Chapter 1

General Introduction

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INTRODUCTION

1. PERITONEAL DIALYSIS: ADVANTAGES, LIMITATIONS AND UNDERUTILIZATION
Renal Replacement Therapy (RRT) is necessary for the survival of patients with End Stage Renal Disease (ESRD), sometimes prior to kidney transplantation in other cases as long-term treatment in patients where kidney transplantation is not an option. Peritoneal dialysis (PD) and haemodialysis (HD) are life-saving RRTs for more than 200,000 patients with ESRD in Europe. The prevalence of people on RRT across Europe increased by 3.3% from 2011 to 2012 to reach 716.7 per million population \(^1,2\). In Europe RRT consumes 2% of overall healthcare expenses for only 0.1% of the population. The total estimated cost of RRT across Europe is €15 billion per year. Moreover, besides to RRT costs there are also additional healthcare costs to treat complications such as transportation to and from the clinic.

Although long-term morbidity and mortality are similar between PD and HD, there is an early patient survival advantage for home-based PD \(^3\). Furthermore, a number of studies have shown that patients on PD have a better quality of life than patients on hospital-based HD \(^4\). PD is an effective treatment for a wide range of patients from children to the elderly and is a more cost effective therapy compared to hospital-based HD \(^3,5\). Nevertheless, only one out of 10 patients on dialysis is treated with PD in Europe. This compares unfavourably with the much higher percentage of patients on PD in other parts of the world e.g. Canada, Australia and Hong Kong \(^6\). The latter suggests that there is a general underutilisation of PD in Europe which in part can be explained by an increased application of kidney transplantation, the introduction of nocturnal HD \(^7,8\) and for other non-scientific factors such as the lack of clinical PD experience and knowledge of PD by nephrologists \(^9\).

PD is a patient performed therapy in which PD fluid (PDF) is exchanged in and out of the peritoneal cavity several times a day or overnight, using the peritoneal membrane for the continuous clearance of uremic toxins and excessive water. Major improvements in patient outcomes in PD have been achieved but additional scientific understanding and application into therapeutic alternatives are required. PD therapy, although performing excellent dialysis and removal of solutes, gives a set of specific challenges to the peritoneal membrane especially when this therapy is applied over many years.

The major limitation of prolonged PD treatment remains its repeated exposure to high glucose containing PDFs. This will result in toxic and inflammatory insults to the cells of the peritoneum that lead both to peritoneal membrane damage and susceptibility to infection, partly due to inhibition of peritoneal host defenses after microbial exposure. Peritoneal membrane failure, especially when characterized by limited ultrafiltration capacity, is detrimental for PD treatment and is a complication that can lead to withdrawal from the therapy and in exceptional cases to death as a result of fluid overload. Other complications associated with the high volumes of
fluid instilled into the peritoneum are lower back pain and hernia or leaking fluid due to high pressure within the abdomen. In the past decade numerous attempts were made to improve PD therapy, by developing more biocompatible fluids such as non-glucose based PDFs, but still PDFs induce pathological changes to the peritoneal membrane and inhibit peritoneal host defenses in the long run.

In addition, there are no established biomarkers (biological characteristics) that would allow timely identification of patients who are at risk of peritoneal membrane damage or infectious complications, early in the course of their PD treatment. Early identification of those patients at risk, may allow individually tailored, specific and novel therapeutic inventions in order to increase treatment and patient survival on PD.

1.1. Causes of PD-induced peritoneal changes

Long term PD treatment can lead to several morphological and functional alterations of the peritoneal membrane as a consequence of repeated exposure to PDF, eventually leading to PD technique failure. Although the pathogenesis of the peritoneal membrane failure is not fully understood, peritoneal damage and its subsequent change in morphology, mainly consists of events as epithelial to mesenchymal transition (EMT) and angiogenesis 10,11. The progressive loss of mesothelium and development of interstitial fibrosis occurring during EMT are early events which cause other abnormalities such as peritoneal thickening, oxidative stress, chronic inflammation and extensive fibrosis 12. On the other hand, alterations in the peritoneal membrane functionality are represented by loss of ultrafiltration (UF) capacity due to peritoneal thickening and changes in the transport status 13. Moreover, bacterial peritonitis is a complication, and in rare cases it can even lead to encapsulating peritoneal sclerosis (EPS) 14, 15.

On a molecular level, the expression of several important cytokines are altered in peritoneal effluents of patients undergoing long term PDF exposure. The main cytokines driving angiogenesis and fibrosis in response to PDFs are, respectively, vascular endothelial growth factor (VEGF) and transforming growth factor β 1 (TGFβ) 16,17. It has been suggested that glucose degradation products (GDPs) and advanced glycation end products (AGEs) are responsible for the upregulation of both cytokines expression as well as for the deposition of collagen and laminin in the peritoneal membrane 18,19,20. Moreover, TGF-β but also VEGF clearly play a key intermediary role in the EMT process. It seems that a kind of positive feedback loop exists where TGFβ enhances the production of extracellular matrix macromolecules (ECM) like collagen, laminin and fibronectin resulting in further new vessel formation 21, 22.

1.2 Cells contributing to peritoneal tissue remodelling

Peritoneal tissue remodeling occurring during PD resembles a chronic low grade of inflammation. Different cells types are involved in this process including mesothelial and endothelial cells, (myo)fibroblast, peritoneal macrophages and mast cells 23.
The main role is played by mesothelial cells (MCs) which, when activated, produce angiogenic and fibrotic factors such as VEGF, TGFβ, fibroblast growth factor 2 (FGF2) and hyaluronic acid (HA). MCs also contribute to leukocytes recruitment by secreting prostaglandins and cytokines. Moreover, they undergo EMT and when dedifferentiated, they can even acquire (myo)fibroblast characteristics, a process altering the structure of the peritoneal membrane. Besides MCs, also endothelial cells (EC) get activated during PDF exposure producing more IL-8 and MCP-1 and as a consequence of that, rolling, adhesion and extravasation of leukocytes are enhanced (see chapter 4). Macrophages are the predominant cell types in the dialysates collected from PD patients. Their number increases in the peritoneum due to the recruitment of
monocytes from the blood during PD. When activated macrophages secrete prostaglandins E2, Interleukin (IL) -1β, IL-6, IL-8, tumor necrosis factor α (TNFα) and monocyte chemoattractant protein 1 (MCP-1), all pro-inflammatory cytokines driving peritoneal inflammation. Recently it was shown that in PD M2 macrophages dominate over M1 macrophages, which may drive EMT. This point of the macrophage subtypes balance is further investigated in chapter 6.

The number of mast cells, which are usually present in the normal peritoneum, increases upon inflammation. These cells can induce angiogenesis by producing pro-angiogenic factors such as VEGF, FGF-2, TGFβ, TNFα and IL-8. Although the role of several cell types and cytokines has been studied, the immunological mechanisms underlying PD-induced peritoneal fibrosis remain largely unknown. Only recently it has been shown, that also T cells, in particular, T helper 17 cells (Th17) might play a crucial role in peritoneal damage. In chapter 5 is demonstrated that the leukocyte antigen CD69, a C-type lectin disulfide–linked homodimer and a member of the natural killer receptor family specifically expressed on Tregs and leukocytes of chronic inflammatory infiltrates, controls tissue fibrosis by regulating Th17-mediated inflammation during PD. In addition, it has been demonstrated in experimental model that pharmacological treatments modulating Th17 response ameliorated peritoneal fibrosis and preserved membrane function. In line with these results, our findings in the uremic mouse model have also shown involvement of Th17 pathway in the prevention of PD-induced peritoneal and angiogenesis.

2. ADDRESSING PERITONEAL DIALYSIS:
IN VITRO MODELS, IN VIVO RODENT MODELS AND CLINICAL TRIALS

2.1 In vitro models
In order to study the effects of PD and associated peritoneal inflammation on the peritoneal cells, a large number of in vitro studies have been performed. In vitro studies are perfectly suitable to study cytotoxicity caused by dialysis fluid and they offer the opportunity to focus on the effects of individual components of these fluids on cellular responses. Furthermore, they allow the identification of cytokines and growth factors produced upon PD fluid exposure. Recently, in vitro models have been applied to investigate the senescence-associated proteome in mesothelial cells, the effect of differently sterilized PD fluids on mesothelial stress responses, and the contribution of mesothelial-to-mesenchymal transition to peritoneal fibroblast expansion.

These studies, although highly intricate and detailed, are limited in order to study the long term effects of PD fluids. Moreover, they do not fully reflect the dynamic situation in PD patients where more cell types interact in the pathophysiological changes of the peritoneal membrane.
2.2 *In vivo* models

When considering an *in vivo* model system there are many factors, which may contribute and need to be taken into account. Therefore, several *in vivo* models have been developed which have examined many different variables within both uremic and not uremic settings, using differing animal species (rabbit, rat, mouse), methods of fluid instillation (injection or catheterisation), length of experiments (hours to months) and the amount of fluid instilled. Animal PD models can be used to study the long term effects of PD fluids on the peritoneal permeability, the peritoneal membrane and mesothelium morphology, cellular composition of peritoneal cavity and functional capacity of peritoneal cells.

In the last decade different animal models for PD fluid exposure have been mainly performed in rats. The model used at the VUMc Amsterdam is world-renowned in PD research and it has been used extensively to study the effects of PD exposure on the peritoneal cavity, including studies that look at different anti-inflammatory interventions, for example heparins, proteins, bio-compatible PD fluids and Vitamin D receptor activation under paricalcitol treatment. These rat models, together with the first established mouse PD exposure model by González-Mateo *et al.* have been used to introduce more biocompatible PD fluids into the market and have allowed to identify important biomarkers driving peritoneal inflammatory mechanisms. To more closely mimic the patient conditions our group recently induced uremia in both the rat and the mouse model and combined it with a long-term PD exposure. Thus, the novel uremic mouse model presented in *chapter 2* was used to elucidate the protective role of Alanyl Glutamine (Ala-Gln) addition to conventional PD fluid against PD-related fibrosis and angiogenesis. The molecular mechanisms of Ala-Gln protective effect is presented in *chapter 3*.

2.3 Clinical trials

Pre-clinical research using cell cultures, biochemistry and animal model testing can only result in suggested therapies that need to be tested for their safety or efficacy. Pre-clinical research needs to be supported by clinical trials, to provide ultimate proof of clinical efficacy.

Clinical studies are mandatory to identify the right PD modality, to study biocompatibility of new PD solutions or new strategies to prevent PD-related peritoneal changes such as the addition of new components to the PD fluids already used in the clinic.

Multicentre studies such as the Euro-Balance, the balANZ trial and many others have allowed the comparison of different PD solutions and provided results having important implications on the existing clinical practise guidelines. In this thesis *chapters 7* and *8* describe the conversion to a bicarbonate/lactate buffered with neutral pH and low GDPs in a 2 years randomized clinical trial.
3. STRATEGIES TO PREVENT PD-RELATED PERITONEAL CHANGES

3.1 Need of biomarkers

In the past decades, evaluation of peritoneal membrane status in patients on long-term peritoneal dialysis has mainly focused on functionality of solute transport. In PD treatment however there is an unmet need for high sensitive and specific biomarkers to identify patients with high risk to develop PD-related complications. These biomarkers could guide personalized interventions and diagnosis thus improving clinical outcomes.

By definition a biomarker is an objectively measured characteristic of a normal or pathological biologic process. Alteration in local or systemic levels of a biomarker usually indicates an inflammatory event or a disease status. Nevertheless, this change could be consequence of increased production or defective clearance of that particular molecule or simply it could reflect changes in distribution or even leakage between different compartments. Moreover, a biomarker can be predictive, diagnostic or a good tool for disease monitoring, alone or most of the time together with others forming a panel of markers useful to guide clinical decisions.

Identification of a valid biomarker is not easy and its interpretation is frequently cumbersome, and requiring careful preclinical and clinical validation.

The identification of biomarkers in PD is usually related to the pathology of the peritoneal membrane. Some serum biomarkers such as albumin have been suggested as indicator of comorbid disease in PD patients but this is likely not so specific. More studies focused on biomarkers indicative of the peritoneal membrane's local integrity and peritoneal infection rather than on the systemic effect of PD treatment.

Several effluent biomarkers have already been identified in PD. Among those for example, Cancer antigen 125 (CA125) and Interleukin-6 (IL-6) respectively markers for mesothelial cell mass and inflammation, have been indicated as good markers for the follow-up of PD patients in many in vitro and in vivo studies. Nevertheless, their use in clinical decisions is still limited for lack of specificity and sensitivity. This also applies for other candidate biomarkers of peritoneal connective tissue turnover (HA), angiogenesis (VEGF) and fibrosis (TGFβ, TNFα). In addition, studies comparing the levels of these markers in a long-term-follow up are often not available in PD patients. Thus, further investigations are needed in order to confirm the role of these factors in the prediction of PD technique survival.

Nowadays, transcriptomics and proteomics represent new promising tools for the identification of novel biomarkers in PD. In this thesis (chapter 8) we used glycomics as a novel and attractive approach for biomarker identification and we showed that changes in the glycosylation profile correlates with PD-related complication such as peritonitis, inflammation and mesothelial loss.
3.2 Biocompatibility aspects of PD fluids

Standard PD fluids contain glucose as osmotic agent, lactate as buffer and sodium, chloride, potassium and calcium in different concentrations in order to maintain electrolyte balance. Formation of glucose degradation products (GDPs) is a consequence of the heat-sterilization process of glucose-based dialysis fluids. The high content of GDPs, together with the low pH and the presence of lactate have been described as the main responsible factors driving the morphological and structural changes of the peritoneal membrane upon PD long term exposure.

During PD treatment, the peritoneal membrane is continuously exposed to unphysiological glucose concentrations, a situation that closely resembles that one found during diabetes mellitus. Glucose induces up-regulation of several proteins such as adhesion molecules and growth factors in both mesothelial and endothelial cells. Moreover, besides being cytotoxic, high concentration glucose is absorbed into the circulation causing systemic consequences as obesity, diabetes and hyperlipidemia. In addition, GDPs too can have a direct cytotoxic effect on cells by inducing EMT or act indirectly via the generation of AGEs. AGEs are end product compounds formed through a series of non-enzymatic reactions ending with the last and irreversible step of oxidations to a divers class of compounds of which only a few have been described so far. AGEs might contribute to endothelial dysfunction and atherosclerosis by binding to specific receptors and causing production of inflammatory cytokines and growth factors.

Glucose degradation and thus GDPs rate formation during heat sterilization depends on pH. Low pH (between 5.2 and 5.5) is applied to prevent glucose caramelization. It reduces glycosilation and GDPs formation. On the other hand, several independent studies have shown that the combination of acidic pH with lactate severely impairs peritoneal mesothelial cells function and have proved the biocompatibility of acidic PDFs.

In the last decades more biocompatible PDFs have been developed and brought into clinical practice. These glucose-containing solutions are sterilized in a different way than by using heat, or glucose is substituted by alternative osmotic agents as icodextrin or amino-acids. Moreover, the amount of GDPs has been lowered and more physiological pH has been introduced. In order to prevent GDPs formation, two chamber-bags dialysis solutions have been developed allowing sterilization of glucose to be performed in an acidic environment (pH 3) and separated from other solution components. The introduction of neutral pH and particularly bicarbonate/lactate-buffered solutions appeared to offer advantages in terms of peritoneal membrane preservation, peritoneal homeostasis control and AGEs formation.

Different solutions with different combinations of buffers and pH are currently available in the market. One of these is the combination of 25mmol/l bicarbonate with 15mmol/l lactate at neutral pH in a glucose based low GDPs content PD solution (Physioneal®, Baxter Healthcare BV), which is currently being used in clinical PD treatment. Previous studies within our group have analyzed the effect of this
solution in both animal and clinical studies. Although those data indicated better preservation of normal peritoneal morphologic features upon bicarbonate/lactate solution, findings regarding peritoneal activation and inflammatory markers were still controversial and sometimes additional mechanisms remained unexplained. A comparison between this solution and a conventional PD fluid (Dianeal®, Baxter Healthcare BV) has been performed in the recently developed uremic mouse PD model (chapter 6) and most importantly in PD patients (chapter 7).

3.3 Therapeutic interventions

A different strategy to prevent or at least reduce the deleterious effects of PD exposure on the peritoneal cavity might be the addition of anti-inflammatory compounds to the conventional PD fluids currently available on the market. PD fluids have been supplemented with different new generation compounds as reviewed by Farhat et al. Pharmacologic interventions against AGEs have been studied by using peroxisome proliferator-activated receptor (PPARG) agonists which have been shown to have a glucose-lowering effects and anti-inflammatory properties. The use of those compounds (Rosiglitazone, Benfotiamine, Aminoguanidine, Alagebrium, Zopolrestat, Pyridoxamine) caused less peritoneal damage during PD but no beneficial clinical effect for PD patients has been proven.

Angiotensin II is an important factor in the development of renal fibrosis probably mediated by TGFβ1. In vitro and in vivo animal data about the use of angiotensin converting enzyme (ACE) inhibitors (captopril and enalapril) showed improved ultrafiltration and less fibrosis but there are not enough data available to use them in the clinic for this indication.

Also agents targeting the peritoneal fibrinolytic system have been tested. Thus the addition of statins to conventional PD solutions might represent a mechanism for removing fibrin deposition in the peritoneal membrane and thereby reversing peritoneal thickening. It has been indeed reported that beside to be designed to lower cholesterol levels, statins increase the synthesis of the fibrinolytic enzyme tissue type plasminogen activator and decrease plasminogen activator inhibitor type 1 in human peritoneal MCs.

Prostaglandins play an important role in the process of interstitial fibrosis, alteration of the mesothelial layer, and new vessel formation in PD. For these reasons the effect of agents targeting prostaglandins such as Celecoxib have been studied. Clinical studies are needed to study efficacy and safety of both statins and prostaglandins use in PD patients.

Other two compounds BMP-7 and sunitinib have been tested previously within our group in animal PD models. It has been shown that BMP-7 has a protective role against EMT and fibrosis. Sunitinib inhibits VEGF and platelet-derived growth factor pathways that regulate new vessel formation representing one of the major causes of ultrafiltration failure. These two compounds however, have never been
used in human PD since the first one did not show any effect on inflammatory responses and the second could cause defects in wound healing and tissue repair where VEGF is the main factor involved.

Recently the projects of our group explored two additional compounds as anti-inflammatory therapies: Vitamin D receptor activators and Alanine-Glutamine dipeptide. Vitamin D3 has anti-inflammatory, anti-angiogenic and anti-proliferative effects and it showed a protective role in peritoneal remodelling during PD \textsuperscript{63,111,112}. Ala-Gln, a dipeptide used in the parenteral nutrition, represents at the moment a novel anti-inflammatory therapy able to prevent from PD induced peritoneal fibrosis and angiogenesis. In this regard \textbf{chapter 3} shows that Ala-Gln supplementation of a conventional PD fluid protect against peritoneal fibrosis via the regulation of IL-17 pathways in both the uremic rat and mouse PD models \textsuperscript{45}. 
4. AIM AND OUTLINE OF THE THESIS

Long term exposure to PDFs is associated with increased rate of peritonitis and morphological as well as functional changes in the peritoneal membrane leading to ultrafiltration failure. PD-related peritoneal changes need to be prevented to avoid or at least delay treatment failure. As a result, patients will be able to undergo PD treatment for a prolonged period. Therefore, the final aim of this thesis was to test new anti-inflammatory drugs and identify new relevant candidate biomarkers of peritoneal inflammation and fibrosis achieving personalized diagnosis and interventions thus improving clinical outcomes.

In chapter 2 we developed a novel uremic mouse PD model allowing to perform long term exposure studies where the clinical situation could be closely mimicked by the combination of uremia and PD treatment.

In chapters 3 and 4 this mouse model was used to investigate the effect of new compounds added to the conventional PD fluids currently used in the clinic. In this regards a protective role of Ala-Gln dipeptide against peritoneal fibrosis and angiogenesis was demonstrated in chapter 3. This study also confirmed the recently identified role of IL-17 as candidate biomarker of peritoneal fibrosis. Moreover, in chapter 4 the effect of Ala-Gln on rolling and adhesion was investigated.

In the same line in chapter 5 we demonstrated that the CD69 lectin expressed in Tregs and leukocytes of inflammatory infiltrates negatively controls tissue fibrosis by regulating Th17-mediated inflammation in both the mouse PD model and in PD patients.

In chapter 6 the uremic mouse model was used to compare bicarbonate/lactate solutions with neutral pH and low GDPs versus lactate and high GDPs content PDFs. Exposure to the first treatment resulted in a less harmful effect in terms of peritoneal membrane deterioration when compared to the latter while it increased recruitment of pro-inflammatory cytokines and macrophages.

Chapter 7 showed the results of a 2 year multi-center randomized clinical trial in which CAPD patients underwent conventional or neutral pH and low-GDP regimes. In this study renal residual function (RRF), ultrafiltration (UF) and peritonitis incidence were measured. In addition, surrogate systemic markers of inflammation and local markers for the health and viability of the peritoneal membrane were analysed.

A novel approach for biomarker identification was used in chapter 8 where the correlation between changes in the glycosylation profile and the expression of putative markers for PD-related peritonitis, mesothelial loss and inflammation in bot serum and peritoneal effluents has been studied.

Finally, in chapter 9 our data from chapter 3-8 are discussed and we come to final conclusions. Moreover, indications for future directions in PD research are given.
REFERENCES


In, ERA-EDTA, p gfp618


