Chapter 10

Summary and Conclusions

Summary

Peritoneal dialysis (PD) is a life saving renal replacement therapy with several advantages compared to haemodialysis (HD) such as increased mobility of patients and better patient survival in the first years. Moreover, it is more cost effective compared to the predominantly hospital based HD[1]. Recent studies suggest that major improvements in patient outcomes in HD are not to be expected using standard three times weekly therapy with current dialysis membranes and therapy regimes[2;3]. In contrast, PD offers many opportunities for improvement. The main bottleneck in PD is, besides peritonitis, the loss of ultrafiltration after long-time treatment as a result of structural changes of the peritoneal membrane.

In **chapter 1** of this thesis, factors contributing to these peritoneal changes including uraemia, peritonitis, volume loading, the presence of a catheter and the PD fluid itself, are described. These factors initiate recruitment and activation of peritoneal cells such as macrophages and mast cells, as well as activation of peritoneal cells including mesothelial cells, fibroblasts and endothelial cells. An overview of cytokines, growth factors and other mediators involved in PD-associated changes is given. Activation of downstream pathways of cellular modulators can induce peritoneal tissue remodelling leading to ultrafiltration loss.

Identification of molecular pathways, and cells and cytokines involved in the development of angiogenesis, fibrosis and membrane failure, may lead to innovative therapeutic strategies which can protect the peritoneal membrane from the consequences of long-term PD.

In the first part of this thesis, the effects of several novel therapeutic interventions are described focussing on the main processes involved in peritoneal membrane damage such as angiogenesis, fibrosis and inflammation.

We studied the effects of heparin on peritoneal tissue remodelling in our PD rat model in **chapter 2**. There are many reports of heparins having other effects beyond anticoagulation, such as immunomodulatory and anti-inflammatory activities, including the binding of cytokines, chemokines and growth factors[4]. However, the effects of heparin administration during PD have been contradictory. Therefore we performed a long term study to elucidate the effects of unfractionated heparin (UFH) as well as well as low molecular weight heparin (LMWH) in our rat PD model on peritoneal tissue remodelling and transport. Both heparins only slightly reduced inflammation and showed to have no beneficial effect on the preservation of the peritoneal membranes (new vessel formation, fibrosis, macrophage influx or epithelial to mesenchymal transition (EMT) of the mesothelial cells), and did not lead

to improved transport parameters. An editorial referring to our paper shows the value of this study for the understanding of chronic heparin use in PD patients.

In **chapter 3** we focussed on the prevention of PD fluid induced EMT and fibrosis during long term treatment. It has been shown that bone morphogenic protein-7 (BMP-7) antagonizes transforming growth factor beta (TGFβ), prevents EMT and can protect against fibrosis[5;6]. In this chapter we showed in vitro that BMP-7 is constitutively expressed by mesothelial cells and down-regulated during EMT. TGFβ-induced EMT in mesothelial cell cultures could be prevented by BMP-7. *In vivo*, addition of BMP-7 to the PDF resulted in a reduced number of trans-differentiated mesothelial cells in the compact zone of the parietal peritoneum as well as in prevention of fibrosis. Furthermore, a reduction in blood vessel formation was seen upon BMP-7 treatment, which correlated to the number of trans-differentiated mesothelial cells, thereby suggesting that EMT is not only involved in fibrosis but also plays a role in angiogenesis.

In **chapter 4**, instead of focussing on fibrosis and EMT, we shifted the focus to the reduction of inflammation and angiogenesis. The cyclooxygenase 2 (cox-2) pathway stimulates angiogenesis and induces prostaglandin synthesis which is involved in inflammatory processes[7;8]. By inhibiting this pathway, Celecoxib prevents the induction of growth factors and cytokines under inflammatory and proliferative conditions.
Interestingly, in our rat PD model inflammatory markers and peritoneal inflammatory cells were not affected by Celecoxib treatment. Angiogenic processes and lymphatic vessel formation, on the other hand, were completely prevented and even a reduction in fibrosis was seen after cox-2 inhibition. The improved morphological appearance of the peritoneal membrane resulted in a preserved ultrafiltration capacity. Since Celecoxib showed different effects we could not identify one single mechanism of action, although the anti-fibrotic and (lymph-) angiostatic properties might mediate its relevant effects.

To investigate whether ultrafiltration failure is directly linked to angiogenesis, we thereafter studied the effects of oral sunitinib in **chapter 5.** Sunitinib is a tyrosine kinase inhibitor, involved in the inhibition of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF)[9;10], growth factors which are both involved in blood vessel formation.
Indeed 5 week treatment with sunitinib in our rat PD model effectively prevented PD induced vessel formation. However, the unaltered ultrafiltration in the PD and

PD+sunitinib group indicates that ultrafiltration loss is not a direct result of increased vascular surface area. Furthermore, sunitinib induced several severe side effects such as weight loss, diarrhoea and overall discomfort, making this treatment difficult to use in clinical settings.

In **chapter 6** we studied the effects of a vitamin D receptor activator (paricalcitol) on peritoneal tissue remodelling. Due to renal failure, PD patients suffer from low vitamin D_3 levels, resulting in a disturbed calcium and phosphate balance. These patients are therefore treated with vitamin D analogues. Long-time experience with the vitamin D_3 analogue paricalcitol has shown that side effects are not to be expected. Since vitamin D_3 has shown to have renoprotective and immunomodulatory properties[11;12], it may also play an important role in the prevention of PD induced peritoneal membrane changes. In chapter 6 we show that paricalcitol preserves the peritoneal membrane by preventing fibrosis as well as neo-angiogenesis in the omentum. Reduced hyaluronic acid levels indicate anti-inflammatory effects of paricalcitol in a PD setting. Altogether these changes prevented loss of ultrafiltration capacity, which is one of the most important complications of PD therapy.

Besides the effects of PDF-induced peritoneal membrane changes on ultrafiltration, the effects on local defence mehanisms are important as well. We therefore studied via videomicroscopy the leukocyte-endothelium interaction upon exposure to different PDF in rats in **chapter 7**. During peritonitis, neutrophils are recruited to the inflammation site by rolling along the endothelium, adhesion and transmigration though the vessel walls[13;14]. In chapter 7 we show that the initiation of neutrophil transmigration during inflammatory conditions is related to the bio(in)compatibility of the PDF. Over time, the degree of PDF induced neo-angiogenesis determines the degree of transmigrating neutrophils through newly immature or activated vessels. We show substantial endothelial activation by conventional PDF wile the newer biocompatible solution resulted in much less endothelial activation and leukocyte transmigration. Therefore we suggest that neutrophil number in peritonitis dialysates can be interpreted as an indirect marker of peritoneal inflammation/activation after chronic exposure to PDF.

Although in rat PD studies peritoneal tissues can be evaluated in detail, this is very difficult in clinical PD. Therefore, dialysate biomarkers that predict outcome would be of great help. By means of micro array analysis (**chapter 8**) and proteomics techniques (**chapter 9**) we try to identify such new biomarkers.
First of all we performed a micro array analysis on RNA taken from the parietal

peritoneum of rats treated for eight weeks with 2 different PD solutions. The microarray analysis showed an impaired immunity and defence pathway for conventional PDF treated rats, but not animals treated with a more biocompatible solution. Analysis of the genes within this pathway showed neutrophil gelatinase associated lipocalin 2 (NGAL) to be a marker for mesothelial damage, which was confirmed by *in vitro* mesothelial cell cultures.
NGAL seems to be involved in the maintenance of the mesothelial phenotype and is expected to prevent EMT. Unfortunately, due to the fact that not only mesothelial cells but also neutrophils are able to produce NGAL, the neutrophils present in the effluents of the rats make it difficult to detect mesothelial damage by measuring NGAL in the dialysates. It is speculated that in the future NGAL may offer promising diagnostic and therapeutic possibilities in the prevention of EMT and further research on exogenously administration of NGAL during PD is warranted.

In **chapter 9** we show our first steps into defining new biomarkers using proteomics techniques. Effluents of patients using either conventional PDF or biocompatible PDF were collected and analysed with LC-MS. Our data show that conventional PDF treatment induces matrix proteins, resulting from enhanced fibrosis. Treatment with a biocompatible solution, on the other hand, enhances anti-inflammatory and anti-angiogenic proteins, indicating that, in line with the results of the micro-array study, biocompatible PDF better preserves peritoneal immunity and defence compared to conventional PDF treatment. Enhanced levels of tissue factor in effluents of patients treated with a biocompatible solution may indicate a better defence mechanism upon vascular damage and prevention of vascular leakage. Tissue factor is therefore seen as an interesting biomarker for further study.

All together, the results described in this thesis lead to the following conclusions and future perspectives.

Conclusions and future directions

It is known that long-term exposure to PDF induces peritoneal damage such as new vessel formation and thickening of the peritoneal membranes. Currently it is thought that these structural changes are mediated by inflammatory processes which lead to a loss of ultrafiltration capacity[15;16]. However, in chapter 2 we have shown that the anti-inflammatory properties of Heparins could only slightly prevent inflammatory parameters, whereas no improvement in peritoneal membrane changes or ultrafiltration was observed.
Cox-2 inhibition in chapter 4 did not prevent inflammation, although in this study (lymp-) angiogenesis and fibrosis were impaired and the ultrafiltration capacity was preserved. These data show that structural changes during PD can be prevented despite pro-inflammatory conditions and the role of inflammation in tissue remodelling seems less significant than previously thought.
Besides inflammatory processes, mesothelial cells are thought to play an active role in tissue damage and failure of the peritoneal membranes[15;17]. Together with the results from chapter 3 in which mesothelial EMT was prevented by BMP-7 treatment, we can indeed conclude that mesothelial EMT plays an important role in tissue fibrosis and peritoneal membrane deterioration.
Nevertheless, a large effective peritoneal surface area is still seen as one of the most frequent reasons for ultrafiltration failure[18]. In chapter 5 we showed however, that under PD conditions new vessel formation itself does not automatically lead to a loss of ultrafiltration. In line with this, prevention of angiogenesis by a tyrosine kinase inhibitor did not improve the PD impaired ultrafiltration. It is therefore assumed that the ultrafiltration capacity of the peritoneal membrane is influenced by a combination of vascular surface area and submesothelial fibrosis as well as glucose absorption, lymphatic absorption rate and Aquaporin-1 (Aqp-1) water channels[19;20]. Animal studies conducted in Aqp-1 knockout mice have shown the role of Aqp-1 in ultrafiltration, and demonstrated that the osmotically driven water transport across the peritoneal membrane was significantly decreased in Aqp-1 knockout mice[21]. These findings suggest that modulating Aqp-1 expression in the peritoneal membrane could be used to prevent ultrafiltration failure[22].

The combination of prevention of angiogenesis, inflammation and fibrosis in chapter 6 by activation of the vitamin D_3 receptor, indeed prevented loss of ultrafiltration capacity. Although inflammatory markers such as hyaluronic acid were decreased, an increase in peritoneal cell numbers was found in this study. The increase in peritoneal cell numbers is often correlated to inflammation and worsening of

peritoneal performance. However, we observed that the peritoneal membranes were preserved despite the increased cell numbers. This trend has also been seen in previous animal studies[23;24]. We therefore speculate that cell type (alternative or classical activated macrophages) more than cell numbers reflect the condition of the peritoneum.

Concluding from our therapeutic intervention studies described in chapters 2-6, we have shown that maintaining ultrafiltration capacity is a complicated process and is dependent on a combination of new vessel formation and fibrosis and to a lesser extent inflammation. We have shown that both Celecoxib and Paricalcitol are able to prevent several of these aspects and lead to a sustained ultrafiltration. We propose that Celecoxib and Paricalcitol are promising candidates for clinical intervention trials and therefore pilot studies are currently set up.
However, when performing studies with Celecoxib, special caution must be paid to cardiovascular safety, since other cox-2 inhibitors like Rofecoxib and Valdecoxib have been associated with cardiovascular toxicity in some studies[25;26]. Due to the possible cardiovascular risks with cox-2 inhibitors, we assume that interventions with the vitamin D receptor activator Paricalcitol will be the most promising strategy for the near future. Besides prevention of PDF induced peritoneal changes, Paricalcitol is also involved in the maintenance of the calcium-phosphate balance and the suppression of PTH[27;28].

Animal and *in vitro* studies are currently set up to further investigate the protective mechanisms of action behind Paricalcitol in the presence of endogenous vitamin D and, more importantly, in a vitamin D deficient environment. Interestingly, in our rat study we observed that paricalcitol treatment enhanced peritoneal cell numbers (mainly macrophages). Future *in vitro* and *in vivo* experiments are therefore directed at the different types of macrophages found in the effluents. The balance between M1 (classically activated) and M2 (alternatively activated) macrophages can be of importance for peritoneal outcome. The hypothesis is that the balance between M1 and M2 macrophages is under the influence of vitamin D receptor activation, and thus in our study positively influenced by Paricalcitol. Reduction in M2 macrophages and an increase in M1 macrophages may be beneficial and is expected to reduce EMT of the mesothelial cells.
Furthermore, future research in animal and human PD studies will focus on whether the prevention of peritoneal worsening upon PDF exposure is only related to the vitamin D analogue Paricalcitol or also to Calcitriol.

Not only therapeutic interventions are important in the preservation of the peritoneal membranes, also the detection of new biomarkers reflecting the status of the peritoneum, can contribute to a pro-longed time on PD therapy. If the status of the peritoneum is known, more specific treatment can be applied and therefore extended research on new biomarkers using proteomic techniques and microarray analysis is necessary in the near future. Further development of microarray and proteomic approaches in PD effluents will provide more information on the complicated processes during PD. These techniques may result in the discovery of new biomarkers which can predict peritoneal worsening or lead to new therapeutic interventions.

In conclusion, this thesis shows that the maintenance of ultrafiltration and the prevention of peritoneal worsening as a result of chronic PD treatment are complicated processes but can, in part, be prevented by therapeutic intervention. The development of new biomarkers can contribute to early detection of membrane failure and lead to a more specific treatment of the patient. Currently, we assume that vitamin D activation by oral Paricalcitol administration is one of the most promising strategies for future treatment to prevent peritoneal membrane loss during chronic PD.

References

1. Collins AJ, Foley RN, Herzog C et al. United States Renal Data System 2008 Annual Data Report. Am J Kidney Dis 2009; 53: S1-374.
2. Rocco MV, Cheung AK, Greene T, Eknoyan G. The HEMO Study: applicability and generalizability. Nephrol Dial Transplant 2005; 20: 278-284.
3. Eknoyan G, Beck GJ, Cheung AK et al. Effect of dialysis dose and membrane flux in maintenance hemodialysis. N Engl J Med 2002; 347: 2010-2019.
4. Casu B, Lindahl U. Structure and biological interactions of heparin and heparan sulfate. Adv Carbohydr Chem Biochem 2001; 57: 159-206.
5. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 2003; 112: 1776-1784.
6. Zeisberg M, Hanai J, Sugimoto H et al. BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 2003; 9: 964-968.
7. Daniel TO, Liu H, Morrow JD, Crews BC, Marnett LJ. Thromboxane A2 is a mediator of cyclooxygenase-2-dependent endothelial migration and angiogenesis. Cancer Res 1999; 59: 4574-4577.
8. Inoue H, Takamori M, Shimoyama Y, Ishibashi H, Yamamoto S, Koshihara Y. Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts. Br J Pharmacol 2002; 136: 287-295.
9. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004; 25: 581-611.
10. Erber R, Thurnher A, Katsen AD et al. Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. FASEB J 2004; 18: 338-340.
11. May E, Asadullah K, Zugel U. Immunoregulation through 1,25-dihydroxyvitamin D3 and its analogs. Curr Drug Targets Inflamm Allergy 2004; 3: 377-393.
12. Zehnder D, Quinkler M, Eardley KS et al. Reduction of the vitamin D hormonal system in kidney disease is associated with increased renal inflammation. Kidney Int 2008; 74: 1343-1353.
13. Vestweber D, Blanks JE. Mechanisms that regulate the function of the selectins and their ligands. Physiol Rev 1999; 79: 181-213.
14. Zarbock A, Ley K. Mechanisms and consequences of neutrophil interaction with the endothelium. Am J Pathol 2008; 172: 1-7.
15. Yanez-Mo M, Lara-Pezzi E, Selgas R et al. Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. N Engl J Med 2003; 348: 403-413.
16. Flessner MF. Sterile solutions and peritoneal inflammation. Contrib Nephrol 2006; 150: 156-165.
17. Lee HB, Ha H. Mechanisms of epithelial-mesenchymal transition of peritoneal mesothelial cells during peritoneal dialysis. J Korean Med Sci 2007; 22: 943-945.
18. Fusshoeller A. Histomorphological and functional changes of the peritoneal membrane during long-term peritoneal dialysis. Pediatr Nephrol 2008; 23: 19-25.
19. Leypoldt JK. Solute transport across the peritoneal membrane. J Am Soc Nephrol 2002; 13 Suppl 1: S84-S91.
20. Rippe B, Venturoli D. Simulations of osmotic ultrafiltration failure in CAPD using a serial three-pore membrane/fiber matrix model. Am J Physiol Renal Physiol 2007; 292: F1035-F1043.
21. Yang B, Folkesson HG, Yang J, Matthay MA, Ma T, Verkman AS. Reduced osmotic water permeability of the peritoneal barrier in aquaporin-1 knockout mice. Am J Physiol 1999; 276: C76-C81.
22. Devuyst O, Yool AJ. Aquaporin-1: new developments and perspectives for peritoneal dialysis. Perit Dial Int 2010; 30: 135-141.
23. Fabbrini P, Schilte MN, Zareie M et al. Celecoxib treatment reduces peritoneal fibrosis and angiogenesis and prevents ultrafiltration failure in experimental peritoneal dialysis. Nephrol Dial

Transplant 2009; 24: 3669-3676.

24. Zareie M, Keuning ED, ter Wee PM, Schalkwijk CG, Beelen RH, van den Born J. Improved biocompatibility of bicarbonate/lactate-buffered PDF is not related to pH. Nephrol Dial Transplant 2006; 21: 208-216.
25. Bombardier C, Laine L, Reicin A et al. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. N Engl J Med 2000; 343: 1520-8, 2.
26. Brophy JM. Cardiovascular effects of cyclooxygenase-2 inhibitors. Curr Opin Gastroenterol 2007; 23: 617-624.
27. Ross EA, Tian J, Abboud H et al. Oral paricalcitol for the treatment of secondary hyperparathyroidism in patients on hemodialysis or peritoneal dialysis. Am J Nephrol 2008; 28: 97-106.
28. Sprague SM, Llach F, Amdahl M, Taccetta C, Batlle D. Paricalcitol versus calcitriol in the treatment of secondary hyperparathyroidism. Kidney Int 2003; 63: 1483-1490.