptide to the PDF. Apart from this a possible involvement of Ala-Gln in preventing alterations of the peritoneal membrane in terms of myofibroblast development and new blood vessel formation was found. Ala-Gln enriched PDF did not cause any significant alteration in terms of fibrosis and new vessels formation when compared with the control group, whereas an increased number of αSMA and CD31 positive cells appeared in the PDF exposed group not treated with Ala-Gln.

Since Ala-Gln is known to play a role in immunomodulation we investigated whether this dipeptide could protect from peritoneal fibrosis by modulating the Th17 response. IL-17, which is the main cytokine produced by Th17 cells, is involved in many inflammatory diseases and is responsible for pulmonary, cardiac and liver fibrosis and for peritoneal damage in experimental PD models. IL-17 injected mice resulted in increased peritoneal thickness and up-regulation of inflammatory cytokines, whereas IL-17 blockade diminished fibrotic responses in the peritoneum. These data point to IL-17 as good candidate for therapeutic strategies. In our study the increase of the submesothelial zone thickness correlated with IL-17 expression in the peritoneal cavity.

Our results showed that Ala-Gln was not only able to reduce peritoneal fibrosis, but it also produced a drastic decrease in IL-17 expression in both peritoneal membrane biopsies and in peritoneal effluents.
Figure 5. Ala-Gln treatment is associated with reduction of inflammatory cell infiltration. Panel (a) shows immunofluorescence microscopy analysis of parietal peritoneal tissue sections, stained for CD4 (green), IL-17 (red) and 4,6-diamidino-2-phenylindole (DAPI) (blue) counterstaining. Double positive cells are marked in yellow colour. (Magnification x200). Graph (b) represents the percentage of T helper 17 cells (CD4+ IL17+) T cells detected by flowcytometry analysis in peritoneal effluent cells collected at the end point. Groups are indicated as follow: healthy control (small squares), PDF group (big squares), PDF ALA-GLN (horizontal stripes). Data are represented as means ± SEM of 5 animals per group. (* P < 0.05).
RORγt is the transcription factor responsible for IL-17 expression and drives Th17 cell differentiation. Our results demonstrated a down regulation of this transcription factor in rats treated with Ala-Gln and this evidence suggests that Ala-Gln plays a protective role by regulating T cell differentiation and subsequently IL-17 production. Our uremic PD model showed elevated expression of the Th17-related cytokines IL-6 and TGFβ and their drastic drop as a consequence of the Ala-Gln treatment. In addition to being a strong pro-fibrotic cytokine, TGFβ also works in a coordinate manner with IL-6 to drive T cells differentiation through the Th17 lineage. Consistent with this evidence, our data showed an analogue pattern for IL-17 levels and the expression of its positive regulators TGFβ and IL-6. Although previous studies observed a synergic function of IL-6 with Interferon gamma (INFγ) in driving peritoneal fibrosis, our results did not showed any significant differences between groups regarding INFγ expression levels. Treated animals appear to have massive induction of INFγ at the protein level but due to the large inter-animal variation, it is not possible to conclude any presence or absence of Th1 cell involvement. Due to contrasting results about IL-4 respectively mRNA expression and protein levels and lack of significant differences between groups, the role of Th2 cells remain still unclear. Taken together, our data suggest that IL-17, partly in synergy with TGFβ and IL-6, is the central player of the Ala-Gln dependent protective effect against peritoneal fibrosis.

In addition to that, in our mouse PD model the number of CD4+ IL-17+ T cells detected in peritoneal effluents significantly increased after PD fluid exposure and decreased in the presence of Ala-Gln dipeptide. This result was also reflected by the presence/absence of CD4+ IL-17+ T cells infiltrated in the peritoneal membrane after PDF/ PDF Ala-Gln treatment. Finally, a positive correlation between the increment of IL-17 levels and peritoneal thickening and the comparable effect of IL-17 blockade and Ala-Gln treatment after injections of recombinant IL-17 or IL-17 antibody, provided direct evidence that Ala-Gln modulates the regulation of IL-17 levels and that it plays its protective role at least in part by means of IL-17 pathway.

In conclusion, exposure to conventional PDFs caused peritoneal fibrosis and increased expression of IL-17. Supplementation of PDF with pharmacological doses of Ala-Gln prevented the peritoneal thickness and preserved original peritoneal vasculature. In our uremic PD models Ala-Gln has a protective role by modulating the IL-17 pathway. The results obtained in the mouse PD model confirmed and strengthened this role and indicated the use of Ala-Gln as a possible therapeutic strategy to preserve the integrity of the peritoneal membrane in PD patients. Further work is needed to study the clinical significance of Ala-Gln-mediated protection against the non-physiological morphological and structural changes occurring in the peritoneal cavity during PD.
ACKNOWLEDGMENTS

This work was supported from European Union, Seventh Framework Program “EuTRiPD” under grant agreement Marie Curie ITN-GA-2011-287813 (EF, GL, TF, MVC). We wish to thank Prof D. Fraser (Cardiff University) for providing English language editing of the final manuscript and N.J. Paauw for his technical help.
REFERENCES


29. Loureiro, J.s., M. Schilte, A. Aguiera, et al., BMP-7 blocks mesenchymal conversion of mesothelial cells and prevents peritoneal damage induced by dialysis fluid exposure. ERA-EDTA. p. gfp618.


Supplementary figure 1. Peritoneal Ultrafiltration Test (PET) after long term exposure to PDF. A slight decrease and subsequent increase in the ultrafiltration capacity of the peritoneal membrane is observed when the PDF group (striped bar) and the PDF ALA-GLN group (white bar) are compared to the control group (grey bar) in both the rat (a) and mouse PD model exposure (b). Differences are not significant.

Supplementary figure 2. Uremia is established by 5/6 nephrectomy in rodent models of Peritoneal Dialysis (PD). Creatinine (a) and urea (b) blood levels are three-fold increased after three weeks from the 5/6 nephrectomy performed in Wistar male rats. Blood samples are taken at day 0 before the surgery and after three weeks from that (day 21). Changes in both urea and creatinine blood levels are showed also in C57BL/6 mice after 15 and 70 days from the 5/6 nephrectomy (c-d). Data are represented as means ± SEM of 5 animals per group. (* P<.05, *** P<.001).
### Supplementary Table 1

**Composition of cells in peritoneal effluents in the rat PD model after 5 weeks of exposure to PDF enriched or not with Ala-Gln.**

<table>
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<th>Cell populations</th>
<th>Control (Mean±SE)</th>
<th>PDF (Mean±SE)</th>
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</table>

* #: p<0.01  @ #: p<0.01  $ #: p<0.05

### Supplementary Table 2

**Composition of cells in peritoneal effluents at the end point in the mouse model after 8 weeks of exposure to PDF enriched or not with Ala-Gln.**

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<th>ALA-GLN (Mean±SE)</th>
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</thead>
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<td>CD4+ (%)</td>
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<tr>
<td>CD8+ (%)</td>
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<td>3.4±1.9</td>
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<td>Macrophages (%)</td>
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<tr>
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### Supplementary Table 3

**Real-time PCR assays sequences**

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<tr>
<th>Gene</th>
<th>Species</th>
<th>Sequence</th>
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</tr>
<tr>
<td>IL-6</td>
<td>rat</td>
<td>R: GTTTCCTTCTGGTATGAC</td>
</tr>
<tr>
<td>RORγT</td>
<td>rat</td>
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<td>GAPDH</td>
<td>mouse</td>
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</table>

### Supplementary Table 3

**Real-time PCR assays sequences**
ALANYL-GLUTAMINE PROTECTS FROM PERITONEAL FIBROSIS
Differences in peritoneal response after exposure of low GDP Bicarbonate/Lactate-Buffered compared to Conventional Dialysis Solution in a uremic mouse model

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ABSTRACT

**Background:** Long-term exposure of conventional Peritoneal Dialysis (PD) fluid is associated with structural membrane alterations and technique failure. Previously, it has been shown that infiltrating IL-17-secreting CD4+ T cells and pro-fibrotic M2 macrophages play a critical role in the PD-induced pathogenesis. Although more biocompatible PD solutions are recognized to better preserve the peritoneal membrane integrity, the impact of these fluids on the composition of the peritoneal cell infiltrate is unknown.

**Material and methods:** In a uremic PD mouse model, we compared the effects of daily instillation of standard lactate (LS) or bicarbonate/lactate-buffered solutions (BLS) and respective controls on peritoneal fibrosis, vascularisation and inflammation.

**Results:** Daily exposure of LS fluid during a period of eight weeks resulted in a peritoneal increase of αSMA and collagen I accompanied with new vessel formation compared to the BLS group. Effluent from LS-treated mouse showed a higher percentage of CD4+ IL-17+ cell population while BLS exposure resulted in an increased macrophage population. Significantly enhanced inflammatory cytokines such as TGFβ1, TNFα, INFγ and MIP-1β were detected in the effluent of BLS-exposed mice when compared to other groups. Further, immunohistochemistry of macrophage subset infiltrates in the BLS group confirmed a higher ratio of pro-inflammatory M1 macrophages over the pro-fibrotic M2 subset compared to LS.

**Conclusion:** Development of the peritoneal fibrosis and angiogenesis was prevented in the BLS exposed mice, which may underlie its improved biocompatibility. Peritoneal recruitment of M1 macrophages and lower number of CD4+ IL-17+ cells might explain the peritoneal integrity preservation observed in BLS-exposed mouse.
INTRODUCTION

Continuous and long-term treatment with peritoneal dialysis (PD) promotes an inflammatory response which eventually leads to a progressive remodeling of the peritoneal membrane. These changes are characterized by the accumulation of extracellular matrix, angiogenesis and other structural alterations of the peritoneum resulting in technique failure and serious clinical complications including encapsulation peritoneal sclerosis. To a large extent, these events seem to be driven by high glucose degradation products (GDPs) content, the low pH and the presence of lactate, typically present in conventional PD fluids. Therefore, the conversion of PD fluids towards more biocompatible solutions is recognized as an urgent unmet clinical need to better preserve the peritoneal integrity. As a consequence, lactate-bicarbonate buffered fluids with more physiological pH, a lower amount of GDPs and alternative solutions in which glucose is replaced by alternative osmotic agents such as icodextrin and amino-acids have been developed.

The introduction of neutral pH and particularly bicarbonate/lactate-buffered solutions seemed to offer advantages in terms of peritoneal membrane preservation and peritoneal homeostasis control. In the Euro balance trial, it demonstrated a significant improvement in effluent markers of peritoneal membrane integrity, a decrease in systemic advanced glycation end products (AGEs) levels, less decline in residual renal function and a decrease in peritoneal ultrafiltration. Moreover, such biocompatible fluids increase mesothelial cell markers, induce less systemic inflammation and reduced the incidence of peritonitis.

Recent studies revealed that immunological responses underlie PD-induced peritoneal injury upon conventional high GDPs lactate PD fluid exposure although the specific mechanism remains unclear. These data pointed to the importance of IL-17-mediated inflammation as a novel player in the PD-induced injury in both PD patients and experimental models. Furthermore, M2 macrophages are suggested to play a key role in the development of peritoneal inflammation and fibrosis. However, while experimental and clinical data suggest a better preservation of peritoneal morphologic and functional features upon bicarbonate/lactate solution compared to conventional PD solutions, the implication on the inflammatory cell population in this novel solution has been poorly defined. To overcome this knowledge gap, our recently developed uremic mouse PD exposure model was used in the present study to compare a pH neutral low-GDP bicarbonate/lactate-buffered solution (BLS) with a standard high GDPs lactate (LS) PD fluid in respect of inflammation, fibrosis and vascularisation. Here, we demonstrate that the enhanced fibrotic and angiogenic response observed in LS–exposed mouse was prevented upon BLS exposure. This preservation of the peritoneal integrity by BLS was accompanied with a lower number of CD4+ IL-17+ cells, higher levels of macrophages-related pro-inflammatory cytokines and with a higher ratio of M1...
macrophages over M2 subset.

METHODS

Mouse PD model

C57BL/6J female mouse (Charles River, Maastricht, The Netherlands) aged 12-14 weeks and weighing approximately 20 g at the start of the study were used. Animals were organized as follow: 1 healthy control group (n=10), 3 PD groups (n=10 per group) daily exposed to 2ml saline or standard lactate-buffered solution (Dianeal®, Baxter) or bicarbonate/lactate-buffered solution (Physioneal®, Baxter) during a period of 8 weeks. Mouse in all the PD groups underwent 5/6 nephrectomy and catheter implantation (Customized mouse catheter MMP-4S-061108A, Access Technologies, Ridgeway, USA). 5/6 nephrectomy consisted in the complete removal of the right kidney and the removal of the anterior and posterior 1/3 part of the left kidney by using a monopolar electric blade as previously described. All the animals were housed under standard conditions and were given food and water ad libitum. Health conditions were checked daily. The experimental protocols were approved by the Animal Welfare Committee at the VU University Medical Center, Amsterdam.

Cell counting

At the end point, following the injection of 2 ml standard PD fluid via a catheter, peritoneal effluents were collected after 30 min, cells were isolated by centrifugation and counted and stained with fluorochrome-conjugated mouse-specific antibodies against CD3, CD4, CD8α, B220, CD11b, Ly6C, F480 and IL-17 purchased from eBiosciences. Before intracellular staining, cells were re-stimulated for 4 h with 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA) and 500 ng/ml ionomycin in the presence of 1 μg/ml BD Golgi Plug (eBiosciences). Samples were analysed in a BD FACS Fortessa (BD Biosciences) flow cytometer and further analyses were performed with FloJo software.

Histology and Immunohistochemistry

Parietal peritoneal biopsies were collected from the opposite side from the catheter installation. The biopsies were fixed in Bouin’s solution, embedded in paraffin, cut into 5 μm sections and stained with Masson’s Trichrome. Peritoneal membrane thickness was determined using a Carl Zeiss Microscope (GmbH, 37081, Göttingen, Germany). Microscope photographs were obtained by using an AxioCam ICc5. The peritoneal thickness of each animal was calculated by the median of measurement taken every 50 μm from one side to the other of the tissue sample.

Biopsies were frozen in Tissue-Teck® (O.C.T.® Sakura) and cut into 5 μm sections. To identify myofibroblasts and vessels samples were stained for anti-rat Alpha Smooth Muscle Actin.
Immunohistochemistry

(αSMA 1A4, DAKO, 1:500) combined with anti-mouse-IgG (H+L) (Invitrogen) and Cluster of Differentiation 31 (αCD31, PECAM, Serotec, Oxford, UK, 1:1000) coupled to anti-mouse-IgG-555 according to the manufacturer’s instructions. Nuclei were stained with DAPI. Fluorescence microscopy was performed with a Carl Zeiss Microscope and photographs were taken with an AxioCam HR R3. The areas positive for CD31 were calculated by CellProfiler software (2.1.1, Broad Institute, UK).

Immunoblotting

Lysates of the peritoneal membrane were prepared by homogenizing of preserved tissue in lysis buffer containing Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). Protein concentrations were determined using the Pierce Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The following antibodies were used: CD31 (Abbiotec; 1:200), Collagen I (Abcam; 1:250), GAPDH (14C10) 1:1000 cell signaling, followed by donkey anti-rat/rabbit conjugated with HRP (Dako; 1:5000). Signal was visualized using enhanced chemiluminescence (Life Sciences) on LAS3000 (Fujifilm, Japan). Image J (NIH, Bethesda, Maryland) was used for analysis.

Quantification of cytokines

Peritoneal effluents collected after 8 weeks of treatment were made cell free by centrifugation (300G, 5mins, RT) and stored at -20°C. Protein levels of mouse Transforming Growth Factor β 1 (TGFβ1), Interleukin-1β (IL-1β), Tumor Necrosis Factor α (TNFα), Vascular Endothelial Growth Factor (VEGF), IL-17A, IL-6, Interferon γ (INFγ), IL-5, IL-4, Macrophages Inflammatory Proteins 1α and β (MIP-1α and MIP-1β) were quantified by ProcartaPlex™ Multiplex Immunoassays (Affymetrix eBioscience).

Statistical analysis

Data were analysed using GraphPad Prism software (La Jolla, CA). Statistical analysis was performed using One-way ANOVA test to compare the groups. A P-value <0.05 was considered statistically significant (‘*’ = P<.05, ‘**’ = P<0.01, ‘***’ = P<.001). Data were shown as means ± SD.

RESULTS

BLS-exposure in mouse prevented the development of both peritoneal fibrosis and angiogenesis with no changes in thickness

In order to mimic the situation in chronic kidney disease patients undergoing PD, a uremic mouse model, performed by 5/6 nephrectomy, was exposed to daily PD fluid during a period of 8 weeks. Nephrectomized groups showed a two-fold increase in both serum urea and creatinine concentrations 15 days after the surgery and remained stable for the duration of
the experiment (data not shown). As shown in Figure 1A, after eight weeks of daily exposure to the PDFs, there was a statistically significant increase (p=0.04) in peritoneal thickness compared to the non-PDF-exposed control (C: 30.63±3.66, S: 41.84±12.05, LS: 78.78±39.62, BLS: 78.11±32.87). However, thickening of peritoneum did not differ between the two PDF compositions (LS or BLS). In contrast, immunohistological analysis of peritoneal biopsies revealed that exposure to BLS significantly prevented (P=0.01) the accumulation of α-SMA positive cells (myofibroblast) in the parietal membrane when compared to conventional LS PDF (C: 0.020±0.016, S: 0.026±0.026, LS: 0.075±0.072, BLS: 0.016±0.014), (Figure 1B-C). Similarly, statistically significant enhanced collagen I protein levels were detected in peritoneal samples after exposure to LS fluid when compared to control whereas upon BLS no significant effect was shown (C: 0.33±0.19, S: 0.40±0.39, LS: 1.24±0.94, BLS: 0.66±0.13), (Figure 1D-E). Analysis of the peritoneal effluents collected after eight weeks of daily exposure to the different treatments performed by standard peritoneal equilibrium test (PET) revealed no differences between the groups regarding the volume of ultrafiltration (data not shown).
Figure 1. Exposure to low GDPs bicarbonate/lactate-buffered PD fluid prevents myofibroblast recruitment but does not protect from parietal peritoneum thickness. Graph (A) represents peritoneal thickness (μm) measurements for each group (n=10). Representative immunofluorescence microscopy (B) and analysis (C) of parietal peritoneal sections stained with αSMA marker (magnification x10; n=10). αSMA positive cells are indicated in red. Nuclei were stained with DAPI (blue). Representative immunoblotting (D) and analysis (E) of Collagen I (Col I) levels in total protein lysates of peritoneal membrane. GAPDH was used as loading control (n=6). (C: control; S: saline; LS: lactate PD fluid; BLS: bicarbonate/lactate PD fluid). Data shows means +/- SD. Differences were considered statistically significant for p<0.05 using one-way ANOVA. *P < 0.05.

Chronic treatment with LS resulted in a significant increment (p=0.02) of new vessel formation in the omentum, represented by the increased abundance of CD31 protein positive cells (indicative of endothelial cells) when compared with the BLS group (Figure 2A-B). These data were confirmed by the detection in the peritoneal tissue of high levels of CD31 protein upon LS fluid which was prevented (p=0.02) in BLS exposed animals (Figure 2C-D). Taken together, these results indicate that LS but not BLS fluid promoted a fibrotic and angiogenic response in the peritoneum although the development of increased peritoneal thickness was not preserved in BLS exposed mouse.

Figure 2. Peritoneal angiogenesis is attenuated in bicarbonate/lactate-buffered-exposed mouse. Immunofluorescence microscopy (A) and analysis (B) of omentum sections stained for vasculature with CD31; (green) marker (n=10). Each value corresponds to an average (% surface staining CD31) of 10 independent values of each mouse omentum taken each time (magnification 20x). Representative immunoblotting (C) and analysis (D) of CD31 levels in total protein lysates of peritoneal membrane (n=6). GAPDH was used as loading control. (C: control; S: saline; LS: lactate PD fluid; BLS: bicarbonate/lactate PD fluid). Data shows means +/- SD. Differences were considered statistically significant for p<0.05 using one-way ANOVA. *P < 0.05.
Analysis of inflammatory cell recruitment in peritoneal cavity upon PD fluid exposure

Exposure to PD fluid caused a numerical increase of the total number of cells in the peritoneal fluid compared to non-exposed animals, which however was not statistically significant (Table 1). No statistically significant variation was found in both CD4 and CD8 positive populations among groups while there was a 9-fold statistically significant increase in CD4/IL-17 double positive lymphocytes (CD4\(^+\) IL-17\(^+\)) found in the LS group compared to the BLS group, in which these double-positive cells remained at the same level as controls. No significant differences were observed between the groups in the percentage of B cells and monocytes (Ly6G\(^-\) CD11b\(^+\)), although the number of the latest increased especially after BLS. As in our previous study in mouse\(^{15}\), a slight increase in macrophages (F480\(^+\) CD11b\(^+\)) was shown after exposure to conventional LS, but this rise was more pronounced in the BLS group (*P=0.04 compared to control). Therefore, exposure to BLS clearly caused profound changes compared to LS primarily in terms of peritoneal inflammatory macrophages recruitment, and IL-17 expressing CD4 cells.

<table>
<thead>
<tr>
<th>Cell populations</th>
<th>Groups (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Total Cells (x10(^6))</td>
<td>2.93±1.31</td>
</tr>
<tr>
<td>CD4(^+) (%)</td>
<td>29±1.14</td>
</tr>
<tr>
<td>CD8(^+) (%)</td>
<td>14.35±3.23</td>
</tr>
<tr>
<td>CD4(^+) IL17(^+) (%)</td>
<td>1.98±2.46</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>7.45±8.7</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>2.03±0.69</td>
</tr>
<tr>
<td>B cells (%)</td>
<td>37.85±19.45</td>
</tr>
</tbody>
</table>

\(^*\)p<0.05

Table 1. Quantification of Total cell number (x10\(^6\)) composition of cells (%) in mouse peritoneal effluents after 8 weeks of exposure. (C: control; LS: lactate PD fluid; BLS: bicarbonate/lactate PD fluid). Data shows Means +/- SD (n=4). Differences were considered statistically significant for p<0.05 using one-way ANOVA. *P < 0.05. (Statistically significant differences were found in LS vs C and LS vs BLS for CD4\(^+\) IL-17\(^+\), and BLS vs C in Macrophages).

Cytokine production changes in BLS treated mouse

We further explored the differences between the PD fluids in terms of inflammatory cytokine responses. As shown in Figure 3A, an increment in TGFβ1 levels was detected upon both BLS (435.9±266.3 pg/ml) and LS (204.1±211.8 pg/ml) treatment when compared to control group (C:28.57±22.85 pg/ml) although this was statistically significant for BLS only (p=0.04). A similar pattern was observed for IL-1β that was slightly enhanced in the LS group.
and further increased upon BLS exposure (Figure 3B), albeit non-significant. Alternatively, IL-6 and VEGF release increased as a consequence of PD fluid exposure but it did not differ statistically between the PD regimens and control (Figure 3C, 3E). On the other hand, a statistically significant increase of TNFα was shown only after BLS exposure but not after LS treatment (C: 10.38±1.25, LS: 33.57±24.82, BLS: 239±186.8 pg/ml; P=0.02) (Figure 3D).

Figure 3. Exposure to a bicarbonate/lactate buffered solution is associated with increase of pro-inflammatory cytokines. Protein levels of the main pro-inflammatory cytokines detected in effluents collected from mouse after 8 weeks of PD fluid exposure. Cytokines levels (pg/ml) are represented as means ± SD (n≥7) as follow: (A) TGFβ1, (B) IL-1β, (C) IL-6, (D) TNFα, (E) VEGF, (F) INFγ, (G)IL-17, (H) IL-4, (I) IL-5, (J) MIP-1α, (K) MIP-1β. (C: control; LS: lactate PD fluid; BLS: bicarbonate/lactate PD fluid). Differences were considered statistically significant for p<0.05 using one-way ANOVA. *P < 0.05.
Emerging evidence points to IL-17 as an important factor in mediating peritoneal inflammation. In this study, the increment found in the CD4+ IL-17+ cell population upon LS exposure was accompanied by a nominal, but statistically insignificant, increase of the levels of IL-17 measured in the peritoneal effluents (Figure 3G). We found upregulation of IL-5 in the LS group which was not shown upon treatment with BLS (C: 18.44±2.40, LS: 53.4±33.09, BLS: 23.44±9.13) (Figure 3I). A similar trend was found for IL-4 (C: 13±0.70, LS: 59.07±51.03, BLS: 18±6.06) (Figure 3H), indicating that involvement of T helper 2 (Th2) cells was more pronounced upon LS treatment over BLS. On the other hand, the statistically significant high levels of INFγ (Figure 3F) detected only after BLS exposure suggested that in BLS regimens an important role may be played by T helper 1 (Th1) cells (C: 23.69±5.65, LS: 42.18±30.2, BLS: 180.3±123.5; P=0.01).

Figure 4. Bicarbonate/lactate-buffered solutions mediates pro-inflammatory macrophages recruitment. Panel (A) shows representative peritoneal membrane macrophages recruitment for each group in the uremic PD mouse model. Peritoneal staining for CD11b (violet), F480 (red), CD11b plus F480 double positive and Dectin-1 (green) are represented in the rows from the top to the bottom panel respectively. Nuclei were stained with DAPI (blue). Column bars represent number of macrophages measured per field in three different pictures taken per mouse for...
The presence of enhanced macrophage population in BLS treated mouse was further substantiated by the rise of MIP-1α and MIP-1β (also known as CCL3 and CCL4) following BLS treatment although significant changes were only found in the MIP-1β measurements (MIP-1β C: 12.81±1.43, LS: 53.43±49.73, BLS: 278.8±246.8; P=0.04) (Figure 3J-K). Taken together, between the two PDFs there are outspoken differences in levels of TGFβ1, TNFα, INFγ and MIP-1β suggesting that macrophages played a crucial role over other cell types in modulating the response of BLS on peritoneal fibrosis and angiogenesis.

**BLS exposure induced the recruitment of pro-inflammatory macrophages in parietal peritoneum**

Given the parallels between the high percentage of macrophages in effluent and the elevated levels of chemokine MIP-1α and 1β, we further explored macrophage subset population in the peritoneum by immunohistochemistry (Figure 4A). Quantification shows high F480 expression, characteristic of classically activated macrophages (M1)\(^\text{16}\), in the peritoneum of BLS treated mouse (Figure 4B). In addition, macrophages with classical morphology display high levels of both F480 and CD11b\(^\text{17}\), which were significantly increased in the BLS group compared to both the control and the LS groups (Figure 4C). On the other hand, staining for Dectin-1, a marker for anti-inflammatory (M2) macrophages, did not reveal any significant differences among groups. (Figure 4D). Overall, our results suggest that pro-inflammatory M1 phenotype modulated the response in the peritoneum induced by BLS exposure.

**DISCUSSION**

The current study is the first one using a mouse PD exposure model comparing lactate and bicarbonate/lactate-buffered solutions in a uremic setting, which more closely mimics the clinic status of PD patients when compared to the non-uremic animal models. Our findings support the notion that bicarbonate/lactate-buffered solution better preserve the peritoneal integrity status when compared with the conventional PD fluid. Specifically, we demonstrated that the enhanced CD4\(^+\) IL-17\(^+\) cell population in effluent detected in LS exposed mouse was prevented in the BLS group although with no significant changes in IL-17 effluent concentrations. Importantly, increased macrophage population together with high levels of chemokines that regulate migration and infiltration of monocytes/macrophages suggested that macrophages played a key role during BLS treatment. While pro-fibrotic M2 macrophages are thought to promote fibrosis and angiogenesis upon conventional PDF fluid...
treatment, in this study it was found that in BLS-exposed mouse the pro-inflammatory (M1) phenotype was dominant in the peritoneal tissue over M2 subset suggesting a difference in inflammatory response between different PD fluid exposures.

The accumulation of extracellular matrix and fibrosis is a characteristic peritoneal alteration induced by PD fluid exposure which leads to a progressive remodeling of the peritoneal membrane. As previously demonstrated, and confirmed by the present study, daily exposure of conventional PD fluid contributes to the fibrotic response by an accumulation of αSMA positive cells in the parietal peritoneum. Consistently, an increment of the extracellular matrix protein collagen I in peritoneal tissues of LS treated mouse was observed. In contrast, daily exposure to BLS fluid prevented the accumulation of both αSMA and collagen I. This is in accordance with previous studies suggesting that the use of low GDPs solutions was associated with less αSMA expression in vitro and less development of fibrotic response in the peritoneal membrane in rats. Importantly, other factors such as the neutral pH present in the more biocompatible fluid may also contribute to the differences observed. In addition, as we reported previously in a rat model with peritoneal dialysis, daily exposure to BLS prevented new vessel formation, which did occur when LS was used, indicating that BLS regimen is more biocompatible in terms of peritoneal angiogenesis. Our findings suggest reduced angiogenesis and peritoneal fibrosis as markers of better preservation of the structure of the peritoneum after exposure of BLS fluid. Despite the differences reported, no changes in peritoneal thickness were detected among the PD-treated groups. Numerous studies have suggested a relationship between peritoneal structural changes and the increment of fibrosis. However, other factors such as the amount of cell infiltrate may contribute to the increment of peritoneal thickness. In addition, daily instillation with normal saline also induces a slight increment of the thickness, indicating that peritoneal remodeling is not exclusive of PD fluid effects, and these additional effects may have masked or overwhelmed any difference between types of PD solution used. Furthermore, we previously reported that uremia perse also contributes to this event. Overall, all those factors may influence the lack of differences in thickness between PD groups.

In vitro experimental research has demonstrated the capacity of neutral pH, bicarbonate/lactate-buffered solutions to maintain mesothelial cellular integrity and function when compared to conventional fluid. Although PD fluid may exert a direct impact on the mesothelial cell stability, evidence points to the inflammatory response as key factor inducing PD-associated pathology. In this regard, in our previous studies, the chronic exposure to standard dialysis fluids resulted in peritoneal Th17 response including elevated IL-17 protein production. Importantly, the modulation of IL-17 during treatment with PD fluid was shown to be an effective therapy for PD-mediated peritoneal fibrosis and angiogenesis. In our study, the increase in CD4+ IL-17+ cell population observed upon LS treatment was
prevented in the BLS groups. Although no significant changes in IL-17 levels were observed between PD fluid exposed animals, it is possible that in this study statistical significance for IL-17 was missed due to too low power.

Alternatively, a striking enhanced INFγ concentration in the effluents of BLS group compared to the LS was found. In parallel, BLS slightly prevented the increment of IL-5 and IL-4 levels detected in the LS group. These findings suggested that inflammatory mechanisms occurring during exposure to bicarbonate/lactate buffer involve Th1 rather than Th2 cell subset while the opposite happened upon exposure to high GDPs lactate solutions. Possibly, this particular inflammatory milieu can explain the differences observed in peritoneal remodeling between PD fluids. In this regard, T helper-related cells interact with many immune cells including macrophages, which also play a crucial role in chronic inflammation-induced fibrosis. Enhanced levels of Th2 cell subsets together with pro-fibrotic cytokine microenvironment contribute to the polarization of peritoneal macrophages towards the anti-inflammatory or pro-fibrotic M2 macrophages subset. Particularly, M2 macrophages subset demonstrated to be dominant in the cellular infiltrate in PD patients and are suggested to drive peritoneal fibrosis. In this study, BLS exposure mediated the increment of the proteins of the chemotactic factors for macrophages MIP-1α and MIP-1β in the peritoneal effluents. It has been shown that in the inflammatory phase, newly attracted macrophages present a more pro-inflammatory (M1) phenotype but only after the switch to M2 they become pro-fibrotic. Interestingly in our experiment, the increase in the percentage of macrophages cell population in the peritoneal effluents after BLS exposure was associated with enhanced recruitment of pro-inflammatory M1 macrophages in the peritoneal membrane. Our results showed indeed an accumulation of macrophages in the parietal peritoneum and prevalence of the pro-inflammatory (F480+) over the anti-inflammatory subset (Dectin-1+) in the BLS group. In line with these findings, inflammatory M1 macrophages secrete TNFα which were significantly enhanced in the effluent of the BLS-exposed mice when compared to the group undergoing standard PD treatment. Furthermore, M1-polarization, or classical activation, is induced by INFγ which also was significantly incremented upon BLS fluid. In contrast, BLS-treated mouse did have high levels of the pro-fibrotic cytokine TGFβ1, also known to be a stimulus of M2 polarization, which, however, as outlined, did not occur. Nevertheless, in some PD studies increased levels of TNFα, TGFβ1 and INFγ have been interpreted as a consequence of improved mesothelial and macrophages cell function as part of a pro-inflammatory process. However, further studies are needed to confirm the effects of these cytokines during longer periods of PD treatment with a more biocompatible fluid. Overall, these findings suggest that the differences observed between the two PD fluids might rather indicate that an inflammatory process led by an influx of M1-macrophages mainly occurs in the BLS subgroup, while a more fibrotic response takes place in the
LS group. Importantly, we previously showed in vitro that only M2 macrophages, and not M1, secrete factors inducing αSMA expression and fibrosis\textsuperscript{31}. So, based on these findings, our present results showing a higher prevalence of peritoneal M1 over M2 macrophages in the BLS treated animals can be interpreted as preventive from the development of fibrosis by BLS exposure as compared to conventional PD fluid.

Our study bears the limitation that the exact mechanism involved in the distinct effects of PD fluid exposure is not defined. Moreover, we did not explore other inflammatory mediators such as T regulatory cells which are important to regulate the activated T-cell expansion\textsuperscript{32}. However, we provide a characterization of the key immunological cells dominating the peritoneal response upon standard high GDPs lactate solution and we compare it with a low-GDP bicarbonate/lactate-buffered fluid. Although previous experimental research already suggested the capacity of bicarbonate/lactate-buffered solution in preserving morphologic parameters when compared with the conventional fluid, here we support previous findings and extend the data by using our well-established uremic model combined with long-term exposure to PD fluid\textsuperscript{14}. As an additional limitation, no differences in the ultrafiltration capacity were detected between groups. This weakness, however, was previously reported in our previous study in uremic mice and rats with PD\textsuperscript{15}. Furthermore, an assumed reduction of the harmful effects of the new generation of PD fluids may explain limited peritoneal thickening after exposure to any PD treatment when compared to relevant effect showed in our previous studies\textsuperscript{33, 34}. This event suggests that longer exposure with PD fluid is necessary to fully explore the different effects in thickness and ultrafiltration. Finally, our model would be closer to the clinical situation by the addition of the drainage of the PD fluid. This feature, however, was not included since rodent models of PD absorb the fluid before 24h post-instillation.

In conclusion, a large difference exists in inflammatory response between conventional and low-GDP bicarbonate/lactate based PD fluids. The use of the latter solution in our uremic mouse model leads to better preservation of the peritoneal membrane in terms of fibrosis and vascularization. Moreover, peritoneal recruitment of M1 macrophages, higher levels of macrophages-related pro-inflammatory cytokines and lower number of CD4\textsuperscript{+} IL-17\textsuperscript{+} cells might explain the response observed in the peritoneal membrane of BLS-exposed mice. Finally, we provide a better understanding of the inflammatory mediators during the exposure of bicarbonate/lactate low GDPs buffered solution which might help to design new therapeutic approaches favoring the PD treatment.
Compliance with Ethical Standards

**Ethical approval:** The animal study protocol was in compliance with animal welfare regulations according to the Dutch law and approved by the Animal Welfare Committee at the VU University Medical Center, Amsterdam.

**Conflict of Interest:** The authors declare that they have no conflict of interest.
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INTRODUCTION

Chronic Kidney Disease (CKD) is associated with a number of serious health complications, of which cellular barrier breakdown in several organs may be an important underlying mechanism. The first part of this discussion addresses studies on CKD-induced structural and functional alterations of the endothelial cell barrier. In addition, the endothelial protective properties of active vitamin D in a CKD setting are evaluated in this part, with specific emphasis on the stabilization of endothelial cell-cell interactions. In the second part, we describe different therapeutic approaches against the damage of the mesothelial cellular barrier of the peritoneal membrane during Peritoneal Dialysis treatment by modulating the inflammatory environment.

PART I – DISRUPTED CELLULAR BARRIERS IN KIDNEY FAILURE: THE ENDOTHELIUM

1.1 Changes in endothelial structure and function following renal failure: beyond classical kidney-related risk factors.

Vascular endothelial dysfunction may trigger or accelerate cardiovascular disease during CKD\(^1\), and thereby be an important component in the pathogenesis of cardiovascular disease in these patients. The progressive loss of the vasculoprotective factors vitamin D and α-Klotho together with the accumulation of Fibroblast Growth Factor 23 (FGF23), uremic toxins, oxidative stress and inflammatory cytokines in the circulation due to kidney disease has been associated with cardiovascular complications\(^2\); however, the specific role of endothelial dysfunction in this relationship is not well established. Considering this knowledge gap, current literature was reviewed (Chapter 1) to provide evidence for the hypothesis that CKD-related factors severely impair the integrity of the endothelial monolayer. Indeed, the vascular abnormalities mediated by these kidney-related factors in experimental uremic animal models and cell-based assays, resemble clinical features observed in CKD patients\(^1, 3, 4\). Thus, this association reinforces the need for more experimental approaches to better understand the mechanisms of kidney-related risk factors on vascular dysfunction.

Given its endothelial protective properties\(^5, 7\), in the present dissertation a special focus was placed on the impact of the progressive decrease of active vitamin D in the CKD-induced endothelial dysfunction. In our uremic rat model, induced by 3/4 nephrectomy (Chapter 2), we observed a disrupted endothelial layer accompanied with an increase in vascular permeability in aortic tissue confirming the impact of kidney disease on the development of endothelial dysfunction in large vessels. This hypothesis is supported by previous associations between the decline of kidney function and arterial stiffness in CKD patients, as assessed by non-invasive techniques such as measurements of pulse wave velocity\(^8, 9\). Similarly, en-
dothelial-dependent aortic dilation, induced by acetylcholine, has been demonstrated to be impaired in different studies with subtotal nephrectomized rat models. Interestingly, when the uremic setting was combined with our recently developed vitamin D deficient model (with both 25(OH)D and 1,25(OH)2D reduced levels), no additional aggravation of endothelial damage by CKD was observed. In CKD patients, low levels of 25(OH)D have been associated with increased arterial stiffness and attenuated brachial artery flow-mediated dilation (FMD) suggesting that vitamin D deficiency plays a critical role in the uremia-induced endothelial dysfunction. Importantly, in these patients, active vitamin D (1,25(OH)2D) production is also expected to be markedly low, and therefore, to contribute to the deterioration of vascular health. Vitamin D status, however, is determined by 25(OH)D concentrations since only a fraction of 25(OH)D is converted to its active metabolite 1,25(OH)2D and serum half-life of active vitamin D is 4-6 h. Interestingly, 1,25(OH)2D circulating levels were reduced in our CKD animal model with both standard and vitamin D deficient diet. Our findings suggest that the reduction of active vitamin D production in CKD might be detrimental for physiological vascular function.

In addition to the role of vitamin D, we evaluated the relevance of disturbed levels of α-Klotho as another endothelial protective kidney-related factor. The several endothelial abnormalities observed in α-Klotho mutant mice, such as insufficient endothelium-derived NO formation, impaired angiogenesis and reduced levels of circulatory endothelial progenitor cells, suggest that α-Klotho is essential to maintain vascular homeostasis. Similarly to other CKD models, and patients with CKD, renal damage in our experimental model resulted in low kidney and circulating levels of α-Klotho. In this study, however, we could not confirm that the disturbed concentrations of circulating α-Klotho contributed to the CKD-induced alterations in the endothelial lining. Yet, there is some clinical evidence that associates low concentrations of circulating α-Klotho with the CKD-induced vascular abnormalities. The impact of α-Klotho deficiency on CKD-induced endothelial damage is still unclear and deserves further experimental and clinical research.

Although Chapter 2 does not resolve how CKD induces endothelial dysfunction, it provides evidence for increased permeability and disrupted endothelial cell monolayer. To overcome this limitation in understanding, as a unique approach, in Chapter 3 we characterized the real-time changes of the human endothelial barrier integrity in a CKD setting by using ECIS (electric cell-substrate impedance sensing). We observed that a uremic environment (medium with 20% plasma from pre-dialysis CKD patients) challenged the stability of the basal endothelial function. When stimulating the uremic conditions-exposed endothelial cells with the pro-permeability factor thrombin or after the induction of a wound, the restoration of integrity of the endothelial barrier was further impaired compared to cells exposed to plasma from healthy patients. Our findings indicated that the uremic environment weakens
the integrity of the endothelial barrier making it more sensitive to barrier disruptive conditions. In parallel to our CKD animals (Chapter 2), we observed reduced 1,25(OH)2D levels (42% lower) in the plasma from uremic patients reinforcing the concept that the presence of active vitamin D might be a positive regulator of the stability of the endothelium. Mechanistically, the damage was driven by impaired cell-cell interactions as a result of decreased membrane expression of adherens junction vascular endothelial (VE)-cadherin, essential for maintaining and modulating the endothelial cell-cell contact. We further showed a remodeling of the cortical F-actin cytoskeleton (characteristic of a quiescent endothelium) towards more stress fiber formation leading to a different cell shape and cell-cell contact destabilization. Previous cell-based experiments confirmed that endothelial cells exposed to uremic media undergo changes in the F-actin cytoskeleton by incrementing the actin depolymerizing protein dextrin and a downregulation of annexin A2, essential for the establishment of adherens junction. The hypothesis that CKD induces disrupted cell-cell interactions between endothelial cells is also supported by Zafeiropoulou et al., demonstrating enhanced levels of matrix metalloproteinase-9 (MMP-9), involved in the breakdown of the extracellular matrix, in endothelial cells challenged with plasma from patients prior to a dialysis session. When using plasma from the same patients, but drawn after dialysis, lower levels of MMP-9 expression in the endothelial cells was demonstrated compared to exposure to plasma taken pre-dialysis. Our data, and others, further indicate that uremic media promotes the disassembly of endothelial cell-cell contact.

Changes in cell-cell interactions and in the F-actin cytoskeleton have been also reported in endothelial cells exposed with indoxyl sulphate and p-cresol, two well-known non-covalently protein-bound uremic toxins associated to cardiovascular complications in CKD. The deleterious effects in the endothelium described for both toxins include vascular leakage, oxidative stress and disturbed endothelial-dependent NO signalling in CKD models and cell-based experimental approaches. The mechanism in a CKD setting seems to be mediated by Rho kinase-dependent pathway. When we explored the impact of the combination of these two uremic toxins on endothelial dysfunction in the ECIS set-up as mentioned earlier, we did not observe a spontaneous loss of basal endothelial barrier function. However, recovery after wounding resulted in impaired restoration of endothelial barrier function. This was driven by low cell-cell interactions as previously observed for uremic media exposure. Our data indicates that high levels of the uremic toxins Indoxyl sulphate and p-cresol are important mediators of the deleterious effects induced by CKD, especially in repair capacity following endothelial injury albeit that also other factors may play a role in the development of endothelial dysfunction.

Another important but neglected cause of cardiovascular disease during renal failure, also explored in this dissertation, is myocardial microvascular dysfunction in CKD. In Chapter 4,
microvascular damage in myocardial tissue from uremic (3/4 nephrectomy) and control rats was assessed by measuring the accumulation of N(ε)-Carboxymethyllysine (CML), a major advanced glycation end product (AGE) which formation is mediated by inflammatory mediators or reactive oxygen species (ROS)\(^{46}\). Both in an arteriosclerotic rat model and in patients, it has been demonstrated that CML depositions can be demonstrated in intramyocardial blood vessels after acute myocardial infarction, and this was associated with an activated endothelium and the production of ROS through the NADPH-oxidase NOX2\(^{46-48}\). Interestingly, our uremic animal model displayed enhanced levels of CML in intramyocardial microvascular tissue as well, suggesting that renal failure has pathological consequences for heart microvascular function. In our study, however, no increment of NOX2 was found in intramyocardial arteries of uremic hearts. It is important to consider that uremia-linked AGEs (including CML) are retained in the circulation of CKD patients due to loss of AGE-clearance because of the decline of renal function, besides increased AGE production, and are considered to be potential uremic toxins for the development of cardiovascular complications\(^ {49,50}\). This might be an important cause for the CML accumulation in tissues observed in dialysis patients which also correlated well with cardiovascular outcomes\(^ {51}\). Moreover, elevated CML in serum in patients is associated with increased arterial stiffness and markers of endothelial dysfunction such as von Willenbrand factor (vWF) and soluble vascular adhesion molecule-1 (sVCAM-1)\(^ {52,53}\). Although we did not measure CML serum levels in our uremic model, it is tempting to speculate that CML accumulation in the heart myocardial tissue was a consequence of a decline in renal function. Besides indoxyl sulphate and p-cresol, CML might be an important factor to consider a causative for CKD-induced vascular damage.

Collectively, these data demonstrate that structural alterations in the endothelial lining induced by a uremic environment are mediated by the disturbance of the concentration of several uremia-related factors. By reducing the endothelial cell-cell contact, the constant exposure to the uremic plasma weakens the integrity of the endothelium making it more susceptible to injury. Long-term effects of uremia on the endothelial monolayer may result in cell detachment and enhanced permeability and thereby contributing to the development of clinical cardiovascular comorbidity in patients with CKD. Thus, limiting the concentrations of the uremic toxins and restoring active vitamin D may be of importance for protecting the endothelium. Those effects might be translated into endothelial junction stabilization and the maintenance of endothelial barrier integrity, ultimately improving clinical outcome.

### 1.2 Active vitamin D treatment stabilizes the endothelial barrier function in CKD.

Altered vitamin D metabolism and reduced levels of the active form (1,25(OH)\(_2\)D) are hallmarks of CKD, as described above, and a condition denoting a high risk for cardiovascular complications\(^ {54}\). Endothelial cells not only express the vitamin D receptor but also respond
to 1,25(OH)2D with cell-specific gene regulation and functional effects. Therefore, activation of vitamin D receptor may have a favourable influence on CKD-associated vascular complications. With this background in mind, we explored the potential endothelial-protective effects of the active vitamin D analogue paricalcitol (19-nor-1,25-OH2 vitamin D2) on the structural vascular alterations induced by uremia as described above.

In Chapter 2, the enhanced vascular permeability and disrupted endothelial cell lining observed in aortic tissue in a CKD rat model was attenuated upon paricalcitol treatment. Previous experimental studies already demonstrated a beneficial effect of active vitamin D treatment in uremic rat models by showing an improvement in the uremia-induced endothelial-dependent aortic vasodilation. Besides a direct effect on the vascular endothelium, active vitamin D may induce its vasculoprotective properties during CKD through modulating the concentrations of the components from mineral-bone metabolism (such as α-Klotho), blood pressure (Renin Angiotensin system) and by protecting kidney function. In our study, however, no association between altered α-klotho concentrations and changes in vascular permeability or endothelial cell detachment were found. Despite the downregulation of renin by active vitamin D there is no convincing clinical or experimental evidence that the protective effects of vitamin D therapy are mediated by blood pressure reduction, but it cannot be ruled out that the Renin Angiotensin system is also involved in the development of kidney fibrosis. The degree of renal failure in our study, however, was not affected by active vitamin D supplementation as indicated by urea and creatinine measurements. Encouraged by the hypothesis of a direct effect of active vitamin D on the endothelium, we explored, by using the ECIS technique, the protective properties of paricalcitol against thrombin, as a pro-permeability factor, and the recovery after wounding (simulating an injury) in human endothelial cells. In addition, the vasculoprotective potential of paricalcitol was also tested against the deleterious effects mediated by human uremic media (Chapter 3). In agreement with our animal experiment, paricalcitol improved the recovery of disturbed endothelial permeability after thrombin stimulation as well as after injury-mediated by an electric wound. In a uremic setting, paricalcitol also attenuated the CKD-induced spontaneous loss of endothelial barrier function and its recovery after the induction of a wound confirming that active vitamin D promotes the recovery of the endothelial barrier integrity in different conditions. The hypothesis that active vitamin D is essential for cell proliferation and wound repair is supported by several previous experimental studies that focused on epithelial barrier function. Indeed, epithelial and endothelial cells with impaired vitamin D signalling are characterized by compromised barrier homeostasis and inflammation. Our results suggest that endothelial vitamin D receptor activation also plays an important role in reducing the effects of a CKD-like environment on endothelial barrier function.
The epithelial-protective effects of active vitamin D, mediated by preserving the structural integrity of junctional complexes may be a prominent, but understudied therapeutic property\textsuperscript{64,65}. In human endothelial cell-based experiments, we were able to find enhanced VE-cadherin-positive adherens junctions together with enforced F-actin with a cortical ring-like organization suggesting a more quiescent endothelial cell state in response to the addition of paricalcitol. These structural modifications were critical to attenuate the thrombin-mediated endothelial gap formation. In parallel, we found that the endothelial-protective effects of active vitamin D in uremia-resembling conditions were also mediated by the stabilization of cell-cell interactions. Here, paricalcitol attenuated the impaired VE-cadherin cell-cell contact and cytoskeleton reorganization towards stress fibers formation induced by CKD (Figure 1). The endothelial-protective effects of active vitamin D supplementation might also implicate changes in other junctional proteins such as Occludin and Claudin as reported in an experimental study with hypoxia-induced damage in brain endothelial cells\textsuperscript{7}. That same study demonstrated that these beneficial effects were mediated by reduced expression of MMP-9\textsuperscript{7}. As suggested in this discussion, this mechanism may also be of importance in endothelial damage as seen in clinical CKD; however, further studies are necessary to fully explore the underlying mechanism that mediates disturbed endothelial cell-cell contact.

**Figure 1:** Schematic illustration of the state of endothelial cell-cell contact integrity in different conditions. VE-cadherin-based adherens junctions are represented in green and F-actin in red. In a healthy situation, endothelial cell-cell contact is maintained by a zipper-like VE-cadherin distribution and strengthened by a cortical ring-like F-actin organization. CKD reduces VE-cadherin presence on the cell surface and mediates the formation of F-actin stress fibers driving endothelial cell contraction which results in enhanced endothelial permeability and impaired barrier stability. The addition of active vitamin D in a CKD condition enforces the disrupted cell-cell contacts by rescuing VE-cadherin-based junctions and enhancing the cortical-like F-actin conformation.
Despite the beneficial effects demonstrated in the endothelium, paricalcitol was not effective in reducing the CML depositions in the myocardial microvasculature, as examined in our uremic rat model (Chapter 4). We hypothesized that CML accumulation was a result of uremia-induced damage in the target tissue, and subsequent deposition of CML derived from the circulation. Whether vitamin D protects against the damage of this uremic toxin in the heart and attenuates the impact of CKD in cardiovascular disease will require additional long-term studies.

From a clinical perspective, active vitamin D seems to protect against cardiovascular disease, but the reported effects of vitamin D on outcomes in CKD patients are still controversial. In randomized clinical trials, active vitamin D therapy did not alter left ventricular mass index or improve certain measures of diastolic function in CKD patients (PRIMO and OPERA trial)\(^{66, 67}\). Recently, firmer evidence for the vasculoprotective properties of vitamin D has been found in patients with CKD where active vitamin D treatment improved both vascular function and structure\(^{68-70}\). These recent clinical trials are in good agreement with animal studies, described in this dissertation, which demonstrated that vitamin D protects against the CKD-induced vascular dysfunction and with the preservation of the CKD-induced structural alterations. With our study, we hope to increment awareness about the beneficial effects of active vitamin D treatment in CKD-mediated endothelial barrier breakdown.

PART II – DISRUPTED CELLULAR BARRIERS IN KIDNEY FAILURE: THE PERITONEAL MEMBRANE

2.1 Morphological changes in the peritoneal membrane upon Peritoneal Dialysis are driven by several factors.

The peritoneal membrane provides a protective barrier and frictionless interface for the free movement of adjacent organs and tissues\(^{71}\). It is composed of a single layer of highly specialized mesothelial cells that line a compact zone of connective tissue containing collagen, few fibroblasts, macrophages and vessels\(^{71}\). In end-stage CKD patients, as a renal replacement therapy, peritoneal dialysis (PD) treatment uses the peritoneal membrane as a semi-permeable barrier across which ultrafiltration and diffusion take place\(^{71}\). As a result of long-term exposure to bio-incompatible fluids, there is a conversion of the mesothelial cells towards a more mesenchymal-like phenotype (EMT – epithelial-mesenchymal transition) and an induction of inflammatory mediators which promotes the progressive remodelling of the peritoneum that jeopardizes its functionality as a semipermeable membrane for dialysis\(^{71}\).
In Chapter 5, we describe the specific challenges that the peritoneal membrane undergoes during PD and its subsequent developed fibrosis and angiogenesis. Although the immunological mechanisms underlying the PD-induced peritoneal damage are not well known, by reviewing current literature we describe that immunoregulatory M2 macrophages and interleukin (IL)-17 secreted by T Helper 17 (Th17) cells are likely important mediators of peritoneal inflammation during PD\textsuperscript{72, 73}. We suggest that diminishing the impact of inflammation-mediated peritoneal damage, driven by bio-incompatible PD fluids, may improve PD treatment efficacy over extended periods of time.

In order to study anti-inflammatory therapeutic interventions for the attenuation of peritoneal morphological changes, we used different PD animal models. Similar to the vascular endothelial cell lining, uremia impacts the stability of the peritoneal membrane\textsuperscript{71}. Indeed, we have previously demonstrated that besides the exposure to PD fluids, uremia per se (induced by 5/6 nephrectomy) contributes to the development of peritoneal thickening and remodelling\textsuperscript{74, 75}. Thus, the uremic setting needs to be taken into consideration when studying the PD-related complications in animal models. Therefore we developed, besides a PD exposure model in otherwise healthy animals, uremic PD exposure models, which we used in most of our studies.

2.2 Preserving the peritoneal membrane integrity by targeting the inflammatory response during PD.

Vitamin D receptor activation regulates the proliferation and function of B and T-cells, macrophages and dendritic cells suggesting that active vitamin D treatment may have immunomodulatory functions\textsuperscript{76}. In Chapter 6, active vitamin D paricalcitol reduced the PD-induced peritoneal thickness and angiogenesis after 5 weeks of treatment. Ultrafiltration capacity, declined upon PD fluid exposure, was restored in vitamin D treated animals. As a plausible explanation for the reduced peritoneal remodeling, we found reduced levels of polarized M2 macrophages, also known as alternative macrophages\textsuperscript{77}, in the omentum of vitamin D treated animals. M2 macrophages contribute to the inflammatory process through the release of TGF-β1, VEGF, IL-10 which promotes controlled wound healing and tissue regeneration\textsuperscript{77}. However, the persistence of the inflammatory insult may lead to the exacerbation of fibrosis and chronic inflammation. The presence of M2 macrophages, indeed, has been shown to be of relevance in the development of peritonitis in PD patients\textsuperscript{73}. Importantly, increased concentrations of C-C motif chemokine ligand 18 (CCL18), characteristic of M2 macrophages, found in effluents of PD patients has been associated in the development of peritoneal fibrosis\textsuperscript{78}. The same study confirmed that paricalcitol reduced CCL18 production from cultured patient-derived macrophages reinforcing the fact that active vitamin D may
be an important inhibitor of M2-induced peritoneal remodeling during PD. In addition to the beneficial effects described here by active vitamin D therapy, recent findings have shown that vitamin D receptor activation modulates the activation of regulatory T cells (Treg) and reduces IL-17 peritoneal levels. Cytokine environment is essential for the differentiation of T-cells representing a delicate balance between tolerance and immune response. The continuous exposure to PD fluid of the mesothelial cell lining induces the production of TGF-β1 and IL-6 which might promote Th17 differentiation. Thus, preservation of mesothelial health is crucial to reduce the feed-forward loop in which the mesenchymal conversion of mesothelial cells induces inflammatory mediators and the inflammation promotes the peritoneal remodelling. Given the recent reports that active vitamin D protects the peritoneal mesothelial cells against the high glucose environment-mediated damage, we hypothesize that the protective effects observed by active vitamin D are not limited to modulating the inflammatory milieu but also encompass preserving the mesothelial barrier function integrity.

Blocking IL-17 responses in a mice PD model was shown to diminish peritoneal fibrosis and inflammation, indicating that neutralization of this pathway may be of importance for the preservation of the peritoneal membrane. As a novel potential therapeutic strategy against IL-17 mediated PD damage, in Chapter 7 we demonstrated that the dipeptide Alanyl-Glutamine (Ala-Glu) drastically reduced IL-17 expression in the peritoneal membrane and in peritoneal effluents from mice treated with conventional PD fluid. Those results were accompanied by a decrease of IL-17 mRNA levels in peritoneal membrane from the transcription factor RORγt, responsible for driving Th17-positive cell differentiation and IL-17 expression, and a lower number of CD4+ IL-17+ T cells in peritoneal effluent. As a result, Ala-Glu-supplemented PD fluid attenuated the PD-treatment induced peritoneal fibrosis and angiogenesis, by its impact on IL-17.

As an additional mechanism, heat shock proteins (which protect from maladaptive stress response) have been suggested to be downregulated in glutamine-starved cells under stress conditions. A comparable downregulation has been shown in PD-exposed mesothelial cells suggesting that glutamine supplementation may be of importance in PD fluid for the preservation of the mesothelial integrity. Given this hypothesis, Ala-Glu has been shown to be an effective treatment to counteract the PD-induced mesothelial cell stress by stimulating cytoprotective processes. Importantly, in our PD-exposed uremic models, we found downregulated levels of IL-6 and TGF-β1 which may suggest that mesothelial integrity was better preserved upon PD fluid supplemented with Ala-Glu. Overall, similar to vitamin D, literature and our data suggest that the protective effects of Ala-Glu in the PD-induced injury are not limited to the regulation of IL-17 pathway, but may, in addition, be protective by its virtue of protecting against the PD-mediated mesothelial stress.
Another strategy for preserving the integrity of the peritoneal membrane includes the replacement or reduction of peritoneal damage-related mediators present in conventional PD fluids such as high glucose degradation products (GDPs) or low pH. In this regard, more biocompatible bicarbonate/lactate PD fluids with physiological pH and lower GDPs emerged as a strategy with improved biocompatibility. In vitro studies and small clinical investigations suggest that these biocompatible PD solutions may cause less structural and functional alterations in the peritoneal membrane than standard solutions. Previously, we have observed that exposure to a bicarbonate/lactate solution in a rat PD model leads to a better preservation of peritoneal morphological features. This finding was supported by better preservation of the mesothelial lining and lower vascularisation found in biopsies from patients after chronic exposure to more biocompatible PD solutions. Despite the benefits reported, a more extensive evaluation of the impact from these novel biocompatible PD solutions, especially in the inflammatory milieu, is still needed. Given this limitation and the importance of the Th17 and M2 pathways in peritoneal remodeling, in Chapter 8 we compared the inflammatory environment of a bicarbonate/lactate PD fluid with a conventional PD solution in a uremic mice model. In a good agreement with previous reports, the development of peritoneal fibrosis and angiogenesis was prevented in mice-exposed with a biocompatible solution supporting the notion that bicarbonate/lactate-buffered PD fluid better preserve the peritoneal integrity status. Biocompatible solution-exposed mice also displayed lower CD4+ IL-17+ cell population with no changes in IL-17 concentrations after exposure compared to baseline, together with dominant M1 pro-inflammatory cell population over M2 subset in the peritoneal membrane. These results suggest that an inflammatory process, driven by an influx of M1 macrophages occurs in the biocompatible treated mice while a more fibrotic response is predominant in the conventional treatment. These results help to better understand the differences of inflammatory mediators released after chronic exposure of different PD solutions which might help to design new therapeutic approaches.

Despite the encouraging data demonstrated in experimental models of PD, the benefits of the treatments described above (and summarized in Figure 2) are still not confirmed in a clinical setting with dialysis patients. Recently, the effects of paricalcitol in peritoneal membrane characteristics were compared to active vitamin D calcitriol in a pilot clinical study. Although no differences were found between two treatments suggesting that both vitamin D forms exert the same effect, the absence of a placebo group precluded any conclusion with regard to active vitamin D as compared to no treatment. More promising data in a clinical trial has been shown with the addition of Ala-Glu in patients undergoing peritoneal equilibrium test, as clinical functional test of peritoneal membrane functionality. Moreover, in a good agreement with previous in vitro and in vivo data, Ala-Glu supplementation improved the resistance of the mesothelial barrier against the PD-induced toxicity by...
increasing the peritoneal cell heat shock protein expression. The supplementation of PD solutions with Ala-Glu also resulted in the downregulation of IL-17 down-stream mediators from peritoneal cells isolated from PD effluents from patients, which is consistent with the hypothesis that Ala-Glu mitigates the inflammatory response. Apart from immune-modulation PD fluid supplements like Ala-Glu, the benefits of more biocompatible PD fluids compared on patients treated with conventional solutions are still unclear. Several randomized clinical trials on biocompatibility in PD have been conducted with conflicting results. Recently, a two-year randomized clinical trial found lower peritonitis rate with more preserved ultrafiltration capacity after switching from conventional PD solution to a bicarbonate/lactate solution. There were, however, no changes in content of inflammatory biomarkers in peritoneal dwells. Overall, more clinical trials are required to investigate the effects of different treatments or PD fluids on the inflammatory response upon standard PD fluid exposure in patients. The current study results suggest that additional improvement of current PD treatment is possible.

**Figure 2:** Summary of the relevant effects of the different immunomodulatory interventions in a PD setting reported in this thesis. The illustration represents some of the effects mediated by the PD conventional fluid in the peritoneal membrane described in Chapter V including macrophage M2 and Th17 infiltration in effluent and tissue, release of inflammatory cytokines, peritoneal membrane remodeling: fibrosis, angiogenesis and thickening. The protective effects from the different interventions in the different PD conventional fluid-induced peritoneal alterations are highlighted (Ala-Glu in blue; Biocompatible PD fluid in green: active vitamin D in orange). The additional benefits from these therapeutic strategies described in literature are not included in this illustration. Alternatively activated Macrophages= M2; T helper 17= Th17; Transforming growth factor β1= TGF- β1; Vascular Endothelial Growth Factor= VEGF; Interleukin 6= IL-6 and 17= IL-17.
LIMITATIONS OF THE STUDIES

The work presented in this thesis has a number of limitations. Despite the description of novel pathological consequences of CKD in the endothelial lining, the underlying mechanism proposed in this dissertation has not been confirmed yet. As described here, CKD might induce endothelial dysfunction mediated by several factors. Although we describe here the impact of some of them, more factors need to be taken into account when the implications of the uremic environment in endothelial stability are studied. Specifically, the role of systemic α-Klotho deficiency in the uremia-induced endothelial dysfunction could not be confirmed. We did, however, demonstrate a specific protection by active vitamin D. In vitro, we explored the protective effects of active vitamin D by using human umbilical vein endothelial cells, but not other sources of endothelial cells. Despite this limitation, we confirmed the benefits of active vitamin D treatment by different approaches (thrombin, wounding and uremic conditions). Furthermore, the effects of uremia in ECIS were assessed by using pooled plasma (20%) from a limited number of patients (n=6). Additional studies with larger samples from patients with different CKD stages and correlation with clinical data including concentrations of renal-related factors will provide a better understanding of the impact of kidney damage in endothelial barrier stability.

In the second part, vitamin D studies in the experimental PD setting were not carried out under uremic conditions and cell subsets such as Th17 and M2 were not explored in the effluents. These limitations, however, were addressed by performing studies with Ala-Glu treatment or when comparing conventional and biocompatible fluids. Given the recent reports that underlined the importance of Th17 and M2 cell populations in the development of peritoneal damage during clinical PD, we focused on studying the implications of these inflammatory cells. Dysregulation of Treg and Th17 balance has been shown to control the inflammatory response upon PD exposure. As an additional limitation, when determining the impact of Th17-mediated inflammation, the identification of Treg infiltrating cells should be considered to better evaluate the immune homeostasis state during the PD-induced inflammatory response. Further, determining other cell types such as Th1 and Th2 also might shed light on the local immune cell subset polarization. However, in spite of such limitations, we think that our data add new and interesting information.

GENERAL CONCLUSION AND FUTURE PERSPECTIVE

Altogether, we demonstrated the importance of uremia in driving endothelial and epithelial dysfunction during renal failure. The impaired endothelial barrier stability induced by a uremic plasma results in disruption of the endothelial lining and enhanced permeabili-
ty after long-term exposure. As important humoral factors of this damage, uremic toxins and decreased active vitamin D levels may be important, thus, restoration to the normal concentrations of these components may carry beneficial consequences for vascular health in clinical CKD. Indeed, active vitamin D treatment stabilized the uremia-disrupted cell-cell contacts which resulted in the attenuation of CKD-induced endothelial damage. As an important therapeutic approach for the development of vascular dysfunction and future cardiovascular complications in CKD, improving the endothelial cell-cell contact and barrier stability should be addressed in future studies. Given the controversy about the clinical benefits of active vitamin D in cardiovascular outcomes, in particular myocardial outcome, we hope to revive the interest in vitamin D as an important treatment for vascular dysfunction in CKD patients.

The benefits from active vitamin D treatment together with other therapeutic strategies have been also explored in another barrier which is challenged during CKD; the peritoneal membrane. Our results showed that active vitamin D was effective in preserving the peritoneal integrity during PD by attenuating the development of peritoneal membrane thickening and angiogenesis. As an additional immunomodulatory effect from vitamin D, we found that its protective effects might be mediated by decreased amounts of pro-fibrotic M2 macrophages. Modulating IL-17 concentrations may be of importance to reduce the impact of PD solution exposure as demonstrated by the Ala-Glu supplementation to PD fluids. In addition, we found that changes in Th17 and M2 cell populations are related, at least in part, to a better preservation of the peritoneal integrity in mice exposed to biocompatible PD solution. Our different therapeutic strategies suggest that immunomodulation of IL-17 and M2-mediated chronic inflammation together with a better preservation of the peritoneal membrane integrity should be taken into account in future studies to prevent peritoneal remodeling upon PD treatment. In a clinical setting, active vitamin D and Ala-Glu therapy might be a potential therapeutic approach to be added in PD fluids. The combination of anti-inflammatory treatment with more biocompatible solutions might result in important benefits for peritoneal membrane health in PD patients.
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ENGLISH SUMMARY

Cellular barrier breakdown may drive important pathological effects in different tissues during renal failure. The endothelium, the first cell line exposed to uremic retention molecules in the circulation, undergoes to structural abnormalities which trigger or accelerate cardiovascular disease. Importantly, the peritoneal mesothelial membrane, which acts as a filter during peritoneal dialysis, can also be compromised not only by chronic kidney disease (CKD) itself but also during the long-term exposure of bio-incompatible dialysis fluids. Protecting these two cell barriers in renal failure may carry important beneficial consequences in the cardiovascular health or in the normal functioning of the peritoneum as a dialysing membrane in peritoneal dialysis. In this dissertation, several therapeutic approaches are presented to maintain the integrity of both endothelial barrier and peritoneal membrane in renal failure.

PART I – DISRUPTED CELLULAR BARRIERS IN KIDNEY FAILURE: THE ENDO- THELIUM

As a consequence of the impaired renal function, there is an increment of blood concentrations of uremic retention solutes together with inflammatory and oxidative stress mediators. Importantly, as an early event in CKD, there is a progressive derangement of the levels of hormones involved in mineral metabolism. The kidney produces a key protein termed α-Klotho, involved in regulation of both calcium and phosphate homeostasis and also the synthesis of active vitamin D (1,25(OH)₂D) occurs primarily in the kidney. Both compounds decline already in early stages of CKD, and thereby contribute to remote tissue damage in CKD. Those changes are accompanied by the elevation of the parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) to maintain calcium and phosphorous homeostasis. The first cellular barrier exposed to these non-physiological conditions is the endothelium, and both functional and structural abnormalities in this inner cell lining of blood vessels may arise. Disrupted endothelial barrier may trigger or accelerate cardiovascular disease, inducing an alarmingly high prevalence of morbidity and increased mortality in all stages of CKD. In Chapter 1, the impact of the disturbed concentrations of the above-mentioned renal factors in the endothelial barrier integrity are highlighted. In this regard, the vascular pathological characteristics observed by these renal-related risk factors in experimental uremic animal models or cell cultures resemble the clinical manifestations observed in CKD patients, suggesting that they are important mediators in the development of uremia-induced endothelial dysfunction in these patients. Emerging studies suggest that vitamin D has important indirect effects via traditional and possibly non-traditional vascular disease risk factors as well as direct effects on vascular cells. In Chapter 2, structural abnormalities (vascular permeability and endothelial cell detachment), present in the aortic tissue of our
CKD rat model, were attenuated by active vitamin D therapy. In agreement with our animal experiment, active vitamin D treatment improved in vitro the recovery of disturbed endothelial permeability after thrombin stimulation as well as after injury-mediated by an electric wound suggesting that the beneficial effect from active vitamin D in the CKD in vivo model was a result of a direct effect on endothelial cells. In addition, the vasculoprotective potential of active vitamin D was also confirmed in an in vitro model that exposed the deleterious effects induced by human uremic plasma (Chapter 3). Here, we observed that plasma from patients with CKD induced a decrease of endothelial barrier function by reducing the membrane expression of adherens junction vascular endothelial (VE)-cadherin, essential for modulating the endothelial cell-cell interaction. This deleterious effect on the cell-cell contact was largely prevented by the addition of active vitamin D in the uremic media. Given the endothelial protective effects reported here in this dissertation, we hope to revive the interest in the importance of active vitamin D treatment for vascular dysfunction in CKD patients. Despite this encouraging data, active vitamin D was not effective in reducing of N(ε)-Carboxymethyllysine (CML) depositions in the myocardial microvascular tissue of our CKD rat model (Chapter 4). We hypothesized that the accumulation of CML in the target tissue was mediated by oxidative stress as a result of CKD condition. This feature, however, was not confirmed in our CKD in vivo model. As an alternative hypothesis, the decline of renal function could retain CML into the circulation leading to the accumulation of this toxin into the cardiac microvasculature. In particular, preventing CML depositions could be a therapeutic target for preventing microvascular abnormalities in CKD.

PART II – DISRUPTED CELLULAR BARRIERS IN KIDNEY FAILURE: THE PERITONEAL MEMBRANE

In parallel to the endothelial lining, the peritoneal membrane may also be compromised during CKD. Peritoneal mesothelial cells are specialized epithelial cells that cover the peritoneal cavity. In end-stage kidney disease, renal replacement therapy is required, one widely applied method of which is peritoneal dialysis (PD). The therapy is based on the ability of the peritoneal membrane to function as a dialyzing membrane, when PD fluid is installed into the peritoneal cavity. However, long-term exposure to bio-incompatible fluids promotes progressive remodelling of the peritoneum and induces fibrosis and angiogenesis. Importantly, this state contributes in several ways to peritoneal malfunction during PD fluid exposure. As an underlying mechanism, the production and secretion of profibrotic cytokines by inflammatory cells such as macrophages and T cells may disrupt the normal homeostasis of the peritoneal membrane. Specifically, immunoregulatory M2 macrophages and interleukin (IL)-17 secreted by T Helper 17 (Th17) cells are likely important mediators of peritoneal inflammation during PD and are described in Chapter 5. Given the potential importance of
modulating immune cells function, in order to protect against the development of peritoneal membrane damage during PD, some therapeutic strategies are proposed in this dissertation. In Chapter 6, active vitamin D treatment was effective in reducing PD-induced peritoneal thickening and angiogenesis in vivo. Here, the presence of M2 macrophages, which can lead to exacerbation of fibrosis and chronic inflammation, was reduced in the omentum of PD-exposed animals when treated with active vitamin D. Another therapeutic approach is described in Chapter 7, demonstrating that Alanyl-Glutamine is effective in reducing the IL-17 expression in the peritoneal membrane and in peritoneal effluents from mice treated with conventional PD fluid. The neutralization of this pathway attenuated the PD-induced peritoneal fibrosis and angiogenesis. Finally, in Chapter 8 we confirmed that the application of a bicarbonate/lactate PD fluid better preserved the peritoneal integrity in a mice PD model when compared to animals exposed to conventional PD fluid. The inflammatory environment of the more biocompatible PD fluid-treated mice was characterized by an M1 macrophage subset over M2 and lower CD4+ IL-17+ cell population with no changes in IL-17 concentrations. Overall, modulating the inflammatory response during PD fluid exposure may preserve the integrity of the peritoneal membrane.

CONCLUDING REMARKS

In this thesis two cellular barriers are studied, which both are jeopardized in patients with severe kidney failure. The first is the endothelial cell layer, which is compromised by the uremic milieu itself, and the second is the peritoneal membrane, which can be damaged by peritoneal dialysis. Understanding the underlying mechanisms of these complications of either the disease or its treatment paves the way to specifically address these aspects, that independently contribute to morbidity of patients affected by this chronic condition. Here, active vitamin D supplementation was shown to protect both cellular barriers by reinforcing the cell to cell junctions in the endothelium upon uremic-induced toxicity or by reducing the M2 macrophage population after bio-incompatible PD fluid exposure. Overall, the favourable effects reported here in this thesis are supportive in considering active vitamin D therapy as a preventive strategy of endothelial cell dysfunction and peritoneal remodelling.

As an alternative approach, two additional therapeutic strategies against the peritoneal damage were also evaluated. Given the rising concern about the key role of IL-17 in peritoneal damage during PD, the potential therapeutic effects of Alanyl-Glutamine against the IL-17 mediated peritoneal inflammation represent encouraging data for improving the peritoneal health in PD patients. In addition, we provided a better understanding of the differences between the inflammatory milieu of the long-term exposure of a biocompatible PD fluid compared to a conventional one which may pave the way to develop preventive strategies.
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CURRICULUM VITAE

Marc Vila Cuenca received his Bachelor’s degree in Biochemistry from the University Autonom-ous of Barcelona in 2011. During this study, he performed an internship at the IDIBAPS center in Barcelona where he learned cell biology techniques applied to the protection of the Alzheimer’s disease-induced oxidative stress. Captivated by cell biological mechanisms, he started in 2012 a Master degree in Cell Biology also including an internship at IMIM in Barcelona evaluating genetic alterations through FISH technique in the chromosome 17 from myelodysplastic syndrome patients.

In December 2013, he was selected as Early Stage researcher for the Marie Curie ITN, Eu-TriPD (European Training and Research in Peritoneal Dialysis) and joined the Department of Molecular Cell Biology and Immunology (MCBI) at the VU University Medical Centre in Amsterdam where he started his PhD-programme. He conducted research to protect through anti-inflammatory interventions the peritoneal remodelling in vivo after long-term exposure of peritoneal dialysis fluid. In addition, he investigated the underlying mechanisms driving the disruption of the endothelial lining during chronic kidney disease and the protective effects of active vitamin D in this feature.

Interested in expanding his knowledge in molecular cell biology techniques, in 2018, he worked as a postdoctoral researcher at the Josep Carreras Leukemia Research Institute in Barcelona focusing in the in vitro characterization of genetic mutations driving erythropoi-etetic diseases.

Since January 2019, Marc continues his research trajectory as a postdoctoral researcher at the LUMC in Leiden in the department of Clinical Genetics and in collaboration with the department of Anatomy and Embryology. Combining his experience in vascular dysfunction and background in characterization of genetic alterations, Marc focuses his research in developing advanced in vitro models to further unravel the pathological mechanism of the hereditary cerebrovascular disease CADASIL.
LIST OF PUBLICATIONS


Amsterdam Cardiovascular Sciences