Chapter 10

Summary and General Discussion
Approximately 200,000 end stage renal disease patients worldwide depend on peritoneal dialysis (PD) as a life-saving treatment. Although the general perception is that PD prevalence is declining, a global trend shows the opposite, in both developed and developing countries. However, the proportion PD relative to total dialysis patients is declining in developed countries except for a few, such as Hong Kong and Mexico, where PD is the first choice in therapy. PD has several advantages over haemodialysis, like being home based, lower costs, increased mobility, and slightly better survival rates in the first 1-2 years upon dialysis. Conversely, some disadvantages can be identified too, such as an increased risk of peritonitis and premature discontinuation of the treatment due to peritoneal remodeling resulting in technique failure. Detailed knowledge of pathological changes induced by PD may lead to novel therapeutic approaches, improving outcomes for patients treated with this technique (Chapter 1). Therefore, in this thesis I aimed to unravel different aspects of PD, PD induced changes, interventions and related studies.

**Angiogenesis in peritoneal dialysis**

Angiogenesis results in a rapid loss of the glucose-driven osmotic gradient of peritoneal dialysis fluid, therefore it is contributing to ultrafiltration failure. Moreover, PD induced vascular wall changes, such as increased permeability and thus increases small solute transport, also contribute to ultrafiltration failure. Different factors associated with PD, such as the presence of the PD-catheter itself, uremia, episodes of peritonitis, glucose, and GDPs, stimulate angiogenesis. In chapter 2, several interventions like COX-2 inhibitor and BMP-7, a TGFβ1 antagonist, as tested in a rat PD model are reviewed. In addition, new data on the prevention of new vessel formation using the drug sunitinib, a tyrosine kinase inhibitor, in our rat PD model are described. Five-week treatment with sunitinib prevented angiogenesis in the omentum as well as the mesentery, although PD-induced VEGF levels were further increased by sunitinib treatment. The increase of VEGF levels could be explained by a lack of degradation of VEGF due to blockage of VEGF receptor signalling. A reduction of mast cells, which are thought to play an important role in angiogenesis, was observed upon sunitinib treatment. Although sunitinib seems promising in our rat model, well-randomised clinical trials showing a reduction in angiogenesis and ultrafiltration failure are lacking and reported side-effects such as hypertension when administered to patients with cancer, must be taken into account when considering this option as future intervention for patients on PD.
Vitamin D deficiency and suppletion

Vitamin D is not only involved in mineral homeostasis but has also reported to be important in fibrosis, inflammation and angiogenesis, all processes involved in peritoneal membrane remodelling. Therefore, vitamin D might have a positive effect on the preservation of the peritoneal membrane. To study the effects of vitamin D in PD, animal models are useful. However, current models face several limitations including time required to induce a vitamin D deficient state, which takes approximately six weeks if for this purpose a vitamin D deficient diet is used. More importantly fluctuations in circulating PTH, calcium, and phosphate levels occur due to the vitamin D deficiency, impending result interpretation. Therefore, we developed a novel vitamin D deficient rat model, based on an established mice model (chapter 3). Wistar rats received three intraperitoneal injections of paricalcitol per week for two weeks and a calcium-enriched vitamin D depleted diet during the whole study. After three weeks both 1,25D and 25D serum levels dramatically dropped, with most values below detection limits, whereas changes in PTH, calcium and phosphate stayed within the normal range.

In chapter 4 the newly developed model described in chapter 3, alongside 1α-OHase knockout mice, was used to test the hypothesis that 1,25D deficiency itself can cause renal injury and if vitamin D treatment could reverse/prevent this injury. Both acquired and hereditary vitamin D deficiency led to reversible glomerular injury and albuminuria, as well as tubulointersitial damage. Six-week treatment with calcitriol or paricalcitol in 1α-OHase knockout mice normalized albuminuria and urinary IgG to wild type levels. NGAL excretion was reduced completely by calcitriol and partly by paricalcitol. In addition, the observed partial foot processes effacement in 1α-OHase knockout mice was reversed in animals receiving either VDRA. Moreover, glomerular injury markers desmin and TRPC6 which were increased in 1α-OHase knockout mice were reduced upon either calcitriol or paricalcitol treatment. Also in acquired vitamin D deficiency in rat, paricalcitol and calcitriol treatment could, partly, prevent albuminuria, increased urinary IgG and NGAL excretion, and upregulation of desmin and TRPC6.

In both chapter 5 and 6 the effect of VDRA on peritoneal remodelling is examined, however, in using a different experimental set up. In chapter 5 we studied the effect of paricalcitol on ultrafiltration, fibrosis and angiogenesis in a non-uremic, vitamin D sufficient, rat PD model. We
showed worsening of ultrafiltration capacity, elevation in inflammation markers, partly increased vascular surface area, and a higher number of cells undergoing epithelial to mesenchymal transition in the animals exposed to PDF, compared to the control situation. Loss of ultrafiltration capacity, increase in ECM thickness, angiogenesis and IL5 levels due to PDF exposure were significantly attenuated by paricalcitol treatment. Moreover, a trend towards decreased glucose absorption, lower HA, TGFβ, VEGF, IL12p70 and IL4 levels, and less ED2 positive macrophage accumulation in the omentum and mesentery was observed in PDF exposed animals upon paricalcitol treatment. Yet, not all factors involved in peritoneal remodeling upon PD, such as total cell number and epithelial to mesenchymal transition, were affected by paricalcitol treatment.

Chapter 6 is a follow up of chapter 3 and 5, and describes results of the comparison between paricalcitol and calcitriol in a vitamin D deficient PD rat model. PDF exposure induced an increase in peritoneal cell numbers, inflammation markers and mesenteric angiogenesis. Moreover, it tended to increase liver mesothelial cell regeneration. However, in this experimental setup, no changes in ECM or ultrafiltration were induced by PD treatment, precluding the possibility to assess protection from active vitamin D intervention. Importantly, those parameters that were changed due to PDF exposure were not modified by either paricalcitol nor by calcitriol.

**Clinical interventions**

Potential clinical pharmacological interventions aimed at the regression of peritoneal damage and prolongation of PD treatment are reviewed in chapter 6. As described, many processes can influence peritoneal remodelling as it includes inflammation parameters, angiogenesis and thickening of the peritoneal membrane. Each process could be a pharmacological target aiming to prevent technique failure. Drugs are described for the following targets: I chronic inflammation, like oral disodium cromoglycate; II AGEs, such as rosiglitazone, benfotiamine, aminoguadine, alagebrum, zopolrestat and pyridoximene; III angiotensin II, for example ACE inhibitors captopril and enalapril and ARB losartan; IV fibrinolytic systems, like simvastatin; V prostaglandins, such as celecoxib and indomethacin; and VI others for example vitamin D, bone morphogenic protein 7 and sunitinib. Most studies are described in animals but well-performed, long-term clinical trials are lacking. However, some drugs might potentially partly reverse the
deleterious effects of long-term PD exposure and therefore might have important clinical value.

In Chapter 8 the outline of a multicenter open-label randomized clinical trial examining the effect of paricalcitol versus calcitriol on peritoneal remodelling in PD patients. Since this study was set up as a pilot study the aim was to obtain preliminary data on clinical important parameters to justify or not the set-up of a larger prospective clinical trial with the aim to test differences in PD-technique survival between patients treated with paricalcitol or calcitriol. We selected four clinical important outcomes; 1) peritoneal transport parameters including ultrafiltration 2) metabolic parameters 3) peritoneal effluent markers 4) peritoneal cells. Eligible subjects were randomized to receive either 1) an oral single-dose paricalcitol capsule of 1 ug/day or 2) an oral single dose calcitriol capsule of 0.5 ug/day for six months. For ethical reasons no patients received a placebo, so the design excluded the possibility to assess the effect of active vitamin D compared to a placebo.

Before the start the study, active vitamin D treatment was stopped for a wash out period of 6 weeks in all subjects. Furthermore, all patients switched to low Ca (1.25 mmol/l) and neutral pH fluids (Balance® or Physioneal®). Data was collected at 0 (baseline), 6, 12, 18, 24 weeks for blood, urine and 24-hour dialysate. A PET was performed, and effluent was recovered, at baseline, 12 and 24 weeks. In total 27 patients were included in the study, of which 14 received paricalcitol- and 13 calcitriol treatment.

**Macrophages**

Studies have shown that macrophages play an important role in fibrosis whereby M2 macrophages correlate with fibrosis in sclerotic skin, pulmonary- and kidney fibrosis. Furthermore, there is accumulating evidence that M2 macrophages are involved in peritoneal fibrosis in PD. Distinctive markers for M1 and M2 macrophages are not known, yet (chapter 9). In addition, different protocols to culture M1 or M2 in vitro are used by various research groups. Therefore, in chapter eight macrophages, grown in vitro under different maturation (NHS, M-CSF, and GM-CSF) and activation (IFN-γ/LPS, IL4, IL10, and dexamethason) protocols, were characterized by comparing macrophage morphology, cytokine production and marker expression. Macrophage morphology was different under the various conditions. IFN-γ/LPS stimulated macrophages appeared
more elongated, whereas IL-4, dexamethason and IL-10 looked more circular. Activation with pro-inflammatory activators IFN-γ/LPS resulted in a significant upregulation of CD40, CD64 and pro-inflammatory cytokines. On the other hand, anti-inflammatory activators, IL-4 and dexamethason, increased MR or CD163 expression respectively. Although not 100% distinctive, CD40 and CD64 expression could be used as indicators for M1, and MR and CD163 expression for M2 identification.

As described by Xu et al. peritoneal macrophages, from PD patients, are characterized by CD163 expression similar to M2 macrophages cultured in vitro, which could possibly be explained by a 2.5 fold higher M-CSF concentration in peritoneal fluid compared to plasma [319].

**Conclusion and future prospective**

In this thesis a bench-to-bedside research approach is described of the effect of VDRAs in kidney disease, with the focus on peritoneal remodelling upon peritoneal dialysis. As known from literature vitamin D is more than a hormone involved in regulating bone metabolism and calcium homeostasis. It also plays a role in the regulation of inflammation, fibrosis, angiogeneis, cell growth, differentiation and apoptosis [47;179]. The latter are all occurring processes during peritoneal dialysis and peritoneal remodelling, and contributing to ultrafiltration failure [63;320]. Therefore, vitamin D deficiency, being highly prevalent in PD patients, may contribute to the pathological changes seen in the peritoneum in patients.

Fibrosis leads to increased barrier resistance and therefore deteriorated ultrafiltration. Increase in vascularisation generates an enhanced loss of osmotic gradient by increased glucose uptake from the PDF. Immune cells, but also EMT can facilitate angiogenesis and thickening of the ECM layer as described in chapter 2.

The effect of paricalcitol on ultrafiltration, inflammation markers, peritoneal cells, ECM, EMT, and angiogenesis were therefore studied in chapter 5A. Despite not totally consistent results from data gathered after five and seven weeks of treatment, promising results regarding ultrafiltration, angiogenesis and ECM thickness were obtained. To continue studying the effect of different VDRAs on peritoneal function and morphology, we developed a new vitamin D and calcitriol rat model to represent the poor vitamin D status in PD patients. The model did not show major
fluctuations in calcium phosphate and PTH levels. However, since we had a short follow-up time it is useful to always monitor the maintenance of calcium, phosphate, and PTH levels to avoid misinterpretations.

In chapter 4, we showed that acquired vitamin D deficiency leads to glomerular and tubulointerstitial injury and proteinuria. VDRA treatment can, partly, prevent/restore this which moreover shows the pluripotent effects of vitamin D. This evidence favours an early start of vitamin D treatment when 1,25D and 25D levels, as in kidney disease patients, drop. In chapter 6 the model is used to study the effect of VDRA, in a vitamin D deficient environment, on peritoneal remodelling. Due to collaborations with other groups we have preliminary data on the effect of vitamin D deficiency on bone morphology. It would be of great value if more consequences of vitamin D deficiency, like the effect on the cardiovascular system, in the model are studied in depth. Despite the possibilities of this model are not fully explored, yet, I think the vitamin D deficient model is valuable tool for research to investigate the sole effects of both 1,25D as 25D deficiencies because no major fluctuations in calcium, phosphate and PTH levels are observed. Moreover, it is a relatively quick and cheap model to use.

In chapter 6 we used the vitamin D deficient model to study effect of paricalcitol/calcitriol on peritoneal remodelling whether or not in combination with PDF exposure. In contrast to chapter 5, where vitamin D sufficient animals were used, we used the vitamin D deficient model to represent the low vitamin D status of CKD patients. A subsequent study could bring the model even more close to human PD by combining vitamin D deficiency with uraemia. This approach would bring the potentially toxic effects of uraemia on the peritoneal membrane into the model. As discussed in chapter 6, the PDF exposure model did not show differences between the PDF exposed and control groups in ultrafiltration and PDF exposure. Therefore, we cannot conclude VDRA does not have a positive effect on peritoneal remodelling and peritoneal function. This is in contrast to the observed results in chapter 5, in which we show better ultrafiltration in the PDF exposed groups treated with paricalcitol, probably due to a less increased EMC and less angiogenesis. Moreover, in mice it’s also observed that paricalcitol treatment reduces fibroses and angiogenesis and, partly, prevents ultrafiltration failure \[186\]. However, in chapter 6 we did observe an increase in cell numbers, HA, IL10, TGFβ, VEGF and mesenteric angiogenesis. Also in these parameters we did not observe an effect of active vitamin D treatment. In contrast to the animals in chapter 5, the rats used in 6 were also 25D deficient and we did not restore 25D levels.
To examine if 25D is necessary to stimulate the positive effect of active vitamin D treatment, an intervention study whereby 25D levels will be restored and active vitamin D treatment is given could be performed.

In chapter 8 the study outline for a pilot randomized clinical trial presented. We aimed to investigate the influence of paricalcitol versus calcitriol in four major clinical outcomes: 1) peritoneal transport parameters including ultrafiltration 2) metabolic parameters 3) peritoneal effluent markers 4) peritoneal cells. Due to ethical reasons, no patient group without receiving VDRAs was added to the study. Unfortunately, therefore we couldn’t investigate whether or not VDRA influenced the studied parameters positively compared to non-VDRA treatment. Twenty-seven patients have been included in the study and data analysis is expected to be finalized in the second-third quartile of 2015. The results will justify or not a larger study set up prospective clinical trial with the aim to test differences in PD-technique survival between patients treated with paricalcitol or calcitriol. Remarkably, no episodes of peritonitis were observed (in the 27 included patients) during the six-month study. Kerschbaum et al. showed prolonged peritonitis-free episodes upon oral active vitamin D treatment (median 16.3 months compared to 5.5 months in patients who didn’t receive VDRA therapy) [321].

To conclude: in the different studies we found arguments in favour of using active vitamin D treatment (improvement of ultrafiltration, and reduction of ECM thickness and partly reduced angiogenesis), however, we also had results that were not supportive for the use of active vitamin D: the treatment didn’t influence the PD-induced effects like angiogenesis and effluent marker concentrations, in full vitamin D deficient animals. We also did not observe any differential effects of paricalcitol versus calcitriol, although we are still awaiting the analysis of the clinical study. Even though, we found contradictory arguments for the use of active vitamin D treatment on functional and morphological changes due to PD, the use of active vitamin D treatment might be beneficial for PD patients for other reasons like all cause mortality since a meta-analysis by Zheng et al. suggest a benefit for vitamin D supplementation on all cause mortality and cardiovascular mortality, also in patients with chronic kidney disease [120;121]. Therefore, longer follow up studies and preferably 25D, 1,25D combinational studies looking at different aspects of PD would be wishful.

Moreover, another treatment strategy could be taken whereby treatment regimes could
be combined. A lot of progress has been made regarding the insight of molecular and immunological processes involved in peritoneal remodelling. Biocompatible dialysis fluids have been developed, although their benefit over conventional fluids has been discussed [322]. Nevertheless, the development of less harmful PD fluids is continued. Promising results have been made regarding the addition of alanyl-glutamine (Ala-Glu) to PDF in vitro [323] as well as in vitro in a mouse model for PD (pending revisions to Kidney International, Ferrantelli et al.). These new PD fluids could possibly be combined with vitamin D treatment to prevent/reverse damage to the peritoneal membrane, and thereby limit PD technique failure which would make the treatment option more attractive for patients with chronic kidney disease.