9 Chapter

Protein identification in effluents of patients treated with different peritoneal dialysis fluids.

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Abstract

Background: Long-term peritoneal dialysis (PD) treatment induces peritoneal changes which ultimately lead to technique failure. Proteins found in the peritoneal effluents may be used to detect peritoneal damage.

Methods: To analyse protein profiles, PD effluents of patients treated with a conventional PD fluid (Dianeal, n=4), or with a biocompatible fluid (Physioneal, n=6) were collected. Abundant proteins were removed by Top 14 IgY spin columns. The flow-through proteins were separated on a 12% gradient SDS-PAGE gel and in-gel digested with trypsin. Proteins were detected by LC-MS and results were searched against the NCBI data base.

Results: A total of 28 proteins were identified in both PD groups, whereas one protein, tissue factor, was found in the Physioneal group only. Of the proteins detected, the majority (30%) were immune system and inflammatory proteins, followed by tissue remodelling proteins (15%). Higher protein sequence percentages of anti-inflammatory proteins, like orosomucoid precursor, were found in Physioneal treated patients, together with proteins involved in vascular integrity. Proteins involved in extra cellular matrix production, like lumican precursor and inter-alpha trypsin inhibitor family heavy chain related protein (IHRP), were mostly detected in the Dianeal group.

Conclusion: The proteins found in the effluents indicate that Physioneal results in less inflammation and angiogenesis compared to Dianeal treatment, whereas Dianeal induces more fibrosis. This is in line with the known morphology of rat tissues after exposure to these PD fluids. The protein profile in the PD effluents therefore gives an indication of the condition of the peritoneum and may be used as a marker of peritoneal damage.

Introduction

Peritoneal dialysis (PD) is a renal replacement technique for patients with end stage renal disease (ESRD)[1]. The therapy is based on the ability of the peritoneal membrane to function as a dialysing membrane, allowing exchange of solutes and waste products between the PD fluid and the circulation. Although PD is effective and offers flexibility and mobility of patients, pathologic changes like the formation of blood vessels and fibrosis of the peritoneal tissues. Together with the induction of inflammatory processes, long term PD treatment can ultimately result in technique failure. Damage to peritoneal tissues cannot be easily identified without invasive techniques. Therefore, non invasive techniques to determine the condition of the peritoneum are needed to early detect and possibly prevent peritoneal damage.

Over the past few years, proteomic analyses have become an increasing area of interest leading to increased understanding of the role of proteins and protein interactions in all aspects of cellular function[2]. Proteomic methods allow separation and identification of single proteins from a complex mixture[3]. Systematic global identification and quantification of proteins can not only improve biomedical understanding of a particular system in healthy or diseased individuals, but may also be used for protein biomarker discovery[4].
Proteomic tools such as mass spectrometry have been widely applied in the study of body fluids, e.g. cerebrospinal fluid, urine and blood, but only a few studies have focussed on peritoneal dialysis effluents[5].

Protein levels found in the peritoneal effluents may provide clues for understanding the mechanism of peritoneal tissue damage caused by PD. Alteration in proteins in the peritoneum may be used as biomarkers for detection of damage. We therefore studied the protein profiles in the PD effluents of patients treated with a conventional or a more biocompatible solution. Compared to conventional solutions, biocompatible fluids are known to induce less pain during infusion and in the long term induce less damage to peritoneal tissues. In this study we have performed proteomic analysis to investigate differences in protein patterns between the two types of dialysis fluids.

Materials and methods

Patient sample preparation

Peritoneal dialysate was collected from 4 patients who had been stably using a conventional dialysis solution (Dianeal, Baxter, The Netherlands) and 6 patients using a biocompatible PD solution (Physioneal, Baxter, The Netherlands) for over 6 months. Patients underwent three daytime PD fluid exchanges with either the conventional or biocompatible solution. During the night, the peritoneal cavity of all patients was exposed to an icodextrin solution (Extraneal, Baxter, The Netherlands). The second of the three daytime exchanges was collected for proteome analysis. None of the patients suffered from peritonitis in the three months prior to collection. Peritoneal effluents were centrifuged to remove peritoneal cells and cell free effluents were stored at -80°C until further use.

Protein preparation

Protein concentration of each effluent was measured by DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). One ml of each sample was pooled for each solution and concentrated (~7.5 times) over a 10kDa column (Amicon filter, Millipore, Billerica, MA, USA).

Of each concentrated and pooled sample, 400 µg was diluted in 500 µl TBS (washing buffer) and added to a prepacked IgY-microbead spin column (Seppro® IgY14, Sigma-Aldrich, St. Louis, MO, USA). Seppro® IgY14 columns separate 14 highly abundant proteins, which can interfere with Liquid Chromatography-Mass Spectrometry (LC-MS), from the samples (Table 1).

Table 1: Proteins depleted by Seppro® IgY14 column.

Albumin
IgG
α1-Antitrypsin
IgA
IgM
Transferrin
Haptoglobin
α2-Macroglobulin
Fibrinogen
Complement C3
α1-Acid Glycoprotein (Orosomucoid)
HDL (Apolipoproteins A-I)
HDL (Apolipoproteins A-II)
LDL (mainly Apolipoprotein B)

Samples (500 µl) were gently mixed with IgY microbeads by inverting the column several times and incubated at room temperature for 15 minutes with rotation on an end-to-end rotator. The sample depleted of the target proteins (flow-through) was collected in a 2 ml microcentrifuge tube by centrifugation. To make sure we collected all non-bound proteins, samples were centrifuged again with 500 µl TBS and also collected in the 2 ml microcentrifuge tube. The bound proteins were four times eluted (500 µl for each elution) with a total of 2000 µl of 0.1M glycine, pH 2,5 (stripping buffer), mixed and incubated with the stripping buffer at room temperature for 3 min. The eluted proteins were collected by centrifugation. After elution, the column was immediately neutralized with 0,1M Tris-HCL, pH 8,0. The bound fractions were pooled and neutralized with 1/10 vol 1 M Tris-HCL, pH 8,0 (neutralizing buffer). Both flow through and eluted samples were concentrated to a volume of <100 µl. Samples were run on a 12% SDS page gradient gel (Invitrogen, Paisley, UK) using reducing conditions. After electrophoresis, the gels were fixed in 50% ethanol containing 3% phosphoric acid and stained with Coomassie R-250. After staining, the gels were washed in Milli-Q water and stored at 4°C until processing for in-gel digestion.

In-Gel Digestion

The gel was processed in a keratin free laminar flow for in-gel digestion according to the whole gel in-gel digestion method. Briefly, the gel was washed and dehydrated three times in 50 mM ABC (ammonium bicarbonate, pH 7.9) and 50 mM ABC + 50% acetonitrile (ACN). Subsequently, cysteine bonds were reduced with 10 mM dithiothreitol for 1 hour at 56°C and alkylated with 50 mM iodoacetamide for 45 min at room temperature in the dark. After three subsequent wash and dehydration cycles, each gel lane was cut in 10 slices and washed once more in 50 mM ABC (pH 7.9) and 50 mM ABC + 50% ACN. Slices were dried for 10 min in a vacuum centrifuge (ThermoFisher, Breda, The Netherlands) and incubated overnight with 6.25 ng/ µl trypsin in 50 mM ABC at 25°C. Peptides were extracted once in 100 µl of 1% formic acid for 15 minutes and subsequently twice in 100 µl of 50% ACN in 5% formic acid for 15 minutes. For protein identification, a protein pellet was obtained using vacuum centrifuge.

Protein identification and database search

Prior to LC-MS the pellet was dissolved in 200 µl 2% ACN of which 20 µl was injected in the LC-MS. The protein tryptic digests were fractionated using a C18 Pepmab Nano LC column (75 µm inner diameter, 15 cm length) with a high-performance liquid chromotography (HPLC) system (Ultimate Dionex 3000,Sunnyvale, CA, USA) coupled to an ion trap mass spectrometer (LCQ DECA XP, ThermoFinnigan, San

Jose, CA, USA). Datafiles were searched against the NCBI human database (http://www.ncbi.nlm.nih.gov/) using the Bioworks search programme. Data is given as protein sequence coverage (= [number of identified residues/total number of amino acid residues in the protein sequence] x100%).

Results

In this study we compared protein levels of stable patients undergoing PD for more than 6 months using either Dianeal or Physioneal. A total of 4 Dianeal and 6 Physioneal patient effluents were collected. The patient and fluid characteristics of these patients are given in Table 2. Total protein levels in the effluent of each patient was measured and given in Figure 1. Physioneal patients tended to have somewhat higher protein concentrations in their effluents compared to Dianeal treated patients.

Table 2: Patient characteristics.

Patient	PDF	Sex [a]	Age (year)	Time on PD (year)	Underlying disease	protein g/l
1	Dianeal	M	82	1.5	Hypertension	0.311
2	Dianeal	F	75	4.8	Hypertension	0.311
3	Dianeal	F	82	0.5	Hypertension	0.463
4	Dianeal	F	31	2	Hypertension	0.612
5	Physioneal	F	61	10	Medullar sponge kidneys with nefrolithiasis	0.626
6	Physioneal	M	79	7	Hypertension	0.551
7	Physioneal	F	81	4.6	Aetiology uncertain	0.580
8	Physioneal	M	63	1.1	Diabetes type II. non-insulin dependent	0.477
9	Physioneal	F	71	1	Ischemic renal disease	0.520
10	Physioneal	M	79	1.1	Wegeners disease	0.495

[a]: M=male, F=female.
PDF= peritoneal dialysis fluid; PD= peritoneal dialysis.

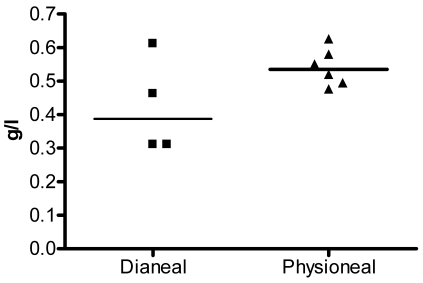

Figure 1: Total protein concentration in g/l of Dianeal and Physioneal treated patients

After concentration of the effluents a pooled sample of each group (Dianeal pool vs Physioneal pool) was run over the IgY top 14 column. The eluate fraction, the bound fraction as well as the total protein was put on gel and run by electrophoresis (Figure 2). To determine the proteins in the bands shown in figure 2, each lane was cut in 10 slices along the 10 marker bands and analysed by LC-MS.

As can be seen in lane F and G, indeed several proteins have been bound by the microbeads of the IgY column (bound fraction Dianeal and Physioneal). A large band is visible around 65 kDa, which proved to be mainly albumin (66,4 kDa). In lane F of Figure 2, in slice 8 at 15-20kDa a band was found which was not seen in lane G, indicating that in the Dianeal pool other proteins were bound compared to the Physioneal pool. However, these proteins could not be identified by LC-MS due to too low protein sequence percentages found.

Of the proteins found in the eluate fractions, a total of 28 proteins were identified in both PD groups, whereas one protein, namely Tissue Factor (TF), was found in the Physioneal group only (Table 3). Table 3 shows the percentage of protein that has been sequenced. To give more insight into the function of the identified proteins, they were classified according to their function in eight different classes. The majority of the proteins were classified as immune system regulation and inflammation proteins

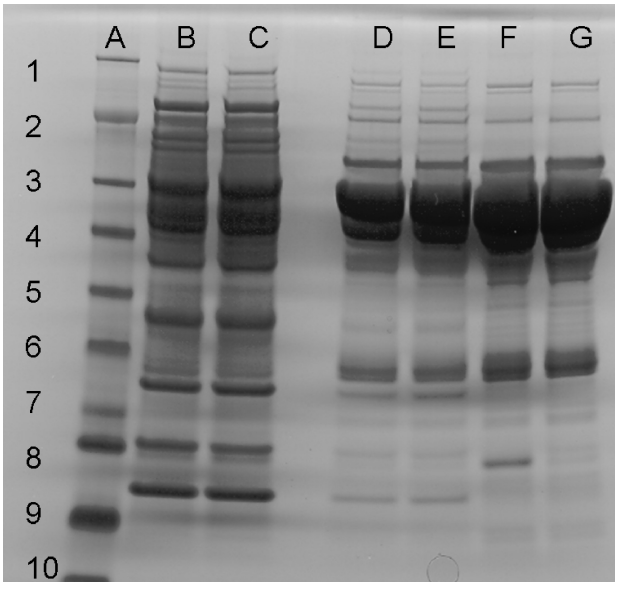

Figure 2: SDS page gel with lane A: marker, B: Dianeal eluate, C: Physioneal eluate, D: Dianeal total protein, E: Physioneal total protein, F: Dianeal bound fraction, G: Physioneal bound fraction. Numbers 1-10 indicate the 10 slices for in-gel digestion.

(10), followed by tissue remodelling proteins (5), transport (3), coagulation (3), blood carrier proteins (3), lipoproteins (2), blood pressure proteins (1), insulin action (1) and other (1) (Table 4).

The immune system regulation and inflammation group contains pro-inflammatory, anti-inflammatory and complement proteins. The majority of these proteins did not differ between the Dianeal and the Physioneal treated patients. However, in the Physioneal group a higher percentage (25.0% in Physioneal treated patients vs. 7.0% in the Dianeal group) of protein sequence was found of the orosomucoid 1 precursor, an anti-inflammatory protein. The pro-inflammatory prostaglandin H2 protein sequence percentage was enhanced from 17.4% in the Dianeal group, to 28.4% in the Physioneal group. Proteins of the complement system were generally found to be enhanced in the Dianeal group.

The inter-alpha trypsin inhibitor family heavy chain related protein (IHRP) is next to being a pro- inflammatory protein also involved in tissue remodelling. Of this protein 22.3% was sequenced in the Dianeal group whereas only 15% was found in the Physioneal treated patients. Of the other tissue remodelling proteins involved in extra cellular matrix production, highest protein sequences were found in the Dianeal group for the lumican precursor (26.5% in the Dianeal group vs. 18% in the Physioneal group). Blood vessel formation was mostly modulated in the Physioneal group due to increased protein sequences of pigment epithelium derived factor (PEDF), known as an inhibitor of angiogenesis. In the Physioneal group 22% of this protein was sequenced whereas only 16.3% was found in the Dianeal group. Another protein involved in blood vessel formation is TF, which was not found in Dianeal treated patients, although 18% of this protein was sequenced in Physioneal treated patients.

Transport related proteins, as well as the coagulation proteins and lipoproteins were down-regulated in the Physioneal group compared to Dianeal treated patients. Blood carrier proteins showed not to be different between the two groups.

Table 3: Proteins found in the eluate, grouped per fraction.
E-01- E-10 indicate slices of gel (see Figure 2), In column Dianeal and Physioneal the sequence coverage (%) is given. The column serum indicates whether the found proteins are normally also found in serum. The column classification indicates the protein function.

Sample	Identified Protein	Dianeal	Physioneal	Serum	Classification
E-01	Not defined	<4	<4		
E-02	Ceruloplasmin precursor	34	25	yes	Transport
E-02	Inter-alpha-trypsin inhibitor family heavy chain related protein	22.3	15	yes	Immune system regulation and inflammation
E-02	Pro-ceruloplasmin	16	8	yes	Transport
E-02	Inter-alpha-trypsin inhibitor family heavy chain H1	9.7	12	yes	Immune system regulation and inflammation
E-02	Inter-alpha-trypsin inhibitor family heavy chain H2 precursor	8	10	yes	Immune system regulation and inflammation
E-03	Gelsolin isoform precursor	20.3	19	yes	Remodelling
E-03	Complement component 1 inhibitor precursor	14	7	yes	Immune system regulation and inflammation
E-03	Ceruloplasmin precursor	12.8	11	yes	Transport
E-03	alpha 1B-glycoprotein precursor	16	11	yes	Transport
E-03	CO4A_HUMAN complement C4A precursor	4	7	yes	Immune system regulation and inflammation
E-03	Hemopexin	12.7	15	yes	Blood carrier protein
E-03	Lumican precursor	15	12	no	Remodelling
E-03	Complement component 9	11	9	yes	Immune system regulation and inflammation
E-04	A chain A, crystal structure of cleaved human alpha 1 anti-chymotrypsin	47.5	40	yes	Coagulation
E-04	Vitamin D binding protein/ group specific component	25.3	23.6	yes	Remodelling
E-04	Serine (cysteine) protease inhibitor Clade A	26.1	16	yes	Coagulation
E-04	Angiotensinogen pre-proprotein	29.1	23	yes	Blood pressure
E-04	Anti-thrombin III variant	22	23	yes	Coagulation
E-04	Alpha 1 B glycoprotein precursor	26.7	10	yes	Transport
E-04	Hemopexin	15.4	12	yes	Blood carrier protein
E-04	Anti-thrombin	41.8	27	yes	Coagulation
E-04	Lumican precursor	11.5	6	no	Remodelling
E-05	Apo-lipoprotein A-IV precursor	34.6	20.2	yes	Lipoprotein
E-05	PEDF_HUMAN pigment epithelium derived factor precursor	16.3	22	yes	Remodelling

Sample	Identified Protein	Dianeal	Physioneal	Serum	Classification
E-05	Alpha-2-glycoprotein 1	24.5	10	yes	Lipoprotein
E-05	Vitamin D binding protein/group specific component	15	8	yes	Remodelling
E-05	Orosomucoid 1 precursor = Lipocalin AGP	7	25	yes	Immune system regulation and inflammation
E-06	Alpha-1-microglobulin/bikunin pre-pro-protein	12	14.2	yes	Other
E-06	Prostaglandin H2 D isomerase	17.4	28.4	low levels	Immune system regulation and inflammation
E-06	Vitamin D binding protein/group specific component	4	4	yes	Remodelling
E-07	A chain A, pro-enzyme of human complement factor D, recombinant profactor D	23.4	24	yes	Immune system regulation and inflammation
E-07	Immunoglobulin kappa light chain VLJ region	7	<4	yes	Immune system regulation and inflammation
E-07	A chain A, crystal structure of the trigonal form of human plasma retinol-binding protein	4	7	yes	Insulin action
E-07	L chain L, crystal structure of Tissue Factor in complex with humanized Fab D3	0	18	no	Remodelling
E-08	Pre-albumin	67	46	yes	Blood carrier protein
E-08	A chain A, structure of pre-albumin	32	33	yes	Blood carrier protein
E-09	Not defined	<4	<4		
E-10	Not defined	<4	<4		

Table 4: Proteins found in the eluate, classified according to function.
In column Dianeal and Physioneal the protein sequence percentage (%) is given as mean of all fractions. In the last column a comparison between Dianeal and Physioneal is made. Differences between Dianeal and Physioneal less than 5% are indicated as equal (=).

Biological process	Protein	Dianeal	Physioneal	Physioneal vs. Dianeal
Immune system regulation and inflammation	Inter-alpha-trypsin inhibitor family heavy chain related protein	22.3	15.0	Down
	Complement component 1 inhibitor precursor	14.0	7.0	Down
	Complement component 9	11.0	9.0	=
	Immunoglobulin kappa light chain VLJ region	7.0	<4	=
	A chain A, pro-enzyme of human complement factor D, recombinant profactor D	23.4	24.0	=
	CO4A-HUMAN complement C4A precursor	4.0	7.0	=
	Inter-alpha-trypsin inhibitor family heavy chain	9.7	12.0	=
	Inter-alpha-trypsin inhibitor family heavy chain H2 precursor	8.0	10.0	=
	Prostaglandin H2 D isomerase	17.4	28.4	Up
	Orosomucoid 1 precursor = Lipocalin AGP	7.0	25.0	Up
Remodelling	Vitamin D binding protein	14.8	11.9	=
	Lumican precursor	13.3	9.0	=
	Gelsolin isoform precursor	20.3	19.0	=
	PEDF_HUMAN pigment epithelium derived factor precursor	16.3	22.0	Up
	L chain L, crystal structure of Tissue Factor in complex with humanized Fab D3	0.0	18.0	Up
Transport	Ceruloplasmin precursor	23.4	18.0	Down
	Alpha-1B-glycoprotein precursor	21.4	10.5	Down
	Pro-ceruloplasmin	16.0	8.0	Down
Coagulation	A chain A, crystal structure of cleaved human alpha 1 anti-chymotrypsin	47.5	40.0	Down
	Anti-thrombin	31.9	25.0	Down
	Serine (cysteine) protease inhibitor clade A	26.1	16.0	Down

Biological process	Protein	Dianeal	Physioneal	Physioneal vs. Dianeal
Blood carrier proteins	Pre-albumin	67.0	46.0	Down
	Hemopexin	14.1	13.5	=
	A chain A, structure of pre-albumin	32.0	33.0	=
Lipoproteins	Apo-lipoprotein A-IV precursor	34.6	20.2	Down
	Alpha-2-glycoprotein 1	24.5	10.0	Down
Blood pressure proteins	Angiotensinogen pre-protein	29.1	23.0	Down
Insulin action	A chain A, crystal structure of the trigonal form of human plasma retinol-binding protein	4.0	7.0	=
Other	Alpha-1-microglobulin/bikunin pre-pro-protein	12.0	14.2	=

Discussion

In this study we elucidated the proteomic composition of the PD effluents of Dianeal and Physioneal treated patients, giving a brief overview of the proteome in PD. The proteome we found in PD effluents of Dianeal and Physioneal patients confirms the occurrence of a number of frequently occurring proteins in the dialysate, like complement factors, coagulation factors and lipoproteins[6-9]. With respect to structural changes during PD, the immune system regulation and inflammation as well as the tissue remodelling proteins are of most interest in preserving the peritoneal membranes.

The most abundant difference between the Dianeal and Physioneal group was the presence of TF in Physioneal treated patients, whereas this protein was not found in Dianeal effluents. TF is best known as the primary cellular initiator of blood coagulation. After vessel injury the TF complex activates the coagulation protease cascade[10]. TF dependent signalling is activated upon a variety of biological processes, including inflammation, angiogenesis, metastasis and cell migration[10]. TF expression has been detected in situ, in an anatomic location where it serves a protective role or a so called barrier function, and safeguards the vascular integrity of tissues following vessel injury[11]. Constitutive TF expression is normally restricted to sub-endothelial cells (like smooth muscle cells and fibroblasts) that only interact with the circulation when the vascular integrity is compromised[11;12]. Moreover, TF expression can be transiently up-regulated in monocytes or macrophages and endothelial cells by growth factors and cytokines[11;12].

It is known that during long term PD, exposure of tissues to PD fluids induce vascular damage and new vessel formation. Studies have shown that more biocompatible fluids, with less glucose degradation products (GDPs) and a physiologic pH, induce less angiogenesis and vascular leakage[13-15]. The TF levels found in the Physioneal treated group may therefore indicate a better defence mechanism upon vascular damage and thereby preventing vascular leakage.

The enhanced activation of the proteins of the coagulation system in the Dianeal treated group, also suggests increased vascular permeability and thereby increased vascular leakage in this group[16].

In line with this, our study showed an increased percentage of sequenced protein for PEDF in the Physioneal group. PEDF belongs to the super-family of serine protease inhibitors and is a potent anti-angiogenic factor as it specifically inhibits the proliferation and migration of endothelial cells[17;18]. PEDF has been described to inhibit advanced glycation end (AGE) product-induced vascular permeability[17].

The effects of PEDF on the prevention of mesangial cell damage by the blocking of nuclear factor kappa B (NF-κB)[19] and its role as an anti-inflammatory factor[20], have been described earlier upon high glucose exposure.

The strongly enhanced percentage of orosomucoid precursor found in the effluents of Physioneal patients compared to Dianeal treated patients, supports the assumption of reduced inflammation in the Physioneal group. Orosomucoid is an acute phase protein and has been shown to have a non specific immunosuppressive activity. It has been described to have anti-inflammatory effects and to prevent tissue damage[21;22]. Other inflammatory proteins found in the effluents are proteins of the inter-alpha-trypsin inhibitor (ITI) family. Similar levels for the ITI family heavy chain 1 and 2 (ITIH1 and ITIH2) were found in the Physioneal and Dianeal group, whereas enhanced protein sequence percentages were found for IHRP in the Dianeal group. IHRP acts as a carrier of hyaluronic acid (HA) and stabilizes the extra cellular matrix (ECM)[23;24]. HA is a pro-inflammatory marker which can induce mesothelial EMT and is strongly up-regulated during PD[25]. It has been shown that patients using a biocompatible solution have significantly lower HA levels in their effluents compared to patients treated with a conventional PD fluid[26;27]. The fact that higher IHRP levels in the Dianeal group were found may therefore indicate enhanced HA levels resulting in enhanced inflammation and fibrosis.

The induction of fibrosis of the peritoneal membranes is a well known complication during PD. Previous studies have shown enhanced peritoneal thickening upon conventional PD fluid treatment compared to biocompatible solutions[14;28]. In line with this are the enhanced levels of lumican precursor found in the Dianeal group. Lumican is synthesized amongst others by fibroblasts and vascular smooth muscle cells and is present in the ECM[29]. Lumican is involved in the maintenance of tissue homeostasis and modulates cellular functions including cell proliferation, migration, and differentiation[30], and thereby participates in the thickening of the ECM.

Furthermore, enhanced levels of transport proteins were found in the Dianeal group, compared to Physioneal treated patients. The main proteins found in this category are ceruloplasmin precursor and pro-ceruloplasmin. Ceruloplasmin is the major copper-carrying protein in the blood, and in addition plays a role in iron metabolism. However, elevated levels of this acute phase protein are found during acute and chronic inflammation[31].

Taken together, our results indicate that long term Physioneal treatment induces proteins involved in anti-inflammatory and anti-angiogenic processes, whereas Dianeal enhances inflammation and matrix proteins and therefore peritoneal fibrosis. These results are in line with the known morphology of rat peritoneal tissues after exposure to conventional or biocompatible solutions[14;28;32]. The proteins found in the PD effluents may give an indication of the condition of the peritoneal tissues, showing the potential of studying the proteome of peritoneal fluid in biomarker analysis. However, more and extended research is needed in the near future to study the full protein profile of PD patients and the influences of complications such as peritonitis and ultrafiltration failure.

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