Clinical and Cytokine Predictors of Outcomes in Peritoneal Dialysis

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Statement of originality

All the work described in this thesis is my own unless stated otherwise.

Measurements of CCL18, MCP-1, IL-6 and angiogenin on the first 100 peritoneal dialysis patients in the West London Peritoneal Dialysis Study were originally made by Dr Sohail Ahmad and Dr Amir Malik. This data is included in the analysis of baseline cytokine levels in peritoneal dialysis patients.

Measurements of albumin, β2 microglobulin, prolactin and IgG in serum and dialysate samples were performed by Dr Mandy Donaldson as these are standard analytes for the clinical service at Hammersmith Hospital.

For the investigation of CT scanning as a screening modality study, expert radiology opinion was required. CT scans were scored by three consultant radiologists: Dr Adrian Lim, Dr Steven Moser and Professor Wady Gedroyc.

Multivariate statistical analysis was performed with Dr Bernard North, Imperial College Statistics service.

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All the members of the renal research group whose dedication and enthusiasm for their work showed me how to be a scientist

ABSTRACT

Background

Changes in the structure and function of the peritoneal membrane limit the duration of PD. Rarely (and unpredictably) these changes progress to severe fibrosis and bowel encapsulation (encapsulating peritoneal sclerosis, EPS) with substantial morbidity and mortality.

Methods

PD fluid and serum samples from 50 patients were added to 100 previously analysed samples (Dr S Ahmad). CCL18, IL-6, MCP-1 and angiogenin were measured by ELISA. CCL15 was measured for the first time in 125 serum and dialysate samples. Fifty one year follow up samples were analysed. Serum cytokines were measured in patients with and without EPS. Peritoneal mesothelial cells were cultured and media cytokine levels measured. CCL15 stimulation of cytokine production was investigated. Protein transfer across the peritoneal membrane by size was investigated. CT scans from 20 pre-EPS PD patients were scored and compared with scans of non-EPS patients.

Results

Levels of CCL18, MCP-1, CCL15, angiogenin and IL-6 in dialysate correlate with clinically important measures such as glucose exposure and D/P creatinine. Mesothelial cells in culture produce MCP-1, IL-6, angiogenin and CCL18. High dialysate levels of MCP-1, IL-6 and CCL15 are found in patients who subsequently developed EPS. High levels of CCL18 are also seen in haemodialysis patients with EPS. CT screening of PD patients used alone does not predict future EPS; in combination with abdominal symptoms CT scans may be of use.

Conclusions

There is local peritoneal production of chemokines such as MCP-1, CCL18, IL-6 and angiogenin, and the correlation of levels of these cytokines with clinically relevant parameters suggests they may be involved in the pathogenesis of long term changes in the peritoneum. At present neither clinical nor cytokine levels can reliably be used to predict future EPS. CT scanning may be helpful in patients at risk of EPS who develop new abdominal symptoms.

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Abbreviations

α-SMA	Alpha smooth muscle actin
ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitor
ADEMEX	Adequacy of peritoneal dialysis in Mexico
AGE	Advanced glycosylation end-product
ALS	Amyotrophic lateral sclerosis
APD	Automated peritoneal dialysis
APKD	Autosomal polycystic kidney disease
ARB	Angiotensin receptor blocker
ATN	Acute tubular necrosis
BAL	Broncho-alveolar lavage
BMP	Bone morphogenetic protein
Ca125	Cancer antigen 125
CANUSA	Canada-USA
CAPD	Continuous ambulatory peritoneal dialysis
CCL15	Chemokine (C-C motif) ligand 15
CCL18	Chemokine (C-C motif) ligand 18
CD	Cluster of differentiation
CI	Confidence interval
CKD	Chronic kidney disease
CRP	C-reactive protein
CT	Computed tomography

CTGF	Connective tissue growth factor
DAB	Diaminobenzidine
DM	Diabetes mellitus
D/P creatinine	Dialysate to plasma creatinine ratio
D/S ratio	Dialysate to serum ratio
DSI	Dialysis symptoms index
EAPOS	European APD outcome study
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-related kinase
EPS	Encapsulating peritoneal sclerosis
ESRD	End stage renal disease
FACS	Fluorescence activated cell sorting
GDP	Glucose degradation product
GFR	Glomerular filtration rate
GN	Glomerulonephritis
HbA1c	Haemoglobin A1c
HD	Haemodialysis
HELLP	Haemolysis, elevated liver enzymes, low platelets
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HPMC	Human peritoneal mesothelial cells
HuVEC	Human umbilical vein endothelial cells

IgG	Immunoglobulin G
IgA	Immunoglobulin A
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
ISPD	International society for peritoneal dialysis
kDa	KiloDalton
MAP kinase	Mitogen activated kinase
MCP-1	Monocyte chemotactic protein-1
MDRD	Modification of diet in renal disease
MIP-1δ	Macrophage inhibitory protein-δ (CCL15)
MMP	Matrix metalloprotease
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MTAC	Mass transfer area coefficient
NECOSAD	Nederlands Co-operative Study Adequacy of Dialysis
NAD	Nicotinamide adenine dinucleotide
NOS	Nitric oxide synthase
OD	Optical density
PARC	Pulmonary and activation regulated cytokine (CCL18)
PBS	Phosphate buffered saline
PD	Peritoneal dialysis

PET	Peritoneal equilibration test
PITPNM3	Phosphotidylinositol transfer protein 3
Q-Q plot	Quantile-quantile plot
RAGE	Receptor for AGE
RhoA	Ras homolog gene family, member A
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RPMI	Roswell Park memorial institute
Smad3	Mothers against decapentaplegic homolog 3
TGF-β	Transforming growth factor-β
THP-1	Human acute monocytic leukaemia cell line
TMB	3,3',5,5' tetramethylbenzidine
TNF-α	Tumour necrosis factor-alpha
Tx	Transplanted
U937	Human leukaemic monocyte lymphoma cell line
UFF	Ultrafiltration failure
VEGF	Vascular endothelial growth factor

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CHAPTER 1:

INTRODUCTION

Worldwide there are an increasing number of people dependent on renal replacement therapy, as their own renal function has declined to a level not compatible with life. Many will receive a kidney transplant, but due to anatomical or co-morbid considerations, age, immunological barriers and the shortage of donor organs, some will remain dependent on dialysis therapy for many years. Renal replacement therapy may be performed by haemodialysis or by peritoneal dialysis.

Peritoneal dialysis is a frequently used mode of dialysis therapy worldwide and is appealing to patients as it may be used easily in the home setting, with no complex machinery. However, the duration of peritoneal dialysis therapy is limited by structural changes in the peritoneal membrane which affect both dialysis adequacy and fluid removal. The mechanisms by which these changes occur over a period of years are not yet fully understood. In addition, in a minority of long term peritoneal dialysis patients, a severe form of peritoneal fibrosis develops. This results in the formation of a cocoon of thick fibrous material that surrounds the bowel and causes obstruction. This condition, termed encapsulating peritoneal sclerosis (EPS), is uncommon yet important as it causes substantial morbidity and mortality. As yet there is no reliable way to predict its occurrence.

1.1 Basic concepts in peritoneal dialysis

1.1(i) Performance of peritoneal dialysis (PD)

In order to perform peritoneal dialysis, a permanent catheter is placed with the tip in the pelvis. The catheter is tunnelled subcutaneously and the end is brought out through the skin at a convenient point (the exit site). To achieve removal of uraemic toxins and excess fluid from the body, dialysate (commonly 1.5 to 2.5 litres) is instilled into the peritoneal cavity via the catheter. The fluid is left within the peritoneal cavity for a variable time period during which solutes diffuse across the peritoneum, which acts as a semi-permeable membrane, into the dialysate. Removal of excess fluid from the body occurs by two mechanisms: there is convective movement of fluid with the transfer of small solutes to the dialysate, and also the dialysate composition is hyperosmotic to blood (containing a high concentration of glucose) so that fluid moves by osmosis into the dialysate. After a specified time the fluid is drained out and fresh fluid is instilled. The excess fluid volume drained out (compared to that originally instilled) is termed the ultrafiltration volume.

Peritoneal dialysis may be performed by manual exchange of fluid carried out usually four times each day – this is termed continuous ambulatory peritoneal dialysis, (CAPD). Alternatively, a machine may perform exchanges during the night. This is automated peritoneal dialysis (APD).The peritoneal cavity may be left empty during the day, or to maximise dialysis opportunity a daytime dwell of fluid may be instilled.

1.1(ii) Mechanism of dialysis and ultrafiltration

The effective barrier to fluid and solute transport is the endothelial cell layer of the peritoneal capillaries. Mathematical modelling based on observed membrane transport characteristics predicted the existence of three types of pore in the peritoneal membrane (Rippe *et al.*, 1991). Firstly, ultra-small pores, small enough (<4A°) to permit only the passage of water, are formed by aquaporin 1. Small pores (40-50A°) are probably formed by clefts between the endothelial cells and permit the passage of small solutes and water. Large pores (250A°) may be inter-cell clefts in venules; the number of these pores is small (<5% of the total) (Nishino and Devuyst, 2008). Macromolecules pass through these larger pores. Recent refinements of this model have demonstrated how fluid and solute movement through these pores may be modified by the interstitial matrix density (Rippe and Venturoli, 2007). The structure of the peritoneal membrane is critical in determining the success or failure of peritoneal dialysis; it is clear that changes in the numbers of pores or the thickness of the membrane will significantly affect dialysis adequacy.

1.1(iii) Adequacy of dialysis

Adequacy of dialysis in the peritoneal dialysis patient is defined in terms of small solute clearance; fluid removal is also of major importance to patient well-being. Creatinine clearance is measured in litres per week and urea clearance as a weekly Kt/V, where Kt is the dialysate urea content/serum urea and V the total body water, usually derived by formulae such as Watson's (Daugirdas *et al.*, 2001). Ultrafiltration is measured using a timed fluid exchange with a defined dialysate glucose concentration. Clinical outcome data has demonstrated a clear relationship between total (peritoneal and residual renal) clearance and patient outcomes (Churchill, 1998), but prospective data from the ADEMEX trial indicated that above a minimum threshold, increasing peritoneal clearance had no outcome benefit (Paniagua *et al.*, 2002); further investigation in anuric APD patients (the EAPOS trial) confirmed this finding (Brown *et al.*, 2003), (Davies and Brown, 2007) and highlighted the importance of adequate ultrafiltration in this group. Residual renal function contributes significantly to overall clearances in many peritoneal dialysis patients and it is residual function which underlies the relationship between total clearance and clinical outcomes.

1.1(iv) Quantifying peritoneal membrane transport characteristics

The time course of fluid and solute movement across the peritoneal membrane is complex and dependent on the physical characteristics of the peritoneal membrane in a particular patient. In clinical practice a standardised assessment of peritoneal membrane transport characteristics is performed - the Peritoneal Equilibration Test (PET) - as developed by Twardowski (Zbylut *et al.*, 1987). For this test a standard glucose concentration bag is instilled for 4 hours and samples of dialysate taken at time 0, 2 and 4 hours. A blood sample is taken at the mid-point of the dwell to measure urea, creatinine and glucose. Small solute transfer is expressed as the ratio of dialysate to plasma creatinine (D/P creatinine) at 4 hours. Historically a 2.27% glucose solution has been widely used but a 3.86% solution is now advocated, which allows more detailed assessment of ultrafiltration capacity since the volumes involved are larger (Mujais *et al.*, 2000). In our institution a 2.27% glucose solution has been used for PET tests throughout the duration of this study. The D/P creatinine result is stratified into four groups – low, low average, high average and high – for clinical purposes. In general high transporters clear creatinine and other small solutes quickly, but experience rapid dissipation of the osmotic gradient for ultrafiltration as glucose is rapidly absorbed. Achieving adequate ultrafiltration may be difficult with the prolonged dwell times of CAPD, since fluid will be reabsorbed from the dialysate later in the dwell. Low transporters will have good UF volumes but will need prolonged dwell times to achieve adequate small molecule clearances. These issues are clinically significant; data from the CANUSA study, derived from patients on CAPD, showed an increased risk of mortality in patients with a high transport rate (Churchill, 1998). The use of APD mitigates this risk (Brown *et al.*, 2003), which may be manifest through the failure to achieve adequate ultrafiltration (Davies *et al.*, 2006).

More detailed information about solute and water transfer can be obtained by using an intra-peritoneal volume marker such as dextran70 and serial measurements of dialysate electrolytes and small solutes – the standard peritoneal permeability analysis. The degree of dilution of dextran70 can be used to calculate the initial ultrafiltration rate. This represents convective movement of fluid and solute through the small pores and osmotic fluid movement through small pores and aquaporin. Because water moving via the aquaporin channels is not accompanied by any solute movement, the dialysate sodium concentration shows an initial fall. It then increases later in the dwell as fluid resorption occurs with loss of the glucose-dependent osmotic gradient. This phenomenon is called sodium sieving and is maximal between and one and two hours into the dwell. The fall in dialysate sodium can be used to calculate the component of total ultrafiltration which is due to free water transfer, although a small correction is required to estimate the effect of sodium diffusion on dialysate sodium concentration (Krediet *et al.*, 2008). Other parameters describing solute and fluid

transport characteristics, such as the membrane reflection coefficient for glucose, can also be calculated. An ideal osmotic agent has a reflection coefficient of 1; for the peritoneal membrane the reflection coefficient for glucose is 0.03. Although aquaporins make up a small proportion of the peritoneal membrane area the reflection coefficient for glucose across aquaporins is much higher and aquaporin-mediated free water transfer makes up a significant (about 50%) contribution to total ultrafiltration.

1.2 Changes in membrane structure and function during PD therapy

1.2(i) Histology

Histologically the peritoneum consists of a mesothelial cell layer and the underlying tissues. Structural changes develop in the peritoneal membrane during time on PD. Williams et al (2002) demonstrated that there is progressive thickening of the submesothelial fibrous level secondary to uraemia and the use of peritoneal dialysis . Non-uraemic patients had a peritoneal thickness of 50µm; this increased to 140µm with uraemia (without peritoneal dialysis) and over 700µm in patients who have been on peritoneal dialysis for more than 7 years (Williams *et al.*, 2002). Fibrin deposition is seen with large proliferative fibroblasts (Honda and Oda, 2005). There is also an increase in the surface area of peritoneal blood vessels (Numata *et al.*, 2003) and a vasculopathy (Sherif *et al.*, 2006) which is similar to that seen in diabetes. These structural changes have functional implications: in a series of papers Davies demonstrated that there are longitudinal changes in membrane function with time on PD and particularly with cumulative glucose exposure (Davies *et al.*, 1996; Davies, 2004; Davies *et al.*, 2001); this was also seen in the EAPOS cohort (Davies *et al.*, 2005). Increasing solute transport status, with resultant loss of ultrafiltration capacity, is a prominent cause of technique failure (Kawaguchi *et al.*, 1997), (Heimbürger *et al.*, 1990) and correlates with blood vessel area (Numata *et al.*, 2003). The structural changes may also render the vessel more permeable to macromolecules; permeability to $\beta_2$microglobulin also correlates with blood vessel area (Numata *et al.*, 2003). Together these structural changes are termed peritoneal sclerosis. Peritoneal sclerosis does not cause abdominal symptoms; the impact on the patient is due to the loss of dialysis adequacy as the structural change of peritoneal membrane affects function.

Epithelial to mesenchymal transition: One of the key histological changes is the epithelial to mesenchymal transition of the cells forming the mesothelial cell layer. This may be the first step in peritoneal damage. Cultures of mesothelial cells derived from peritoneal effluent can take an epithelioid, transitional or fibroblast-like form and the pattern observed varies with the time on PD (López-Cabrera *et al.*, 2006; Yáñez-Mó *et al.*, 2003). Within the first two years on PD, before the development of vasculopathy and often before much submesothelial thickening has occurred,

submesothelial cells are present in peritoneal biopsies which express both epithelial (cytokeratin) and mesothelial (α-smooth muscle actin) markers. Combined with loss of the mesothelium this is suggestive of epithelial-to-mesenchymal transition (EMT) (Del Peso *et al.*, 2008). The process of EMT was reviewed by Aroeira et al; during EMT, peritoneal mesothelial cells lose tight junctions, adherent junctions, desmosomes and cellular polarity. The cell cytoskeleton is reorganised and the cell takes on mesenchymal morphology and expresses mesenchymal markers. There is degradation of the basement membrane and migration of cells into the interstitium (Aroeira *et al.*, 2007). In a study of peritoneal biopsies the presence of myofibroblasts, positive for the mesenchymal marker α smooth muscle actin (α-SMA), was noted in biopsies from peritoneal dialysis patients. In addition modified mesothelial cells were seen, also expressing α-SMA, which suggests the source of the myofibroblasts was the mesothelium (Jiménez-Heffernan *et al.*, 2004). EMT is recognised as part of the pathology in renal fibrosis, where TGF-β is thought to be an important mediator (Liu, 2004). Using adenovirus-mediated transfer of TGF-1β to the rodent peritoneum it has been shown that peritoneal fibrosis and EMT can be induced (Margetts *et al.*, 2005). There are likely to be multiple upstream and downstream mediators involved in this process – for example, although advanced glycosylation end-products (AGE) are implicated, blockade of the AGE receptor only partly reduces peritoneal fibrosis in a uraemic rat model (De Vriese *et al.*, 2006). Methylglyoxal, a glucose degradation product found in the dialysate, can also induce EMT (Hirahara *et al.*, 2009), but it is not clear whether this is via advanced glycosylation end-product (AGE)-mediated activation of the AGE receptor, or via a direct oxidant action. Other candidate factors involved in the causation of EMT include angiotensin II, fibroblast growth factor and connective tissue growth factor (CTGF) (Liu, 2010).

The precise molecular pathways that mediate EMT are the subject of investigation and are likely to be multiple. For example, smad3 knock-out mice were protected from fibrosis and angiogenesis induced by TGF-β, but an attenuated EMT was still observed, suggesting a parallel pathway not involving smad3 (Patel *et al.*, 2010). TGF-β may also act via smad2, Notch (Zhu *et al.*, 2010) and RhoA (Liu, 2004). Other pathways, such as the integrin and β-catenin signalling pathways may also be important (Liu, 2010). In the renal tubule cell model, production of matrix metalloproteases (MMP) is necessary for EMT to occur (Cheng *et al.*, 2006) and inhibition of MMP2 had a beneficial effect on fibrosis and angiogenesis in a chlorhexidine-induced peritonitis model (Ro *et al.*, 2007). Matrix metalloproteases may act by disruption of the basement membrane, permitting cell migration and invasion of the interstitium.

1.2(ii) Encapsulating peritoneal sclerosis (EPS)

EPS is an uncommon but devastating complication of long term PD in which there is massive deposition of fibrin on the surface of the bowel, forming a thick encasing layer. The incidence increases with time on PD (Maruyama and Nakayama, 2008) and hence much of the literature originates from Japan, where there is a large cohort of long term PD patients. The clinical consequences are severe with the development of bowel obstruction associated with vomiting, ascites and abdominal pain. Malnutrition and septicaemia may lead to death. Total parenteral nutrition may be required; surgical release of bowel from the fibrotic cocoon is technically highly demanding and is successful only in specialist hands (de Freitas *et al.*, 2008; Kawanishi *et al.*, 2008). Whether EPS represents an extension of the sclerotic process seen in long term peritoneal dialysis, or whether it is a separate entity, has been debated (Nakayama *et al.*, 2005; Garosi *et al.*, 2006). It is not unique to peritoneal dialysis patients, being associated with the use of practolol (Eltringham *et al.*, 1977), intra-peritoneal contamination with chlorhexidine (Lo *et al.*, 1991) and is seen in patients with chronic ascites (Mekeel *et al.*, 2009). The frequently seen changes of peritoneal sclerosis and EMT seen in most long term PD patients fortunately do not usually progress to EPS and it is unclear what triggers some patients to develop the severe, symptomatic fibrosis of EPS. Honda and Oda proposed a "two-hit" model, in which the abnormal peritoneum post-long term PD undergoes a second, inflammatory, insult which triggers EPS. They suggest that the severity of the second insult required to cause EPS would vary depending on the level of chronic damage which had already occurred (Honda and Oda, 2005).

The source of fibrin may be the abnormal blood vessels seen in the peritoneum, but it is not clear how closely the processes of neoangiogenesis and fibrosis are linked. Margetts et al were able to reduce fibrosis (using the TGF-β inhibitor decorin) and angiogenesis (using angiostatin) selectively (Margetts *et al.*, 2002). The cessation of PD itself aids fibrin deposition as there is no longer wash-out of fibrin by the process of dialysis. This has led some to advocate continued peritoneal lavage after change in renal replacement modality to attempt to reduce the risk of EPS (Yamamoto *et al.*, 2010); there are no randomised trials and the risk of peritonitis is a concern.

There are currently no validated screening methods to identify which patients will develop EPS, although a number of risk markers (such as very severe peritonitis and duration of PD) are recognised (Yamamoto *et al.*, 2005). The overall incidence of EPS in PD patients is low (1.8 per 1000 patient years in the Australian data (Johnson *et al.*, 2010) and 2.5% of patients in the Japanese EPS Registry (Kawanishi and Moriishi, 2005)), but after 8-10 years of peritoneal dialysis as many as 20% (Brown *et al.*, 2009a; Brown *et al.*, 2009b) of patients may develop EPS. The incidence of EPS in the Japanese data after 3, 5, 8, 15 and longer than 15 years on PD was 0%, 0.7%, 5.9%, 8.6% and 17.2%,

demonstrating the effect of PD duration. EPS frequently manifests clinically after change of renal replacement modality – whether to haemodialysis or, perhaps increasingly, post transplantation (Fieren *et al.*, 2007; Korte *et al.*, 2007). Early elective modality change remains controversial: haemodialysis is not possible in all patients, and not all countries offer both peritoneal and haemodialysis due to resource constraints. Changing to haemodialysis after a set period of PD is not advocated by the ISPD for all patients (Brown *et al.*, 2009a) as some, particularly the elderly and those not suitable for transplantation, may be better remaining on PD. Conversely, a young patient who has chosen PD to accommodate work commitments may be reluctant to change dialysis modality despite an increasing risk of EPS – yet there is limited information available which would allow assessment of individual EPS risk and tailored advice.

EPS is a serious condition. The mortality as well as the incidence increases with the preceding PD duration; in the Japanese registry no patient who had performed PD for less than five years died of EPS but after eight years on PD those patients who developed EPS had a mortality of 8.3%, increasing to 28.6% when EPS followed ten years of PD, 61.5% after fifteen years and 100% if more than fifteen years of PD had preceded EPS. The Pan-Thames group found 53% of 111 EPS patients died (again risk of death was related to duration of PD) (Balasubramaniam *et al.*, 2009). EPS can be a particularly bitter blow post transplantation. In a recent large study of all (1241) transplanted PD patients in the Netherlands (Korte *et al.*, 2011), the incidence of EPS was only 3% but nearly half of these patients died during the follow-up period (compared to 16% of non-EPS transplant patients).

1.2(iii) Ultrafiltration failure and loss of dialysis adequacy

PD patients can experience failure of dialysis technique after a variable period on PD, so that even increasing the amounts of dialysis fluid used and optimising the timing of exchanges does not confer adequate clearance of solutes, salt and water. Loss of ultrafiltration (along with peritonitis) is one of the leading causes of technique failure. Serial PET results can give some warning of loss of dialysis adequacy, which may be impaired small solute clearance or more frequently loss of ultrafiltration, facilitating timely modality change before significant symptoms develop. The current definition of ultrafiltration failure is less than 400mls ultrafiltration from a four hour dwell with 3.86% glucose (Mujais *et al.*, 2000). This phenomenon has been considered to have four possible underlying mechanisms which reduce the net transfer of fluid from patient to dialysate (Krediet *et al.*, 2000):

1. A large vascular surface area – this results in a high transport state and rapid loss of the osmotic gradient for fluid transfer

2. A decreased osmotic conductance to glucose. The osmotic conductance is related to the hydraulic permeability of the membrane, the surface area and the reflection coefficient for glucose. It would be expected to decrease with aquaporin loss or membrane fibrosis.
3. Increased re-absorption of fluid via lymphatic channels
4. Small peritoneal area – rare, and could be caused, for example, by adhesions

When a standard peritoneal permeability analysis is performed on patients who have ultrafiltration failure the sodium sieving is lost and the reflection coefficient is reduced, suggesting that aquaporin dysfunction may be involved in ultrafiltration failure (Smit *et al.*, 2004). Other factors reducing ultrafiltration are a high MTAC (mass transfer area coefficient, the maximal theoretical diffusive clearance of a substance at t=0) with rapid loss of the osmotic gradient for absorption and high effective lymphatic absorption rate. A high MTAC for creatinine and urea is associated with a high MTAC for glucose and rapid dissipation of the osmotic gradient. However La Milia et al found reduction of ultrafiltration was present even in the early stages of a dwell (La Milia *et al.*, 2007). This would not be expected if the structural change was predominantly neo-vascularisation leading to increased small solute transfer rate, since early in the dwell there would be increased ultrafiltration. The incidence of ultrafiltration failure increases with increasing duration of PD but lymphatic re-absorption has not been shown to increase consistently with duration of PD (Michels *et al.*, 2004), making it unlikely that increased lymphatic resorption is the major factor. Analysis of ultrafiltration in long term (>5 years) patients by Parikova et al showed a low total ultrafiltrate volume and reduced free water transport. The osmotic conductance to glucose was reduced in this group, without any change in the reflection coefficient (Parikova *et al.*, 2006). Modelling by Rippe et al (Rippe and Venturoli, 2007) has shown how an increase in fibrosis in the interstitium can lead to these changes in membrane permeability: diffusion is related to the square of pore size but convective transport is related to pore size to the power four. A decrease in the interstitium pore size due to fibrosis thus affects small solute diffusive movement less than convective movement.

It appears that loss of ultrafiltration is a complex process, sometimes related to an increase in membrane transport status, but the underlying pathology may differ from one patient to another and different structural changes such as fibrosis, neo-angiogenesis or loss of aquaporin function may be responsible. Hence clinical markers alone may not be sufficient to predict either the timing of technique failure or the later development of EPS. It is possible that a biochemical marker which predicted incipient ultrafiltration failure might also predict future EPS, since some aspects of the pathology are similar. However it is important to note that patients with poor ultrafiltration may be particularly heavily exposed to high dialysate glucose levels and large dialysate volumes and that

these factors may predispose to both worsening membrane function and also to EPS after the cessation of PD.

1.2(iv) Predicting EPS risk and technique failure

For the above reasons, a clinical, radiological or biochemical screening tool which would aid the clinical decision to change dialysis modality could be of great value. However, at present there are no screening tools available to clinicians. There are potential problems with the use of peritoneal biopsy and CT scanning has not been validated as a screening tool. Although clinical features are useful, there are no factors which can be used to reliably predict EPS in the individual patient, as discussed below.

Biopsy: As well as being an invasive procedure, biopsy findings do not differentiate between asymptomatic PD patients and EPS patients. When biopsy specimens from 12 EPS patients were compared with those of non-EPS patients, perivascular bleeding, capillary angiogenesis, inflammatory mononuclear cells and swollen, proliferating fibroblasts were seen in both groups (Honda *et al.*, 2003). Immunohistochemical markers of peritoneal sclerosis such as α-smooth muscle actin (α-SMA), fibroblast growth factor and macrophage inhibitory factor were also investigated. The expression of these markers was more prominent in the EPS patients, but all the markers could be found in the non-EPS group. Fibrosis is a patchy process and biopsy of a limited region of parietal peritoneum may not be representative of the visceral peritoneum. It is therefore difficult to distinguish EPS from uncomplicated peritoneal sclerosis by biopsy alone.

Radiology: It is not currently known whether radiological screening by CT scanning can predict incipient EPS and facilitate timely modality change. Tarzi et al (Tarzi *et al.*, 2008) examined the role of CT scans in the diagnosis of EPS, using a scoring system to assess CT features of EPS such as peritoneal thickening, peritoneal calcification, bowel wall thickening, bowel dilatation and bowel tethering. CT was found to be a useful adjunct in the diagnosis of EPS, with good inter-observer variability between radiologists. Other imaging modalities such as MRI (Hüser *et al.*, 2006) and cine MRI (Wright *et al.*, 2011), fluorodeoxyglucose positron emission tomography (Tarzi *et al.*, 2006) and contrast studies of the small bowel (Hur *et al.*, 2004) have been used to image EPS patients and PD patients but are much less readily available. CT scanning is the imaging modality of choice in most PD units when EPS is suspected on clinical grounds. However it was noted by Tarzi et al that many of the patients with a firm diagnosis of EPS had had CT scans within the last year which did not show any changes. It is therefore important to examine the issue of whether CT screening for EPS, which is widely practised in PD units, has any basis by examining a larger group of pre-diagnostic CT scans.

Clinical features: little is known about symptoms experienced by patients on peritoneal dialysis. Available evidence, such as that from the NECOSAD population (Thong *et al.*, 2009; Ring *et al.*, 2000) suggests that physical symptoms are common and do not always correlate with expected clinical variables. The Dialysis Symptoms Index (DSI) has been used in a study of haemodialysis patient symptoms (Weisbord *et al.*, 2005) and it was demonstrated that the format used (a Likert scale where 1= "not at all bothersome" and 5= "bothers very much") showed good test/re-test repeatability (Weisbord *et al.*, 2004). A similar set of symptoms was used in the MDRD cross-sectional symptoms survey (Rocco *et al.*, 1997), although the emphasis was perhaps more firmly on physical symptoms. To establish the frequency of physical symptoms in our PD population we developed a modified version of the DSI (see Appendix A), which had a greater emphasis on abdominal symptoms. Each symptom was scored for frequency and severity. Ethical approval was obtained and questionnaires were administered at routine PD clinic visits by the peritoneal dialysis nursing team.

The purpose was to establish the frequency of symptoms in the peritoneal dialysis population and to determine whether abdominal symptoms are frequent in stable peritoneal dialysis patients. Clearly if abdominal symptoms are very common it is unlikely that the presence of minor abdominal symptoms could be used as a risk marker for future EPS. We found that abdominal symptoms were not infrequent in stable PD patients, but not usually severe (data presented at EuroPD 2009, Strasbourg; see Appendix C); thus monitoring the patient population for new onset abdominal discomfort, distension, constipation and loss of appetite may at least provide an early warning of more severe symptoms. However even if only mild symptoms have become apparent in a stable PD patient symptomatic EPS might occur later, after cessation of PD; more marked abdominal symptoms may be a late warning sign and therefore of little use as a screening tool.

Other clinical data such as membrane functional changes - ultrafiltration failure and high membrane transporter status - may correlate with symptoms and relate to technique failure, but have not always successfully predicted which patients will develop EPS. The risk of EPS was found to be high (50%) in a group of patients who continued on PD for more than three years after a diagnosis of ultrafiltration failure (Sampimon *et al.*, 2010a) (in general this would not be usual clinical practice). The membrane transport characteristics of this group of patients with ultrafiltration failure were studied in detail and the only potential marker for future EPS was a low effective lymphatic absorption rate. This test requires the use of an intra-peritoneal volume marker and is not currently part of routine peritoneal membrane assessment. The Stoke group noted an early disproportionate decrease in ultrafiltration capacity, with only a later increase in dialysate to plasma creatinine ratios,

in nine pre-EPS patients and hypothesise that this pattern of change in peritoneal function is due to fibrosis and hence may predict EPS (Lambie *et al.*, 2010).

The current lack of well-defined screening tools and predictors of EPS has prompted interest in a biochemical marker of EPS. In addition, finding a biochemical marker for EPS risk may help elucidate the pathogenesis of this condition and hence lead to a method of preventing or treating EPS in the future.

1.3 Potential causative factors for changes in peritoneal membrane function and EPS

1.3(i) Glucose

One of the most important factors in the development of peritoneal fibrosis and vasculopathy is likely to be the effect of chronic exposure to the high glucose concentrations found in dialysate. Historically glucose has been used as the osmotic agent to promote ultrafiltration of fluid into the dialysate. Data from the European APD Outcomes Study showed that patients with greater exposure to glucose experienced more rapid changes in membrane transport function and decline in ultrafiltration capacity (Davies *et al.*, 2005). Dialysate is heat treated to sterilise it and this process generates glucose degradation products (GDPs) such as methylglyoxal and 3-deoxyglucosone. These reactive carbonyl compounds may themselves be toxic to the peritoneum as well as mediating the production of advanced glycosylation end-products (AGEs). In a rat model the GDP methylglyoxal was shown to increase the formation of matrix metalloprotease (MMP), collagen IV and vascular endothelial growth factor (VEGF) (Hirahara *et al.*, 2009). High concentrations of methylglyoxal even induced the formation of an abdominal cocoon, the key feature of EPS. Although the concentrations required were higher (20mmol) than those typically found in PD fluid (range 2-33μmol), the duration of exposure was much shorter. Intraperitoneal N-acetyl cysteine acts to prevent the formation of reactive oxygen species by GDPs and alleviates the increases in TGF-β, VEGF and collagen I production caused by conventional dialysate (Noh *et al.*, 2006).

AGEs are formed by non-enzymatic glycosylation of proteins; a reducing sugar condenses with a free amino acid to form a Schiff base. Rearrangements produce a stable ketamine called an Amadori adduct. Dehydration and fragmentation then result in the formation of stable covalent adducts; these are AGEs. Rapid AGE formation occurs if a protein reacts with a glucose auto-oxidation product such as methylglyoxal (Mandl-Weber *et al.*, 2001), but high glucose concentrations alone can result in the accumulation of AGEs. AGE are known to be formed in dialysate (Lamb *et al.*, 1995)

and are found deposited in the abnormal microvasculature of the peritoneum. This deposition is more marked in patients with low ultrafiltration (Honda *et al.*, 1999). This may be due to the increased amounts of glucose exposure needed to achieve adequate ultrafiltration in these patients, but it seems possible that AGE are involved in the generation of fibrosis and hence are causative in the development of ultrafiltration failure.

Further evidence suggests that AGEs are important in peritoneal fibrosis; de Vriese et al showed that in uraemic (sub-total nephrectomised) rats there were high levels of AGE and the AGE receptor RAGE. Peritoneal fibrosis had developed by the time the rats were sacrificed at 6 weeks. Histologically, uraemic rats had developed EMT, with co-staining of cytokeratin and alpha SMA seen in the submesothelial layer; these changes were reduced if an anti-RAGE antibody was administered (De Vriese *et al.*, 2006). Anti-RAGE antibody also partially blocked the uraemic up-regulation of TGF-β. AGE also promote neo-angiogenesis via the release of VEGF from endothelial cells (Boulanger *et al.*, 2007).

Glucose metabolism via the polyol pathway and by glycolysis generates reduced nicotinamide adenine dinucleotide (NADH) and increases the NADH/NAD^+ ratio (the ratio of reduced to oxidised nicotinamide adenine dinucleotide). This mimics cellular ischaemia (pseudohypoxia) and prompts angiogenesis (van Westrhenen *et al.*, 2008). Lactate buffer in the dialysate also increases flux via the polyol pathway since the conversion both of pyruvate to lactate and NADH to NAD^+ are reduced. Glucose induces the pro-fibrotic mediator TGF-β and the chemokine MCP-1 in mesothelial cells, possibly by the induction of aldose reductase and the intracellular accumulation of sorbitol (Wong *et al.*, 2003). This pathway is implicated in the generation of diabetic complications and the vasculopathy seen in the peritoneum resembles the pathological changes of diabetic vasculopathy.

1.3(ii) Lactate and other causes of poor dialysate biocompatibility

Standard dialysate bags have an acidic pH and contain lactate as a buffer; both of these factors may cause damage to the peritoneum and more modern solutions have utilised a pyruvate buffer and a more physiological pH (van Westrhenen *et al.*, 2008; Chan and Yung, 2007). There is evidence that more biocompatible solutions may have benefits in terms markers of inflammation in the dialysate and systemic circulation: Pajek et al used a randomised cross-over design to investigate the effects of changing between “conventional” dianeal and the more biocompatible physioneal. They found increased mesothelial cell HLA expression in the physioneal group, which they hypothesise is a marker of mesothelial cell well-being. Ca 125, which is a marker of mesothelial cell mass, was found at higher levels in the dialysate using physioneal and dialysate IL-6 levels were lower. No differences were found in the serum levels of CRP or IL-6, which may reflect the fact that there are many other

factors driving inflammation in dialysis patients – uraemia, infection and carbonyl stress, for example (Pajek *et al.*, 2008). Clinical studies show a similar D/P creatinine with the conventional and newer fluids (Choi *et al.*; Fang *et al.*) but ultrafiltration is sometimes less. Where bicarbonate is used with pyruvate as a buffer this would be expected because bicarbonate has a lower reflection coefficient than lactate, reducing the effective osmolarity. Inflow pain is consistently less. There is some evidence that peritoneal immune function is improved (Libetta *et al.*, 2011), and one single centre study has seen a reduction in peritonitis rates (Ahmad *et al.*, 2006); this was however not confirmed in the Euro-Balance study (Williams *et al.*, 2004), a multicentre cross-over trial.

1.3(iii) Uraemia

Even if PD is never used, uraemia itself is associated with thickening of the mesothelial zone. This was demonstrated by studies of peritoneal biopsies (Williams *et al.*, 2002); there is also a vasculopathy seen in association with uraemia. Chronic uraemia alters peritoneal permeability. This may occur due to the increased carbonyl stress associated with uraemia (Miyata *et al.*, 2000) and alterations in nitric oxide synthase (NOS) expression (Combet *et al.*, 2001).

1.3(iv) Infection

The role of infection – peritonitis – in the development of peritoneal damage and hence failure of peritoneal dialysis seems intuitive. However systematic review of available RCTs by Wiggins et al (Wiggins *et al.*, 2007) did not find evidence that repeated episodes of peritonitis had a measurable impact on mortality. Nor is there a higher frequency of peritonitis in patients who develop EPS than in those who do not (Johnson *et al.*, 2010). In fact many EPS patients have no history at all of peritonitis (Johnson *et al.*, 2010; Brown *et al.*, 2009b; Balasubramaniam *et al.*, 2009). This may be because EPS is associated with a long duration of PD and those patients who repeatedly develop peritonitis may be withdrawn from PD at an earlier stage. Although EPS may not be associated with a high total number of peritonitis episodes, in an individual patient an episode of peritonitis may accelerate changes in peritoneal membrane structure. Peritonitis triggers the release of inflammatory cytokines such as interleukin 1-β (IL-β) and tumour necrosis factor α (TNFα). This increases the production of TGF-β by mesothelial cells above the basal level induced by chronic dialysate exposure (Kang *et al.*, 1999). If peritonitis is recurrent, levels of cytokines are higher at each subsequent infection, with a sustained alteration in peritoneal cytokine regulation (Fielding *et al.*, 2008). Certain pathogens are regarded as both difficult to treat and more likely to lead to technique failure – fungi in particular (Lui *et al.*, 2007). Gram negative organisms are also associated with increased risk of adverse outcome (Szeto and Chow, 2007). The duration of unresolving peritonitis

seems to be the key factor, with white cell count and CRP at 72 hours post onset of peritonitis being predictors of adverse outcomes (Moon *et al.*, 2008).

1.4(v) Angiotensin

The angiotensin system is well established in the pathogenesis of fibrosis in the renal and cardiac systems (Kim *et al.*, 2011; Kagami, 2011; Shahbaz *et al.*, 2010).

The use of angiotensin blockade to modify the progression of peritoneal membrane changes has been investigated by several groups. Nakamoto et al used olmesartan in a rat model of peritoneal fibrosis, and found that animals which had olmesartan added to an acidic dialysate did not develop the peritoneal inflammation and fibrosis seen in control rats (Nakamoto *et al.*, 2008). Similarly Chun-Yen Ke et al showed that aliskiren reduced peritoneal fibrosis and TGF-β production in a rat model of chlorhexidine induced peritoneal fibrosis (Ke *et al.*, 2010). Angiotensin was shown by Chang et al to induce EMT in rat peritoneal mesothelial cells, an effect which could be attenuated by either losartan (an angiotensin receptor blocker) or NADPH oxidase inhibitors (Chang *et al.*, 2011). This suggests that the effects of angiotensin may be mediated through the production of reactive oxygen species. Noh et al also found that either anti-oxidants or losartan reduced peritoneal membrane thickening, TGF-β production and VEGF production in a rat model of peritoneal dialysis (Noh *et al.*, 2006). The same group found that human peritoneal cells express all the components of the renin-angiotensin system and application of angiotensin II increases the expression of TGF-β, fibronectin and cellular reactive oxygen species (Noh *et al.*, 2005). Inhibition of oxidant activity prevented the increase in TGF-β, as did losartan. Thus beneficial effects of angiotensin blockade may occur via attenuation of ROS production; however, an alternative mechanism of action was proposed by Sauter et al (Sauter *et al.*, 2007), who found that angiotensin converting enzyme inhibitors and angiotensin receptor blockers reduced both the basal level of VEGF production from human mesothelial cells and the increase in VEGF production which occurs when peritoneal cells are stimulated by the inflammatory cytokines TNFα and interleukin-1α. Angiotensin may therefore be involved in the development of neoangiogenesis in the peritoneum as well as contributing to fibrosis.

An observational study by Kolesnyk et al (Kolesnyk *et al.*, 2009) looking at peritoneal membrane characteristics in the NECOSAD (Netherlands Cooperative Study on Adequacy of Diabetes) population found that the use of ACE inhibitors or angiotensin receptor blockers reduced the increase in dialysate/plasma creatinine ratio seen over time in peritoneal dialysis patients.

1.3(vii) Fluid flow stress

During peritoneal dialysis there will be an impact of the dialysate fluid on the peritoneal cells which is related to fluid flow stress, generated by patient movements and peristalsis. Aoki et al (Aoki *et al.*, 2011a; Aoki *et al.*, 2011b) investigated the response of rat peritoneal mesothelial cells grown under conditions of enhanced fluid shear stress (cultured in a rotary shaker). The cells cultured in the shaker showed mesothelial cell hypertrophy and hypercellularity, whatever the concentration of glucose in the culture medium, whereas those not exposed to fluid stress only developed mesothelial expansion when a high glucose (4500mg/L) culture solution was used. EMT was demonstrated in all cells cultured under the fluid stress conditions. The mechanism of mesothelial hyperplasia was thought to be via the inhibition of the p38 mitogen activated kinase (MAP kinase) pathway by fluid stress. Of note, the authors hypothesised that the effects on the peritoneal MAP kinase system might be different under the influence of dynamic as compared to static cultures (where low glucose was associated with lower levels of MAP kinase activity). The effects of fluid flow stress on protein phosphatase activation were found to persist after reversion to static culture conditions; thus previous fluid flow stress might continue to pose a risk of EPS for some time after peritoneal dialysis has ceased. The role of fluid stress in the generation of peritoneal fibrosis may explain the observation that some patients with cirrhotic ascites develop encapsulating peritoneal sclerosis with no history of exposure to PD.

1.4 Cytokine mediators of peritoneal fibrosis and vasculopathy

A large number of cytokines have been suggested as potential mediators of the processes of peritoneal fibrosis and vasculopathy. Hence these cytokines are also candidates as biomarkers for the development of EPS and for technique failure. Some, such as vascular endothelial growth factor (VEGF), TGF-β and interleukin-6 (IL-6) are well known to have roles in angiogenesis and inflammation. IL-6, VEGF and cancer antigen 125 (ca125) have already been investigated as biomarkers of EPS (Sampimon *et al.*, 2010b). It was found that VEGF did not predict EPS risk, but a combination of low levels of ca125 and high levels of IL-6 had a specificity of 89% for later EPS. However the sensitivity of this test was only 70%. The actions of TGF-β, VEGF and IL-6 within the peritoneum are first summarised and then the possible actions of less extensively investigated cytokines are discussed.

1.4(i) Transforming growth factor β (TGF-β)

TGF-β is established in the literature as a potential cause of peritoneal membrane changes. TGF-β is implicated in epithelial to mesenchymal transition (EMT), one of the earliest histological changes in the peritoneal membrane (see section **1.2**). In addition it has been shown to have a role in fibrosis and angiogenesis. Using an adenovirus gene transfer model, Margetts et al showed that TGF-β causes submesothelial fibrosis, increased matrix production and increased VEGF production (Margetts *et al.*, 2001). Part of the angiogenic response to TGF-β may be the hypoxic conditions in induced fibro-proliferative material. Sekiguchi et al showed that there is both direct TGF-β induced VEGF production and hypoxia induced VEGF; the hypoxia-induced pathway is mediated by hypoxia inducible factor (HIF)1α (Sekiguchi *et al.*, 2011).

TGF-β production by human peritoneal mesothelial cells is increased by high glucose concentrations and this then leads to an increase in fibronectin levels which can be blocked by anti-TGF-β antibodies (Medcalf *et al.*, 2001). This shows that TGF-β is one possible link between glucose exposure and changes in the peritoneal membrane. Antagonism of the action of TGF-β by bone morphogenetic protein 7 (BMP-7) via smad-5 prevents EMT in a rodent model; antagonism of the action of TGF-β by blocking peptides also preserved mesothelial integrity in a mouse model of peritoneal sclerosis (Loureiro *et al.*, 2011).

It is difficult to demonstrate any association between the levels of TGF-β measured in spent dialysate and clinical outcomes. This may be because TGF-β is found in both an active and inactive form and total TGF-β levels do not reflect TGFβ activity adequately. Additionally there are three forms of TGF-β and two major families of receptors. TGF-β signalling is further modulated by the co-factors betaglycan and endoglin (Santibañez *et al.*, 2011), making assessment of overall activity levels more difficult.

1.4(ii) Vascular endothelial growth factor (VEGF)

Levels of VEGF are higher in the dialysate than in serum, suggesting local production (Selgas *et al.*, 2001). VEGF has been shown to be a mediator of angiogenesis and increased capillary density. Human umbilical vein endothelial cells (HuVEC) which are co-cultured with human peritoneal mesothelial cells (HPMC) or with supernatant from HPMC show capillary tube formation that can be prevented by anti-VEGF antibodies (Boulanger *et al.*, 2007). Interestingly a genetic polymorphism of VEGF has been shown to have an impact on the longitudinal changes in peritoneal transport – patients with the genotype of note had high dialysate VEGF levels and a progressive increase in transport status (Szeto *et al.*, 2004), indicating the clinical relevance of VEGF levels. In a rat model in which streptozocin was given to cause diabetes, an increase in peritoneal transport was seen which

was blocked by anti-VEGF antibodies. Anti-VEGF antibodies prevented the formation of new irregularly arranged dense vascular networks in the peritoneum and blocked an increase in membrane albumin permeability (De Vriese *et al.*, 2001). This suggests that VEGF could mediate the changes in blood vessels secondary to the chronic exposure to high dialysate glucose concentrations. Glycated albumin also increases the amount of VEGF mRNA production by human peritoneal cells (Mandl-Weber *et al.*, 2002), and activation of the mesothelial receptor for AGE by AGEs enhances VEGF release (Boulanger *et al.*, 2007). There is evidence that VEGF production increases in mesothelial cells that have developed a non-epithelioid morphology as part of the process of EMT, thus providing a link between the fibrotic and the vasculopathic elements of peritoneal damage. The MTAC creatinine has been shown to correlate with VEGF levels (Aroeira *et al.*, 2005), suggesting VEGF has a role in the development of an increased vascular surface area. Measurement of total VEGF levels could be complicated by the existence of multiple splice variants of VEGF, some of which may have anti-angiogenic effects (Woolard *et al.*, 2004).

1.4(iii) Interleukin-6 (IL-6)

IL-6 has an established role as a mediator of both acute and chronic inflammation. In both peritoneal and haemodialysis patients systemic IL-6 levels are a risk factor for mortality (Pecoits-Filho *et al.*, 2002b). IL-6 levels rise as renal function deteriorates due to reduced renal excretion of IL-6; thus systemic IL-6 levels would be expected to rise with duration of peritoneal dialysis as the majority of patients experience a decrease in residual renal function over a long time period on PD. Raised IL-6 is also seen in fluid overload and congestive cardiac failure and thus again systemic IL-6 levels might be predicted to rise in long term PD patients who had developed ultrafiltration failure and might be raised in such patients without having a direct role in the development of peritoneal membrane failure. Dialysate IL-6 levels are theoretically more likely to be relevant; effluent IL-6 levels have been shown to reflect a chronic inflammatory state in the peritoneum as demonstrated by increased peritoneal permeability to macromolecules (Kawanishi *et al.*, 2005). Both dialysate and plasma VEGF and IL-6 levels are associated with peritoneal transport rate (Pecoits-Filho *et al.*, 2002a) and IL-6 can be found at higher levels in the dialysate than plasma, indicating local production may determine IL-6 levels. IL-6 is produced by peritoneal mesothelial cells (Topley *et al.*, 1993).

A further role has been suggested for IL-6 in the switch from early phase leucocyte responses to chronic inflammation. IL-6 mediated responses are modified and regulated by the soluble IL-6 receptor (sIL-6R). IL-6 binds sIL-6R and this permits it to induce a response from cells which would not normally respond to IL-6 alone. For example, IL-6 levels were shown to be high in dialysate early in the course of peritonitis associated with an influx of neutrophils. The levels of sIL-6R increased at

around 72 hours post diagnosis and this was followed by a change to increasing dialysate mononuclear cell levels and chemokine levels (Hurst *et al.*, 2001).

Mesothelial cells may not possess a cognate IL-6 receptor, since increased chemokine production in response to IL-6 by human peritoneal mesothelial cells was noted only in the presence of soluble IL-6 receptor (Hurst *et al.*, 2001).

Cytokines of interest for further investigation:

Other cytokines are currently less well characterised with respect to any role within the peritoneum. Our group has had an interest in the role of small inflammatory molecules called chemokines (chemotactic cytokines). These molecules have the ability to induce the migration of inflammatory cells into the peritoneum. When a multiplex antibody array was used to investigate the cytokine profile in dialysate, it was noted that some cytokines were present at particularly high levels (Ahmad *et al.*, 2010). Chemokines selected as of potential interest as mediators of the changes in the peritoneum were monocyte chemotactic protein-1 (MCP-1), CCL15 (leukotactin), and CCL18 (also known as pulmonary and activation regulated cytokine, PARC). These chemokines were chosen on the basis of high levels in the dialysate and the known actions of these chemokines, which could plausibly be involved in the development of fibrotic or angiogenic changes in the peritoneal membrane. Angiogenin was also noted to be present in dialysate in the multiplex array; this small protein (as its name suggests) is important in angiogenesis. In the following sections the rationale for the investigation of each of these chemokines in the dialysate effluent and serum of PD patients is discussed.

1.4 (iv) Monocyte Chemotactic Protein (MCP-1)

MCP-1 is a chemokine with monocyte specificity which is readily detectable in the dialysate effluent (Malik *et al.*, 2007). It has been shown that glucose – found at high levels in dialysate to permit ultrafiltration - increases MCP-1 mRNA expression in the supernatant of cell cultures of peritoneal mesothelial cells. This supernatant can be shown to have increased chemotactic ability for monocytes (Lee *et al.*, 2001). In the presence of high concentrations of glucose, glucose is converted to sucrose by aldose reductase (the rate limiting step) and metabolised via the polyol pathway. The effect of hyperglycaemia in causing MCP-1 and TGF-β secretion can be blocked by the addition of aldose reductase inhibitors, suggesting that polyol pathway metabolites are important in the release of MCP-1 and TGF-β (Wong *et al.*, 2003). Lactate (previously used as a buffer in dialysate) promotes the polyol pathway and potentiates the effects of glucose. Hyperosmolality may have a similar but smaller effect to induce MCP-1 (and TGF-β) (Wong *et al.*, 2003).

Thus the high glucose levels found in dialysate might cause high levels of MCP-1 production. MCP-1 could then trigger migration of circulating monocytes into the peritoneum and these may be a source of fibrogenic factors. In support of a role for MCP-1 in the pathogenesis of peritoneal membrane changes, Malik et al (work from Dr Tam's group) found that dialysate MCP-1 correlated with the D/P creatinine, the duration of PD and the number of previous peritonitis episodes (Malik *et al.*, 2007). Dialysate MCP-1 levels are higher than those of many other cytokines and are similar in magnitude to those seen in serum.

MCP-1 is known to have a role in fibrogenesis, for example after lung injury (Moore *et al.*, 2006) and may be important in fibrocyte regulation (Sakai *et al.*, 2006; Wada *et al.*, 2007). In a mouse model of chlorhexidine-induced peritoneal fibrosis, levels of MCP-1 mRNA were up-regulated. A p38 mitogen-activated protein kinase (MAP kinase) inhibitor reduced both the degree of fibrosis and the expression of MCP-1 in a mouse model of peritoneal fibrosis (Kokubo *et al.*, 2011). Similarly a MAP-kinase inhibitor attenuated EMT in light-chain induced renal interstitial fibrosis as well as reducing MCP-1 secretion (Li *et al.*, 2008); in this model EMT was independent of the action of TGF-β.

MCP-1 is a 76 amino acid protein with a molecular weight of 8.7kDaltons in the non-glycosylated form, but is usually found glycosylated with a molecular weight of around 11kDa. As such it is somewhat smaller than albumin, and might potentially pass through the small pores. It could be argued that the high levels of MCP-1 in dialysate originate from the circulation, but other chemokines of a similar size do not have the same high dialysate concentrations as MCP-1. Additionally, mesothelial cells in culture have been shown to produce MCP-1 (Wong *et al.*, 2003), implying that there is local production within the peritoneum. The question would be whether dialysate MCP-1 concentrations are determined by circulating MCP-1 levels or by the amount of MCP-1 produced locally. Dialysate [MCP-1] correlates strongly with transporter status (Malik *et al.*, 2007), which might confer a relationship with circulating MCP-1 levels. Alternatively, circulating [MCP-1] may be influenced by the same factors that predispose to peritoneal fibrosis and increase intraperitoneal MCP-1 levels. Further investigation of MCP-1 levels in dialysate and serum is indicated to establish whether the levels of MCP-1 found in the dialysate are determined by local production or by diffusion across the peritoneal membrane from the circulation, and whether MCP-1 levels in the dialysate can be used to predict the later onset of EPS.

1.4(v) CCL18 (Pulmonary and activation-regulated cytokine, PARC)

CCL18 is a 7.8kDa chemokine produced by monocytes, macrophages and dendritic cells (Schutyser *et al.*, 2005). The receptor is as yet uncharacterised but was thought probably to be a G-protein coupled protein. However in a recent paper by Chen et al (Chen *et al.*, 2011) it was shown that

CCL18 binds to a membrane-associated phosphotidylinositol transfer protein called PITPNM3 on breast cancer cells. The localisation of CCL18 on the cell surface was abrogated when expression of PITPNM3 was blocked using a silencing RNA. Interestingly this potential receptor for CCL18 was not found on investigation of a gastric cancer cell line, so it is possible that CCL18 acts via a different membrane protein in non-breast cancer cells. CCL18 production can be up-regulated by bacterial compounds such as peptidoglycan and lipopolysaccharide (so CCL18 could perhaps be up-regulated by intraperitoneal infection). CCL18 is produced by alternatively activated macrophages and Th2 pattern cytokines such as IL-4 (perhaps as part of a tissue repair response) increase CCL18 production. Within germinal centres CCL18 has a role in the development of both the immune response and also tolerance induction. CCL18 recruits T lymphocytes (Hieshima *et al.*, 1997). Monocytes stimulated by CCL18 differentiate into macrophages with the M2 phenotype (in the absence of IL-4). These cells have increased production of MCP-1 and other cytokines associated with inflammation and the attraction of further inflammatory cells, but also produce the anti-inflammatory cytokine IL-10 (Schraufstatter *et al.*, 2011).

There is some evidence that CCL18 may be involved in angiogenesis; it is produced by early pro-angiogenic cells which are involved in neovascularisation (Urbich *et al.*, 2011) and is found at lower than normal levels in the placentas of women with HELLP syndrome (with endothelial and angiogenic dysfunction) than those with normal pregnancies (Tranquilli *et al.*, 2007).

CCL18 has been detected in numerous pathological conditions in humans (reviewed in (Schutyser *et al.*, 2005)). For example, it is produced by alternatively activated macrophages and involved in chronic inflammation and fibrogenesis in chronic sinusitis (Peterson *et al.*, 2012). In tumours CCL18 can have both an immunoactivating effect (gastric cancer) or be associated with a tumour tolerogenic state with suppression of the host anti-tumour immune response (ovarian cancer). It is also found at high levels in response to staphylococcal infections such as septic arthritis.

However in humans the best characterised and most relevant situation is the role of CCL18 in pulmonary fibrosis/systemic sclerosis. CCL18 acts via an extracellular signal-regulated kinase (ERK) mediated pathway to increase collagen mRNA production in adult lung and dermal fibroblast cells (Atamas *et al.*, 2003). In a mouse lung model, Luzina et al showed that over-expression of CCL18 by an adenovirus vector resulted in T cell infiltration and accumulation of collagen. This increase in collagen was blocked if the T cell influx was abrogated, suggesting that the action of CCL18 was mediated via the T lymphocyte influx (Luzina *et al.*, 2006a). CCL18 may have a direct effect on the fibroblast. CCL18 treated pulmonary fibroblasts do not show an increase in TGF-β protein or mRNA production and TGF-β blocking antibody does not prevent CCL18-mediated up-regulation of collagen production (Luzina *et al.*, 2006b) suggesting CCL18 acts either downstream of TGF-β or via a separate

pathway. Interestingly, as well as the amount of CCL18 in broncho-alveolar lavage fluid, systemic serum levels of CCL18 have been shown to correlate with the activity of lung fibrosis in patients with systemic sclerosis (Kodera *et al.*, 2005). Here it may be a marker of activated alveolar macrophages. Similarly plasma levels of CCL18 produced by glycolipid-laden Gaucher cells in the spleen can be used to monitor the response to treatment in Gaucher's disease (Boot *et al.*, 2004). This raises the possibility that serum/plasma levels of CCL18 may mirror the fibrosing process in the peritoneum. A serum or plasma marker of the fibrosing process would be of use for those patients who have transferred renal replacement modality (either to haemodialysis or transplantation) and who had symptoms suspicious of ongoing peritoneal fibrosis.

Work from Dr Tam's group suggests CCL18 is detectable in dialysate. CCL18 was measured in the dialysate of over 100 peritonitis patients and was found to correlate with duration of PD, membrane transport properties and glucose exposure (Ahmad *et al.*, 2010). The number of patients who developed EPS was naturally small, and the role of CCL18 as a biomarker for future EPS requires further investigation in a larger group of patients. CCL18 mRNA production has been demonstrated in alternatively activated macrophages from human peritoneal effluent (Bellón *et al.*, 2011); the same group detected CCL18 in the dialysate of 25 patients. Levels of CCL18 were highest in patients who went on to develop EPS, but again the number of patients was small (6 EPS, 19 non-EPS) (Bellón *et al.*, 2011). It is not known whether mesothelial cells can produce CCL18, or indeed whether the majority of CCL18 detected in the dialysate effluent has entered from the circulation or been produced within the peritoneum.

1.4(vi) CCL15 (MIP-1δ, leukotactin 1)

CCL15 is also known as macrophage inhibitory protein-1δ (MIP-1δ) and leukotactin 1. It is a relatively novel (Youn *et al.*, 1997) chemokine which has been shown to be involved in endothelial differentiation and angiogenesis (Hwang *et al.*, 2004). Coupled with apparently high levels in an initial screen of dialysate using a multiplex antibody array for multiple cytokines (Dr Ahmad, data not published) this suggests it could be involved in angiogenesis within the peritoneum of PD patients. In addition CCL15 has an interesting pattern of activity as a chemoattractant. The 92 amino acid form is chemotactic for neutrophils (not a usual property for a CC chemokine). This 92 amino-acid form is then truncated by neutrophil cathespin G to produce a shortened (approximately 68 amino-acids) form which is instead chemotactic for monocytes/dendritic cells/T lymphocytes, and which has far greater activity in the activation of lymphocytes (Richter *et al.*, 2005; Forssmann *et al.*, 2001). The shortened form is not detected in human serum or plasma and it is thought that the conversion process is a localised process (quantum proteolytic processing) occurring only in the presence of

activated neutrophils expressing proteases such as cathespin G or elastase; such a process would be expected to be limited by plasma-derived inhibitors of neutrophil proteases. The shortened form is found in haemofiltrate (Forssmann *et al.*, 2001), which has been exposed to activated neutrophils. Activation of CCL15 has also been demonstrated by matrix metalloproteases (Starr *et al.*, 2011) in human synovial fluid; the authors suggest that this is a mechanism by which monocytes may be selectively attracted into an inflamed joint. CCL15 could therefore be involved in the initial inflammatory response to dialysate exposure as a neutrophil chemoattractant. Local activation to the shortened form might then be involved in transition to a chronic inflammatory state involving monocyte influx. CCL15 induces the production of cytokines such as MCP-1 by monocytes (the human acute monocytic leukaemia cell line (THP-1) cell line) that might be involved in a more sustained, chronic inflammatory response (Lee *et al.*, 2002). CCL15 levels (as measured by an assay detecting both the truncated and non-truncated forms) may be expected to be high in dialysis patients with ongoing chronic inflammation and in patients with an acute inflammatory process, but would not necessarily remain high once the later, fibrosing phase of disease was established.

In humans CCL15 was found at high levels in the broncho-alveolar lavage (BAL) fluid of patients with sarcoidosis. CCL15 levels were high in those with a more advanced disease stage, at which time a high number of neutrophils is usually found in the BAL fluid, and high levels of CCL15 were associated with disease progression (Arakelyan *et al.*, 2009). This is consistent with a role for CCL15 in a disease known to cause human fibrosis, and also with the possibility that CCL15 is associated with the presence of neutrophils. Up-regulation of CCL15 is also seen in arthritis (Haringman *et al.*, 2006), being found in synovial tissue in patients with rheumatoid arthritis. CCL15 has been shown to induce increased expression of matrix metalloprotease-9 (MMP-9) from activated macrophages (Kwon *et al.*, 2008); this may also be relevant given the role of MMPs in the initiation of EMT. There is no reference in the literature to the measurement of CCL15 in dialysate effluent.

1.4(vii) Angiogenin

Angiogenin is a 14.4kDa protein originally isolated from colon cancer cells (Fett *et al.*, 1985). It is a potent angiogenic factor. It binds to α-actin on vascular endothelial cells. The angiogenin-actin complex can activate tissue plasminogen activator and hence start the process of generating collagenases to degrade the basement membrane and allow endothelial cells to migrate and form new blood vessels (Strydom, 1998). In these invading endothelial cells where there is a low cell density, a specific angiogenin receptor is expressed which facilitates endocytosis of angiogenin and transfer to the nucleus. This is necessary for endothelial proliferation. Angiogenin also causes arterial smooth muscle cell proliferation after binding to α-actinin-2 (Tello-Montoliu *et al.*, 2006). Angiogenin

is essential for the action of fibroblast growth factor and VEGF on blood vessels (Kishimoto *et al.*, 2005); loss of proliferation induced by these factors was seen in an angiogenin knock-down model. In humans angiogenin is known to have a role in the pathogenesis of many tumours – for example lung cancer (Yuan *et al.*, 2009) and breast cancer (Nilsson Åberg *et al.*, 2011). It is found at increased levels in the serum of ALS patients (Cronin *et al.*, 2006), and in the induced sputum of asthmatics (Kristan *et al.*, 2009). Angiogenin may be a marker of endothelial damage, being found at high levels in patients with vascular disease (Lip and Blann, 2004). Interestingly angiogenin has been found at high levels in young patients with diabetes, and correlated with HbA1c and the degree of microvascular complications; reduction in glucose levels was associated with a fall in angiogenin levels (Chiarelli *et al.*, 2002). This suggests that the high glucose levels of dialysis fluid could prompt neo-vascular development in the peritoneum via the action of angiogenin. However another study in type II diabetics found lower levels of angiogenin with increasing HbA1c (Siebert *et al.*, 2010).

In patients with EPS, tamoxifen has been used as a medical treatment (del Peso *et al.*, 2003; Korte *et al.*, 2010). It is thought to have beneficial effects on EMT, angiogenesis and fibrosis (Selgas *et al.*, 2006). Tamoxifen may act at least partly via blockade of the action of angiogenin (Nilsson *et al.*, 2010; Nilsson Åberg *et al.*, 2011), although it may have other important effects mediated by other pathways.

Summary

It is likely that the structural changes in the peritoneal membrane are driven by an inflammatory, fibrotic and vasculogenic response to the presence of dialysis fluid within the peritoneum. The exact pathways involved are not yet fully defined in the literature and may well be multiple. Many factors may be damaging to the peritoneal membrane – glucose exposure, infection, uraemia, angiotensin system activation and mechanical stresses on the peritoneum have all been implicated. Epithelial to mesenchymal transition is an important step in the development of a thickened and denuded peritoneum. Once these changes have occurred over a long period of time on peritoneal dialysis, the patient is at risk of developing encapsulating peritoneal sclerosis, especially after the cessation of peritoneal dialysis. Currently this is an unpredictable event; the majority of long term peritoneal dialysis patients do not develop EPS. For those who do, however, it is a serious complication. The ability to assess EPS risk more accurately would be of great help to the clinician and patient, but CT based screening may not be reliable and requires investigation. There is great interest in the possibility of predictive biomarkers which could be measured in stable peritoneal dialysis patients. While IL-6, VEGF and TGF-β are already implicated in the inflammatory, angiogenic and fibrotic changes within the peritoneum, other cytokines are also found in the dialysate effluent and might be

potential biomarkers for the future development of EPS. Whether or not a cytokine is directly involved in the pathogenesis of EPS it may still be able to act as a biomarker if dialysate effluent concentrations are found to differ in patient who do and do not subsequently develop EPS. The advantage of measuring cytokine levels in the dialysate is that it is a non-invasive procedure, with dialysate samples readily available from PD patients.

Cytokines such as CCL15 and MCP-1 could play a role in the progression of inflammation and recruitment of inflammatory cells. Angiogenin is a well-known promoter of neo-vascularisation in tumours and areas of hypoxia which might be involved in new vessel development in the membrane. CCL18 is a mediator of fibrosis; the development of thickening and fibrosis of the membrane is another key change in long term PD patients. Cytokines could therefore plausibly play a role in the development of membrane changes, but it is not known whether the quantities of these cytokines detected in the dialysate have any correlation with the present or future clinical features of the patient. It is also unclear whether cytokines are produced within the peritoneum or are diffusing into the dialysate from the circulation. In addition, levels of cytokines within the dialysate may not reflect the biological effect or activity of the cytokine in tissue; low levels of a cytokine in the dialysate effluent do not exclude an important role for the cytokine in the peritoneum, especially if there is evidence for local production of the cytokine by cells of the peritoneum.

CHAPTER 2:

AIMS OF THE STUDY AND HYPOTHESIS

As part of the West London Prospective PD study, our group (Dr S Ahmad) has previously measured the levels of cytokines (CCL18, angiogenin, monocyte chemotactic protein-1 (MCP-1) and interleukin 6 (IL-6)) in dialysate fluid and serum in a group of 106 patients. The number of patients with EPS in this group was small at that time and a larger group of patients have now developed the condition. This study further investigates the possibility that cytokines are mediators in the development of the long term peritoneal damage which is thought to lead to later EPS.

Aim: assess whether cytokine levels correlate with clinical data and membrane function

The first aim of the study is to investigate whether cytokines may have a role in the development of pathological changes in the peritoneal membrane. The work done by Dr Ahmad (Ahmad *et al.*, 2010) and Dr Malik (Malik *et al.*, 2007) indicates that the cytokines CCL18 and MCP-1 are present in the dialysate at levels which correlate with clinically relevant data. CCL18 appeared to correlate with the D/P creatinine and glucose exposure in a group of 106 patients (Ahmad *et al.*, 2010), while MCP-1 correlated with the past peritonitis history in a group of 45 patients (Malik *et al.*, 2007). Other cytokines which were shown by Dr Ahmad using a multiplex antibody array to be present in the dialysate at apparently high levels were angiogenin and CCL15. As discussed in the introduction, the known biological effects of MCP-1, CCL18, CCL15 and angiogenin means they are plausible candidates as mediators of peritoneal damage. IL-6 is well documented in the literature as important in inflammation both generally (Pecoits-Filho *et al.*, 2003) and specifically within the peritoneum (Hurst *et al.*, 2001).

In this study the original West London prospective peritoneal dialysis study patient group has been extended and a further 45 baseline patient samples have been collected to be analysed together with the results of cytokine measurements made by Dr Ahmad in the original 106 patients. In addition, measurements of the chemokine CCL15 (leukotactin) (which has not previously been investigated in dialysate effluent) have been made on 126 of the patient samples. The aim is to discover whether the previously observed correlations still hold in the larger patient group and whether any new associations between cytokine levels and clinical results emerge. It is known from longitudinal studies of membrane function that factors such as fluid type, glucose exposure and residual renal function are important in technique survival; an association between high glucose

exposure, for example, and higher cytokine levels would suggest that the cytokine might potentially be involved in mediating the effect of high glucose levels on the peritoneum.

Aim – establish the source of cytokines detected in the dialysate

The second aim of the study is to establish whether cytokines are produced within the peritoneum or enter by passive diffusion from the circulation. Human peritoneal cells from used overnight dialysate have been collected and cultured. Cells have been stained for epithelial and mesenchymal markers to demonstrate typical features of mesothelial cells in culture. The culture medium has been analysed to assess whether there is evidence that peritoneal cells produce the cytokines of interest. Passive transfer of cytokines from the circulation might also determine dialysate levels of chemokines; in order to establish whether this could be the case the transfer of small proteins, of the same range of molecular weights as cytokines, from the circulation to the dialysate is investigated.

Aim – assess the role of cytokines as biomarkers for later EPS

The third aim of the study is to investigate whether cytokine levels can improve our ability to predict the onset of EPS. In clinical practice a method for identifying which patients are at higher risk of EPS is greatly needed. There is now a larger group of patients in our database who have developed EPS after the cessation of PD. Cytokine levels measured while all the patients were stable on PD are analysed to discover if high dialysate levels of cytokines predict whether a patient will subsequently develop EPS. Serum cytokine levels in EPS patients who are no longer using peritoneal dialysis are also measured and compared with levels in non-EPS patients who have never used peritoneal dialysis to assess whether there is further evidence for a role of these molecules in the ongoing fibrotic pathology.

Although a new biomarker would be of great potential clinical usefulness, it is important to be clear whether it actually represents an improvement over the available risk markers. There are well-known clinical risk factors for EPS, the most secure being duration of dialysis. Patient demographics such as age (Johnson *et al.*, 2010) have been significant risk factors in at least one study; markers of membrane damage such as raised D/P creatinine and loss of ultrafiltration are more consistent risk factors for later EPS. Hence clinical measurements are also investigated to determine whether patient demographics, dialysis history, adequacy data or peritoneal membrane function tests can be used to predict future EPS.

Aim – assess the roles of radiological screening for later EPS

Computed tomography (CT) scans are used in some peritoneal dialysis units to screen for the future development of EPS. As discussed in the introduction (Tarzi *et al.*, 2008; Vlijm *et al.*, 2009), there is good evidence to show that CT scans are useful in the diagnosis of symptomatic EPS. However, the basis on which CT scans are used to establish the likelihood of future EPS in a current PD patient has not previously been investigated. Although CT is used in many PD units it requires validation and may not be an appropriate screening method.

Hypothesis:

Cytokines – MCP-1, CCL18, IL-6, CCL15 and angiogenin - are important in the process of peritoneal membrane damage. Peritoneal cells from dialysis patients produce these cytokines. Cytokine levels in dialysate and perhaps serum of peritoneal dialysis patients:

1. **will reflect clinically important measures of ongoing pathological processes in the peritoneal membrane and**
2. **may predict adverse outcomes such as ultrafiltration failure and development of EPS**

Clinical and radiological findings may also aid prediction of clinical outcomes.

Definitions

EPS is defined clinically as a syndrome of persistent or recurrent bowel obstruction with vomiting, abdominal distension, abdominal pain and weight loss (Woodrow *et al.*, 2009) in a patient with a history of long term peritoneal dialysis. This may be accompanied by evidence of inflammation such as raised CRP, fever and hypoalbuminaemia. The characteristic fibrous cocoon of EPS is seen at laparotomy only and the majority of our patients did not undergo surgical treatment. As well as the typical clinical picture, most EPS patients underwent CT scans showing the typical changes of EPS – peritoneal thickening, abnormal bowel pattern due to tethering, bowel dilatation, peritoneal calcification and fluid loculation.

UFF is defined as either

- less than 750mls of fluid removal in 24 hours - the target for fluid removal in the EAPOS trial (Brown *et al.*, 2003) - in a patient still on PD (measured as part of adequacy assessment) or
- cessation of PD due to clinical fluid overload (resistant oedema requiring transfer to HD)

Ultrafiltration capacity data is not available for all patients in the study and PET tests are performed in our institution on an annual basis. Hence a clinical definition must be included to avoid underestimating the number of patients with technique failure due to limited ultrafiltration.

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CHAPTER 3:

METHODS

Prospective collection of clinical data and samples of serum/plasma and dialysate from PD patients has been undertaken as part of the West London PD study and this is continued in the extended patient group. Ethical approval was given for this study (Riverside Research Ethics Committee, Charing Cross Hospital; REC reference number 08/H0706/20). Patients are recruited when they attend the PD unit for a PET test; the prolonged nature of this visit allows time for informed written consent to be obtained.

Clinical data and samples of serum/plasma from patients with established EPS, and from a control group of patients matched for age, sex, treatment modality (HD v. transplant) and duration of end stage renal failure that have never used peritoneal dialysis have also been collected.

Overnight dialysate has been collected as a source of shed peritoneal cells for culture.

3.1 Clinical data

Demographics: age, sex, renal diagnosis, duration of dialysis

Medication history and biochemistry results on day of sample

Stoke co-morbidity score: (for symptom study patients) - this is a semi-quantitative co-morbidity score developed in the Stoke renal unit which incorporates seven disease domains: malignancy, ischaemic heart disease, peripheral vascular disease, left ventricular dysfunction, collagen vascular disease and other life-expectancy limiting pathology. Patients are scored from 0 to 2 where 0 = no co-morbidities, 1 = one or two co-morbidities and 2 = three or more co-morbidities. This scale has been shown to be predictive of mortality in PD patients independently of age, residual renal function and membrane function (Davies *et al.*, 2002).

Peritoneal dialysis history: dialysis modality (CAPD v. APD), glucose exposure (mmol/24 hour in the current PD prescription), peritonitis history, icodextrin use

Adequacy data: kT/V, creatinine clearance, and residual renal function

Membrane transport data: PET result and ultrafiltration volume

Outcome post cessation of PD: EPS v. no EPS

Ultrafiltration failure as the cause of PD cessation

3.2 Sample Collection

3.2(i) Dialysate and serum samples for cytokine measurement

For patients currently on peritoneal dialysis samples were taken at the time of PET which provides the opportunity for a standardised blood sample at the midpoint of the 4 hour dwell. Dialysate samples were taken from the fluid drained at the end of the PET test. The volume of this fluid was measured. The peritoneal appearance rate can then be calculated from the chemokine concentration and the dialysate volume; this is the quantity of the cytokine produced during the 4 hour dwell. This permits comparison of results between patients using differing dwell volumes and durations. PET tests in our institution are performed annually and after an episode of peritonitis; samples were taken only when a PET test was performed routinely and at least six weeks after the last episode of peritonitis (since cytokine levels increase during peritonitis and for at least some weeks afterwards (Lai *et al.*, 2000; Oymak *et al.*, 2003)). The protocol used during a PET test includes patient movement to facilitate mixing of the dialysate within the abdomen, to aid collection of a representative sample (Zbylut *et al.*, 1987).

For patients on haemodialysis blood samples were taken from the dialysis lines after discarding any anticoagulant lock. Samples were taken prior to the initiation of dialysis as haemodialysis can cause a rise in the levels of cytokines due to exposure to bacterial products (Schindler *et al.*, 1996) and components of the dialysis circuit (Pereira *et al.*, 1995); alternatively some cytokine levels may fall post-dialysis session (Tarakçioğlu *et al.*, 2003).

For patients with a renal transplant blood samples were taken at the time of other blood samples at routine clinic visits.

All samples were collected and placed onto ice then spun at 4°C and frozen (-20 to -80^{o}C) within 2 hours.

Blood samples: serum in gold top vacutainer (contains a clot activator and gel for serum separation)
plasma in purple top vacutainer (contains EDTA, an anti-coagulant)
Both spun at 3000rpm (1107g) at 4^{o}C for 10 minutes and aliquoted for storage

Dialysate samples were taken in plain (Universal) tubes and spun at 3000rpm (1107g) at 4^{o}C for 15 minutes, then aliquoted.

3.2(ii) Samples for cell culture

For collection of cells for culture the overnight dialysate bag was used. A 50ml aliquot of fluid was spun and stored and the remainder of the bag was hung in an incubator at 37°C for two hours to allow the cells to settle prior to cell collection.

3.3 Cytokine Assays

3.3(i) ELISA technique

All cytokines were measured using a sandwich ELISA technique which allows quantification of small amounts of antigen. The principle of this method is that the plate is coated with a "capture" antibody specific for the molecule of interest. The sample is added and binds to the coating of capture antibody in the well. After washing, a biotinylated detection antibody is then added which binds to the molecule of interest. Streptavidin (horseradish peroxidise) binds avidly to the biotin and the bound streptavidin then drives the colour change in the 3,3',5,5' tetramethylbenzidine substrate.

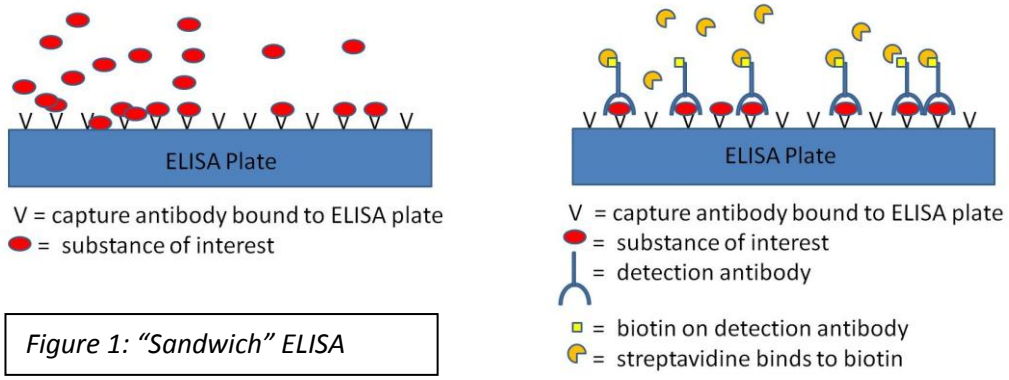


Figure 1: "Sandwich" ELISA

The general protocol for MCP-1, CCL15, CCL18 and angiogenin is as follows:

A 96-well ELISA plate is coated with 100µl to each well of the capture antibody in phosphate buffered saline (PBS) and refrigerated overnight at 4°C. It is washed and 300µl/well of blocking solution applied for one hour. On each wash the plate is rinsed three times with PBS/tween. The ELISA plate is kept on a rotary platform in between the applications of antibodies. All standards, samples and antibody solutions are mixed on the whirlimix before adding to the ELISA plate. A standard curve is constructed using a serial dilution technique. The block is washed off and 100µl/well each of samples (diluted appropriately) and standard are applied to the plate in duplicate. After two hours the plate is washed and a detection antibody applied for two hours. The plate is washed and streptavidin (Invitrogen, Life Technologies, Paisley, UK) applied for twenty minutes. This is washed off and 3,3',5,5' tetramethylbenzidine (TMB), (Cambridge Bioscience, Cambridge, UK) substrate reagent added. Once a deep blue colour has developed in the high standard the reaction is stopped using 50µl of 2N sulphuric acid/well and the optical density measured at 450nm using a plate reader (Anthos Labtec, Wals, Austria).

For dialysate samples the same diluent solution was used with the detection antibody, the standard curve and the sample dilution (see below). For serum and plasma samples solution S was used to make the standard and dilute the samples.

The appropriate dilution for each cytokine and each type of sample was established using a series of dilutions to be as follows:

	Serum	Dialysate
CCL15	1:20	1:10
MCP-1	1:2	1:1
CCL18	1:200	1:10
Angiogenin	1:5000	1:50
IL-6	1:1	1:10

Table 1: Dilutions for serum and dialysate

Samples from cell culture experiments were measured undiluted using solution S as the diluent for the standard curve. Antibodies were purchased from R&D Systems (Minneapolis, USA); the same antibody clones were used throughout the study. For IL-6 the quantities found in human serum are too low to measure using this technique and a high sensitivity Quantikine ELISA kit (R&D Systems) was used to measure the levels of IL-6 in both dialysate and serum. This uses the same basic technique, but is supplied with the plate pre-coated with the capture antibody and the necessary solutions pre-prepared. This ELISA uses a different colorimetric system, developing a violet colour and is therefore read at 490nm. The lower limit of detection of IL-6 using this kit (HS600B, R&D systems) is 0.156pg/ml.

3.3(ii) Reagent preparation method for MCP-1, CCL15, CCL18 and angiogenin ELISAs.

PBS (phosphate buffered saline) g/L
1. Sodium chloride NaCl 8.0
2. Potassium chloride KCl 0.2
3. Disodium hydrogen phosphate Na_2HPO_4 $2H_2O$ 1.44
4. Potassium dihydrogen phosphate KH_2PO4 0.2

BLOCKING SOLUTION
1. PBS 200ml
2. Sucrose 10g
3. BSA 4g (bovine serum albumin)

STOP SOLUTION
10ml H_2SO4 into 90mls of water

WASH BUFFER (use within the same day)
500µl of Tween 20 in 1000ml of PBS

DILUENT (0.1%BSA, 0.05% Tween 20 in tris-buffered saline pH 7.3)

1. Water (Millipore)	500ml
2. NaCl	4.4g
3. Trisma base F.W.121.1 Sigma T-1503 (Dissolve completely, and then add HCL until pH 7.3)	1.2g
4. BSA	0.5g
5. Tween 20	0.25ml

SOLUTION S (for dilution of serum samples and standards)

1. PBS	95ml
2. NaCl	5g
3. BSA	1g
4. Fetal Calf Serum	5ml (filtered and aliquoted in sterile tissue culture hood)

Stock solution details and reconstitution protocols:

MCP-1

MCP-1 (standard) 279-MC, 10µg, R&D systems: Reconstitute with sterile Phosphate Buffered Saline (PBS) containing 0.1% Bovine Serum Albumin (BSA) to make a stock solution of 10µg/ml.

MCP-1 (capture Ab) MAB679 500µg, R&D systems: Reconstitute with 1ml sterile PBS to give a stock antibody concentration of 500µg/ml.

MCP-1 (detection Ab) BAF279, 50µg, R&D systems: Reconstitute with 1 ml sterile Tris-buffered saline pH 7.3 (20mmol Trizma base, 150mmol NaCl) containing 0.1% BSA.

CCL18

CCL18 (standard) 394 - PA, 10µg, R&D systems: Reconstitute with sterile PBS containing at least 0.1% BSA to make a stock solution of no less than 25µg/ml. Use 0.4ml of PBS with 0.2% BSA (0.2g BSA in 100mls of PBS)

CCL18 (capture Ab) MAB394, 500µg, R&D systems: Reconstitute with 1 ml sterile PBS; the antibody concentration will be 500µg/ml.

CCL18 (detection Ab) BAF394, 50µg, R&D systems: Reconstitute with 1ml sterile Tris-buffered saline pH 7.3 (20mmol Trizma base, 150mmol NaCl) containing 0.1% BSA to give antibody concentration of 50µg/ml.

CCL15

CCL15 (standard) 363-MG-025, 25µg, R&D systems: Reconstitute with 1ml sterile PBS with 1% BSA to make a stock solution of 25µg/ml

CCL15 (capture Ab) MAB363, 500µg, R&D systems: Reconstitute with 1ml sterile PBS; the antibody concentration is 500µg/ml

CCL15 (detection Ab) BAF363, 50µg, R&D systems: Reconstitute with 1ml sterile Tris-buffered saline pH 7.3 (20mmol Trizma base, 150mmol NaCl) containing 0.1% BSA to give antibody concentration of 50µg/ml.

Angiogenin

ANG (standard Ab) 265-AN-050, 50 ug, R&D systems: Reconstitute with 1 ml of sterile 0.1%BSA in PBS; the stock concentration is 50µg/ml

ANG (capture Ab) MAB265, 500 ug, R&D systems: Reconstitute with 1 ml of sterile PBS to give a stock concentration of 500µg/ml.

ANG (detection Ab) BAF265, 50 ug, R&D systems: Reconstitute with 1 ml of sterile 0.1% BSA in Tris-buffered saline pH 7.3 (20mmol Trizma base, 150mmol NaCl); the stock concentration is 50 µg/ml.

3.3(iii) Calculation of cytokine levels

The standard curve is plotted as corrected optical density (corrected OD) versus log [cytokine concentration] and the sample concentrations derived from the measured optical density. GraphPad Prism version 4 software (GraphPad Software, La Jolla, USA) was used to plot all standard curves. A typical standard curve is shown below:

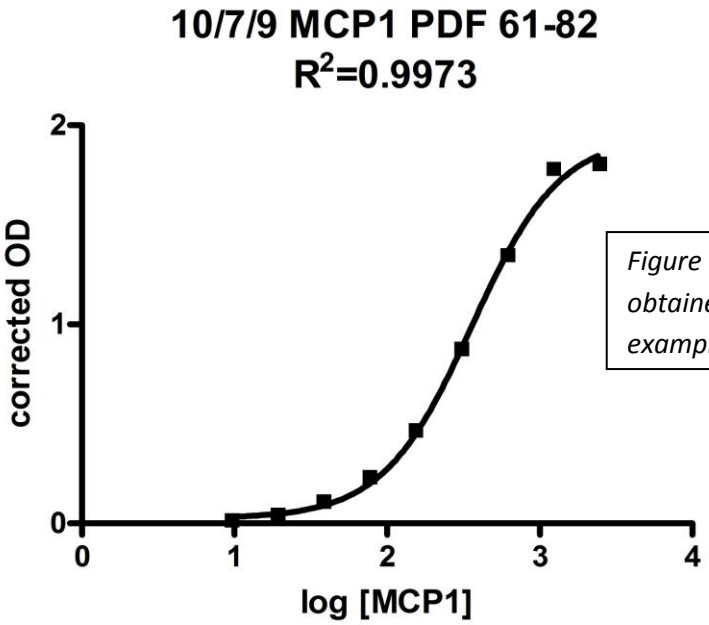


Figure 2: Typical standard curve obtained using MCP-1 as an example

A typical Excel spreadsheet showing how the optical density (OD) readings are used to calculate the standard curve and the sample concentrations is shown below:

Sample pairs which differ by more than 10% are highlighted in yellow. There are also repeats of samples on this plate to enable calculation of intra-assay variability.

Figure 3: Typical ELISA results spreadsheet

Label	[h MCP-1]	log [h MCP-1]	OD-1	OD-2	Mean	Cor. OD	*Log[h MCP-1]*	*anti Log MCP-1*	*dilution*	*[h MCP-1]*
	0		0.149	0.163	0.1560	0.0000				
	9.76	0.989	0.192	0.202	0.1970	0.0410				
	19.53	1.291	0.227	0.249	0.2380	0.0820				
	39.0625	1.592	0.308	0.323	0.3155	0.1595				
	78.125	1.893	0.426	0.454	0.4400	0.2840				
	156.25	2.194	0.678	0.692	0.6850	0.5290				
	312.5	2.495	1.113	1.177	1.1450	0.9890				
	625.0	2.796	1.615	1.648	1.6315	1.4755				
	1250.0	3.097	1.923	1.915	1.9190	1.7630				
	2500.0	3.398	2.055	2.075	2.0650	1.9090				
60.1			1.224	1.276	1.2500	1.0940	2.554677	358.655091	1	**358.655**
59.1			1.056	1.089	1.0725	0.9165	2.446382	279.500121	1	**279.500**
58.1			0.913	0.930	0.9215	0.7655	2.350754	224.261127	1	**224.261**
57.1			1.115	1.230	1.1725	1.0165	2.507500	321.736253	1	**321.736**
55.1			2.102	2.068	2.0850	1.9290			1	**>**
54.1			0.727	0.754	0.7405	0.5845	2.223256	167.207595	1	**167.208**
60.1			1.210	1.235	1.2225	1.0665	2.537912	345.073811	1	**345.074**
59.1			1.033	1.038	1.0355	0.8795	2.423456	265.128247	1	**265.128**
58.1			0.877	0.889	0.8830	0.7270	2.325172	211.432624	1	**211.433**
57.1			1.111	1.116	1.1135	0.9575	2.471541	296.169955	1	**296.170**
55.1			1.928	2.087	2.0075	1.8515	3.241006	1741.830938	1	**1741.831**
54.1			0.672	0.732	0.7020	0.5460	2.192983	155.949146	1	**155.949**
53.1			1.209	1.215	1.2120	1.0560	2.531523	340.034513	1	**340.035**
52.1			0.877	0.893	0.8850	0.7290	2.326521	212.090395	1	**212.090**
48.1			0.406	0.429	0.4175	0.2615	1.884698	76.682807	1	**76.683**
47.1			0.750	0.725	0.7375	0.5815	2.220962	166.326711	1	**166.327**
37.1			0.975	0.978	0.9765	0.8205	2.386298	243.387349	1	**243.387**
34.1			1.048	1.008	1.0280	0.8720	2.418775	262.285934	1	**262.286**
33.1			1.022	1.076	1.0490	0.8930	2.431849	270.301839	1	**270.302**
17.1			0.772	0.728	0.7500	0.5940	2.230520	170.027825	1	**170.028**
14.1			0.605	0.609	0.6070	0.4510	2.110702	129.033358	1	**129.033**
11.1			0.822	0.818	0.8200	0.6640	2.281778	191.327766	1	**191.328**
4.1			0.613	0.634	0.6235	0.4675	2.125935	133.639549	1	**133.640**
3.1			1.234	1.223	1.2285	1.0725	2.541564	347.987785	1	**347.988**
2.1			0.655	0.717	0.6860	0.5300	2.179954	151.340094	1	**151.340**
1.1			1.342	1.379	1.3605	1.2045	2.622941	419.701963	1	**419.702**
37.1			0.962	0.922	0.9420	0.7860	2.364141	231.281556	1	**231.282**
34.1			0.957	1.078	1.0175	0.8615	2.412223	258.358646	1	**258.359**
33.1			1.057	1.077	1.0670	0.9110	2.442992	277.326902	1	**277.327**
17.1			0.752	0.742	0.7470	0.5910	2.228226	169.132084	1	**169.132**
14.1			0.615	0.579	0.5970	0.4410	2.101262	126.258900	1	**126.259**
11.1			0.729	0.841	0.7850	0.6290	2.256578	180.541896	1	**180.542**

3.3(iv) Intra and Inter assay variability:

This has been determined as a coefficient of variation for each cytokine using a minimum of ten repeated results pairs for each measure:

	Serum		**Dialysate**	
	Inter assay	**Intra assay**	**Inter assay**	**Intra assay**
MCP-1	7.73%	6.19%	11.86%	6.33%
CCL18	7.89%	7.68%	6.23%	4.14%
CCL15	7.02%	6.58%	5.14%	5.63%
Angiogenin	7.89%	7.23%	11.01%	6.03%
IL-6	10.95%	9.83%	9.6%	5.59%

Table 2: Inter- and intra-assay variability

3.3(v) Consistency of the standard over time

In order to exclude a systematic change in the standards used over the course of the study, the median levels in both dialysate and serum were compared in the first, second and third fifty patients. Cytokine levels were also assessed for significant correlations with time since study commenced to investigate whether there was any evidence of a progressive change in median measured cytokine levels which might be artefactual. A problem with the standard would be expected to affect both serum and dialysate levels.

The only cytokine for which both serum and dialysate levels showed a statistically significant difference between samples from early, mid and late points in the study was CCL15. CCL15 was higher in the middle period of patient recruitment for both serum (Kruskal-Wallis test, $p = 0.0021$) and dialysate ($p = 0.0016$) levels. This is unlikely to be due to a change in the standard as CCL15 was measured on the majority of samples over a short period of time using the same preparation for the standard, and is more likely to represent true higher levels in these patients.

There were no cytokines for which both serum and dialysate levels showed a significant correlation with time since study commenced.

3.3(vi) Serum and plasma levels

Simultaneous plasma levels were taken and samples analysed for CCL15, CCL18 and MCP-1, to assess whether the nature of the blood sample (serum v. plasma) was important. Angiogenin is known to be found at very similar levels in serum and plasma samples (McLaughlin *et al.*, 2010), and IL-6 is found at slightly (not statistically significantly) higher levels in serum than plasma (O'Neill and Bowie, 2001).

For all three cytokines, a linear relationship was observed between serum and plasma levels:

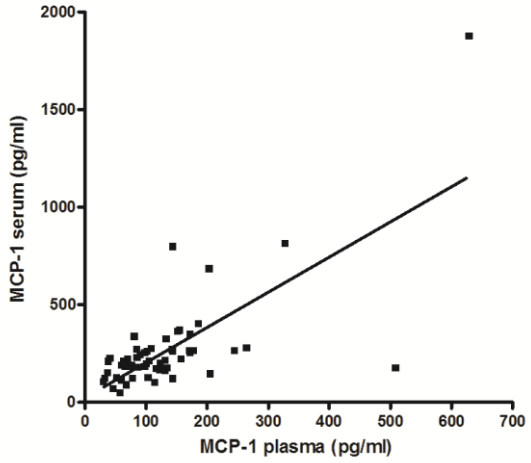


Figure 4: There is a linear relationship between serum and plasma levels of MCP-1

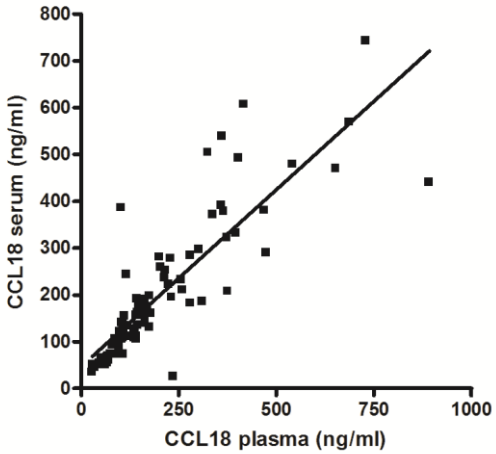


Figure 5: There is a linear relationship between serum and plasma levels of CCL18

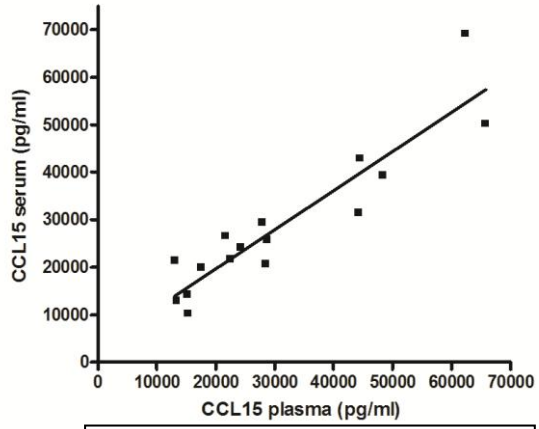


Figure 6: There is a linear relationship between serum and plasma levels of CCL15

There is a linear relationship between serum and plasma levels for all three cytokines. However it is important that one type of sample is analysed consistently, as the serum levels of MCP-1 are nearly twice as high as the plasma levels (205 v.109 pg/ml, P<0.001). There was no significant difference between levels of CCL18 and CCL15 in plasma and serum. In this study serum levels are used throughout.

3.4 Cell culture techniques

3.4(i) Peritoneal mesothelial cell culture method

Cells were cultured from the overnight dialysate bag. Any aliquots of fluid required for cytokine studies were removed and the bag hung for two hours at 37^{o}C for 2 hours.

All but 200mls of the dialysate was aspirated off. This was then pipetted into four 50ml flasks. These were centrifuged at 1500rpm at 4°C for 20 minutes and the dialysate discarded. The four cell pellets were re-suspended in 10mls of Hank's balanced salt (calcium-free) solution (Sigma, Gillingham, UK) and spun again for 20 minutes at 1500rpm and 4°C. The Hank's solution was aspirated off and the

cells re-suspended in 10mls of fresh Hank's solution. This was spun again for 20 minutes at 1500 rpm at 4^{o}C. The Hank's solution was aspirated off and 1ml of culture medium (M199 Earl's base with L-glutamine, 10% fetal calf serum (PAA, Yeovil, UK) and 10mls penicillin-streptomycin (Invitrogen) was added. A 10µl aliquot was taken and tryptan blue used to stain the cells for cell count. A further 6.5ml more culture medium was added and the cells cultured in a flask in 5% CO_2 at 37°C in an incubator (Wolf Laboratories, York, UK).

Cells were split using cell dissociation solution (Geneflow, Fradley, UK) when confluent and samples of the culture medium taken to investigate cytokine production by the cells in culture. Cells in culture were photographed using a Nikon TS100 camera to demonstrate the variability in morphology of peritoneal cells.

Once sufficient cells were present, the cells were re-suspended and transferred to a twelve well culture plate for the stimulation experiments, or used to produce a cytospin preparation for staining and characterisation by immunohistochemistry.

3.4(ii) Immunohistochemistry to characterise mesothelial cells

Cells cultured from dialysate effluent are known to show varying morphology, in keeping with an ongoing process of EMT (Yáñez-Mó *et al.*, 2003). Cells were therefore stained for:

- The epithelial marker cytokeratin 18
- The mesenchymal marker vimentin
- Alpha smooth muscle actin (α-SMA), which labels smooth muscle cells and myofibroblasts.

All antibodies were supplied by Dako (Ely, UK) and were mouse anti-human antibodies used at 1 in 50 dilution.

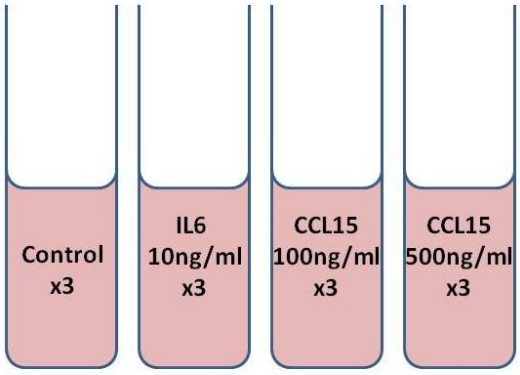
The slides were air-dried then rinsed with PBS. The staining was done with the slides in a humidified chamber to prevent over-drying. The cells were ringed with wax and 100µl of Marvel block (1g Marvel milk powder in 10mls PBS) applied for 15 minutes. The Marvel was then tapped off and the primary antibody added to one cell spot and 100µl of Marvel added to the other cell spot on the slide as a negative control. The slides were left for one hour and then rinsed twice with PBS.

The Dako mouse EnVision kit was used to visualise the primary antibody distribution. Diaminobenzidine (DAB) was added to each cell spot; 50µl of DAB at a concentration of 1 in 50 and observed until brown staining was seen. The slides were then covered with PBS and washed in running water. The cells were counterstained with haematoxylin for 30 seconds then washed in a water bath. The slides were then dipped in 1% acid:70% alcohol and returned to the water bath until blue. The slides were then dipped in ethanol and xylene for 3 minutes twice and mounted with Pertex.

3.4(iii) Mesothelial cell stimulation experiment – investigating the action of CCL15

Cells were transferred to a 12 well culture plate. Baseline samples of culture medium were taken once the cells were established. The cells were then stimulated with IL-6 and CCL15. Human peritoneal mesothelial cells may however be IL-6 receptor negative (McLoughlin *et al.*, 2004) and have been shown to respond to IL-6 in the presence of soluble IL-6 receptor only. It was not therefore clear whether mesothelial cells would be expected to respond to IL-6. The peritoneal mesothelial cells were stimulated with CCL15 and IL-6 to investigate whether these cytokines caused a change in cytokine production by the mesothelial cells. CCL15 has been shown to induce an increase in MCP-1 mRNA production in THP-1 cells (Lee *et al.*, 2002) but the response of peritoneal mesothelial cells has not previously been investigated.

Details of stimulation experiment:



Mesothelial cell stimulation experiment

Figure 6: Mesothelial cell stimulation experiment
3 wells control
3 wells IL-6 10ng/ml
3 wells CCL15 100ng/ml
3 wells CCL15 500ng/ml

Levels of MCP-1, CCL18, CCL15 and angiogenin were measured in the control culture medium for comparison with levels in the wells stimulated by IL-6 or CCL15 after 48 hours. The concentrations of CCL15 used were chosen with reference to the concentrations used by Lee et al (Lee *et al.*, 2002) and that of IL6 to the concentrations used by McLoughlin et al (McLoughlin *et al.*, 2004).

3.4(iv) U937 cell culture

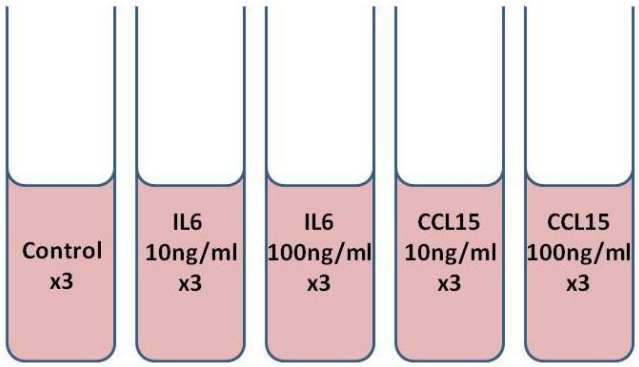
U937 cells (European collection of cell cultures, Salisbury, UK) were grown in RPMI 1640 medium (Gibco, Life Technologies) with penicillin-streptomycin and 5% FCS. After the second passage cells were characterised initially using flow cytometry (FACS). The U937 cells were labelled with antibodies for CD14 and CD68 (monocyte/macrophage markers) and also for CCL18. Initial FACs data indicated a heterogenous cell population, and a new culture was established.

Samples of the culture medium were taken to establish which cytokines were produced. After the second passage, these cells were split and half were transferred to serum free medium for the

stimulation experiment; the remaining cells were used to produce a cytospin preparation for immunofluorescence to characterise the cells using an antibody to the monocyte marker CD68.

3.4(v) U937 cell stimulation experiment – investigating the action of CCL15

After 24 hours in serum-free medium, a sample of tissue culture fluid was taken for cytokine ELISAs. The cells were spun down and washed twice with Hank's solution then re-suspended in 16 mls serum-free medium. 1ml/well of culture medium and cells was added to each of 15 wells and cytokines added as follows:



U937 cell stimulation experiment

Figure 7: U937 cell stimulation experiment
3 wells control
3 wells IL-6 10ng/ml
3 wells IL-6 100ng/ml
3 wells CCL15 10ng/ml
3 wells CCL15 100ng/ml

IL-6 was chosen as a positive control to confirm that the experimental conditions permitted an increase in cytokine production levels at 48 hours; IL-6 is known to induce MCP-1 release from monocytes without requiring the addition of the soluble IL-6 receptor.

After 48 hours samples of culture medium were taken for measurements of cytokine levels.

3.5 Comparison of serum and dialysate protein levels

In order to investigate the relationship between serum and dialysate levels of cytokines, the transfer from blood to dialysate of proteins which are known not to be produced within the peritoneum was investigated for a number of proteins of varying molecular weight. This allows the construction of a graph of dialysate: serum ratio versus molecular weight. The blood samples were taken at the mid-point of the four hour PET test and the dialysate samples taken at the end of the PET test as for cytokine levels. The cytokines range from 7.8 to about 20kDa (Daltons) in molecular weight:

Protein	Molecular weight (kDa)
CCL18	7.8
MCP-1	8.7
CCL15	10
Angiogenin	14
IL-6	20.3

Table 3: Molecular weights of chemokines

The following proteins were chosen as molecular weight markers:

Protein	Molecular weight (kDa)
Creatinine	0.113
B2-microglobulin	11
Prolactin	24
Albumin	68
IgG	155

Table 4: Molecular weights of proteins

These proteins were measured by the biochemistry laboratory (Dr M Donaldson and Dr J Bailey) as they are routinely measured analytes in our institution. All the analytes were measured on the Abbott Architect analyser (Abbott Laboratories, Maidenhead, UK) except for β2-microglobulin which was measured by a microparticle immunoassay on the Abbott Axsym analyser. Twenty paired serum and dialysate samples were used to construct a graph of dialysate to serum (D/S) ratio v. molecular weight of protein. This was used to calculate an expected D/S ratio for each cytokine, which could then be compared to the measured D/S ratio.

3.6 Statistical methods

All data was analysed using Graph Pad Prism version 4 and SPSS versions 17 and 19 (IBM, New York, USA).

The distribution of continuous variables was checked for normality using histograms, normal Q-Q plots and the Shapiro-Wilk and Kolmogorov-Smirnov tests. Any variable not found to be normal in distribution was analysed using non-parametric statistics. The Mann-Whitney test is reported for comparison of independent samples (Kruskal-Wallis test for three or more groups) and the Wilcoxon Signed Rank Test for comparison of related samples (follow-up samples at one year). Two-tailed p values are reported. Spearman's rho is reported for tests of correlation.

Multivariate analysis was performed with the help of Dr Bernard North using SPSS v.19 and the R project version R.13. Variables were log transformed to a more normal distribution to satisfy model requirements. Model fit was assessed by residual plots including Q-Q plots for normality of residuals and residuals vs fits plots for linearity of effect of the predictors on the log transformed cytokine level. Linear regression was used to analyse the factors determining dialysate cytokine levels; logistic regression was used to explore whether EPS could be predicted from clinical and cytokine data.

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CHAPTER 4

RESULTS: Levels of cytokines in dialysate and serum

4.1 Peritoneal dialysis patient cohort: baseline levels of cytokines in dialysate and serum

4.1(i) Demographics of the patient group

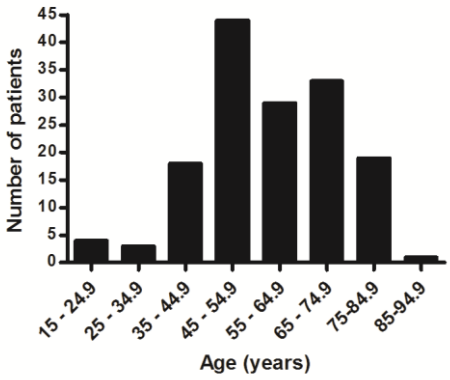


Figure 8: Age distribution of peritoneal dialysis population

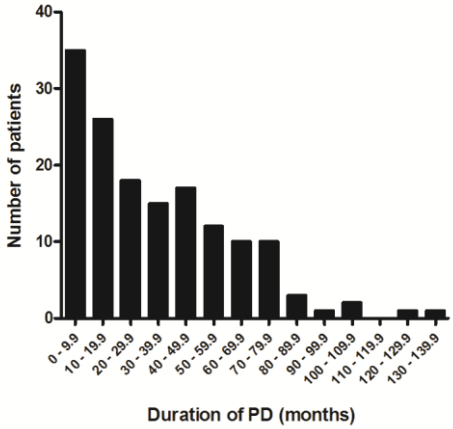


Figure 9: Duration of PD at time of sampling

Baseline samples and data were collected from 151 patients. The mean age of the patients was 58 years (95% confidence interval 55.75 – 60.24 years). The median duration of peritoneal dialysis at the time of sampling was 27 months (range 0.7 – 134.8 months). The distribution of time on dialysis is skewed; this is likely to be due to patients transferring to haemodialysis or obtaining a renal transplant; however the population contains a significant number of patients who had been using peritoneal dialysis for more than five years at the time the baseline samples were taken (28 of 151 patients). Samples have not been collected from all incident patients during the period of the West London PD study; some patients may not have had a PET test and a researcher may not always have been able to attend the test. All patients approached consented to take part in the study and were enrolled.

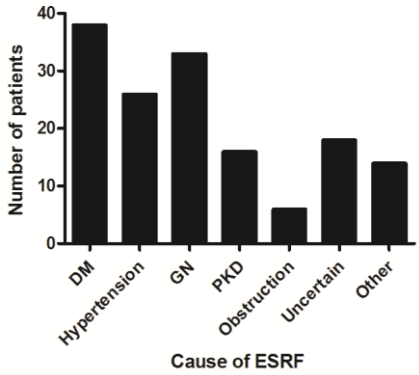


Figure 10: Cause of ESRF in peritoneal dialysis patients

The major causes of ESRD in the patient group are as would be expected: diabetes, glomerulonephritis, hypertension and vascular disorders and polycystic kidney disease. Other diagnoses include Alport's syndrome, pyelonephritis, reflux and scleroderma.

4.1(ii) Peritoneal dialysis data in the baseline patients

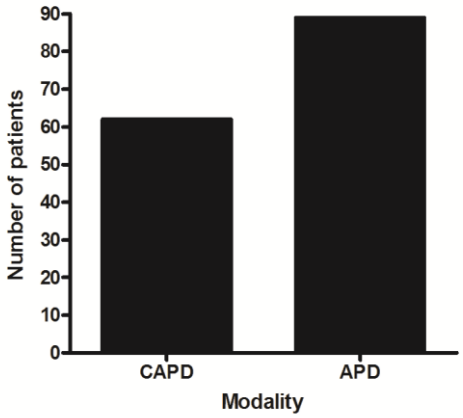


Figure 11: Modality of peritoneal dialysis

The proportion of patients using APD did not alter greatly during the course of the study, being 52% of the first 50 patients, 60% of the second 50 patients and 60.7% of the final 51 patients (in chronological order of recruitment).

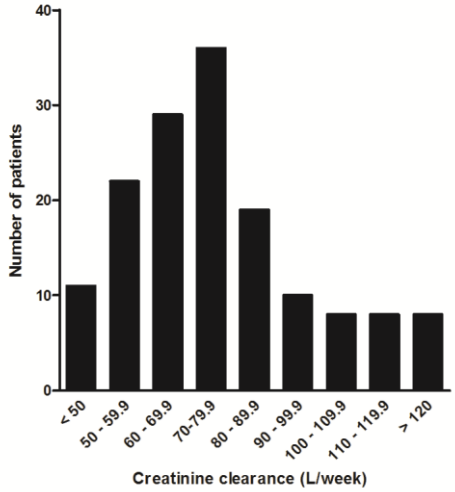


Figure 12: Creatinine clearance results in peritoneal dialysis patients

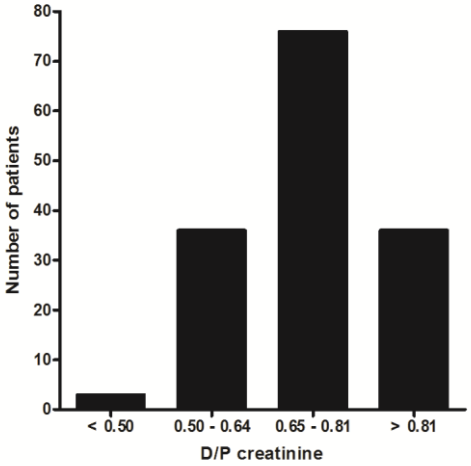


Figure 13: Distribution of PET results (D/P creatinine) in peritoneal dialysis patients

This is a well-dialysed group with a mean total creatinine clearance of 74.6 L/week (range 41.2 – 185.8 L/week). The mean D/P creatinine was 0.72 (95% confidence interval 0.7 - 0.74).

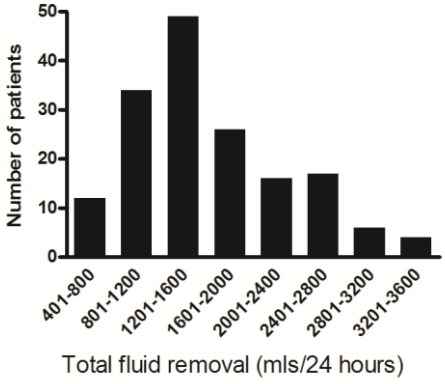


Figure 14: Total fluid removal from peritoneal dialysis patients

The mean total fluid removal was 1607.5ml/24 hours (95% CI 1482-1733mls/24 hours). There is a small group of patients who at baseline had poor 24 hour fluid removal results. This might reflect a damaged peritoneal membrane with loss of ultrafiltration capacity, so that patients were unable to achieve an acceptable amount of fluid removal. Alternatively these patients may have had the ability to achieve greater 24 hour fluid removal, but were not using an appropriate prescription at the time.

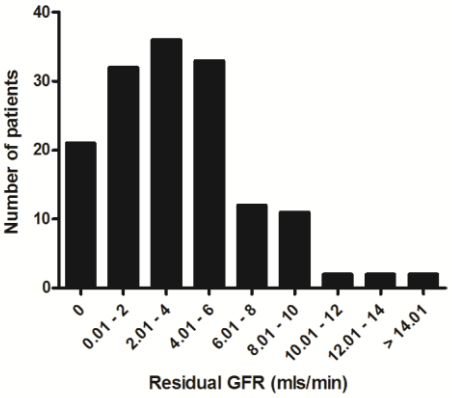


Figure 15: Residual GFR in peritoneal dialysis patients

The median residual GFR in the peritoneal dialysis population was 3.2mls/min (range 0 – 20.4mls/min). The GFR is calculated by the Adequest (Renalsoft version 2.0) software used in our department as the mean of urea and creatinine clearances, an approximation that helps to minimise the error inherent in the calculation of creatinine clearance in patients with very little renal function.

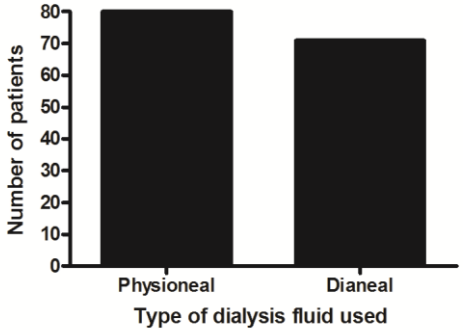


Figure 16: Types of dialysis fluid used in peritoneal dialysis patients

Physioneal (Baxter, Deerfield, USA), a relatively new and potentially more biocompatible dialysis fluid, was used in 80 of the patients at the time of sampling. There was a change (an increase) in the proportion of patients using Physioneal over the period of the study. This means that later-recruited patients are more likely to have used Physioneal than patients recruited earlier in the study. This might be important in the analysis of risk factors for future EPS.

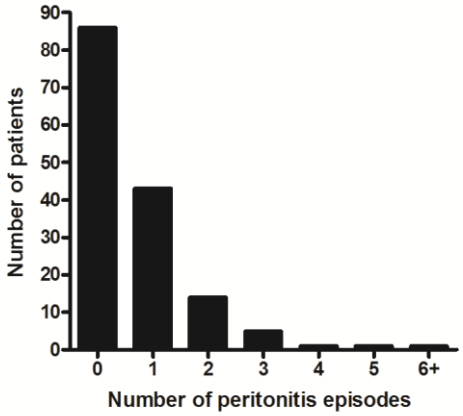


Figure 17: Number of previous peritonitis episodes at time of sampling PD patients

The rate of peritonitis episodes per patient-treated-month did not vary significantly during the study period, and was similarly low for patients recruited at the beginning and the end of the study.

Peritonitis is not a common event in our population. To time of sample collection, 102 episodes of peritonitis were treated in 5129 patient-months, giving an overall rate of 1 episode/50 patient-months. This is considerably lower than the acceptable limit of 1 episode/18 patient-months cited in the ISPD guidelines of 2005 (Piraino *et al.*, 2005).

4.2 Dialysate levels of cytokines are not just a reflection of serum levels

4.2(i) Median serum and dialysate concentration of cytokines

Cytokine	Serum concentration (pg/ml)		Dialysate concentration (pg/ml)		Peritoneal appearance rate (ng/4hrs)	
	Median	Range	Median	Range	Median	Range
CCL18	121815	24825 – 470788	2438	104 - 17583	6509	261 - 35856
MCP-1	244.7	33.6 - 745.8	199.1	76.5 - 1294.5	413	100.4 - 2718.4
IL-6	4.22	0.55 – 86.5	21	1.7 - 423.2	46.7	3.8 - 1860.5
Angiogenin	832397	311702 – 1681535	45977	7551 - 159222	104544	13592 - 318444
CCL15	21649	10021- 91386	2242	302 - 9698	4390	726 - 19394

Table 5: median concentrations of cytokines in serum and dialysate

Inspection of the median serum and dialysate levels shows that while some cytokines show considerable higher serum than dialysate concentrations, there is a higher concentration of IL-6 in the dialysate than in the serum.

4.2(ii) Dialysate cytokine concentrations do not always correlate with serum cytokine concentrations

If the dialysate concentration of a cytokine was primarily determined by the serum concentration, with the cytokine entering the dialysate by diffusion through pores in the peritoneal membrane, a strong correlation would be expected between the serum and dialysate concentrations. In order to correct for the varying volumes of dialysis fluid at the end of the PET test, when discussing cytokine production in the peritoneal space, all dialysate cytokine concentrations are corrected for the drained volume at the end of the PET test and expressed as the amount of cytokine (in ng) present after the 4 hour dwell (the peritoneal appearance rate). However when considering whether a cytokine may have entered the peritoneal space by diffusion from the serum, the dialysate concentration is more relevant and this is used here.

For all correlations between cytokines and clinical factors the Spearman's rho, or r value, is reported as the distribution of cytokine values is not normally distributed.

The relationship between serum and dialysate CCL15 concentrations shows this expected close correlation and to a lesser extent so does the relationship between serum and dialysate CCL18:

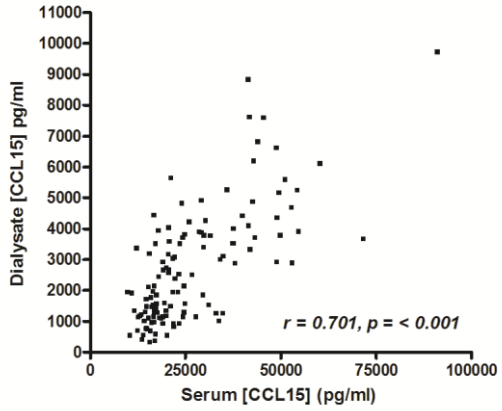


Figure 18: There is a close correlation between serum and dialysate CCL15 levels

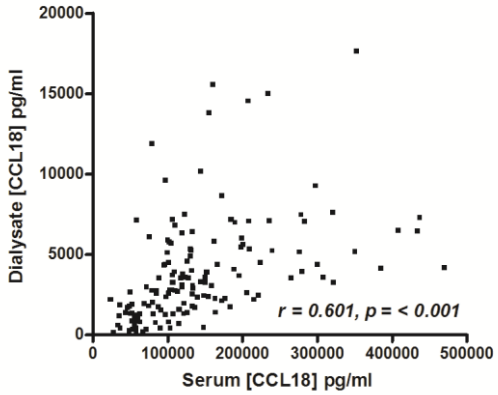


Figure 19: There is a less marked correlation between serum and dialysate CCL18 levels

However, this is not the case for angiogenin, MCP-1 and IL-6:

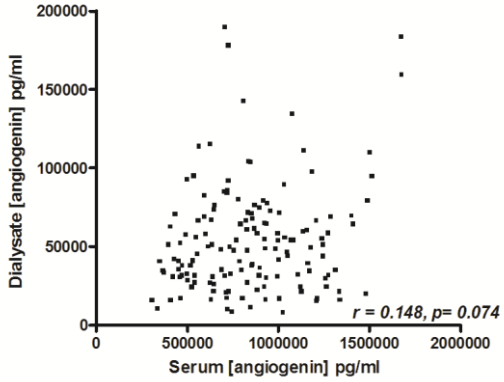


Figure 20: There is little correlation between serum and dialysate angiogenin levels

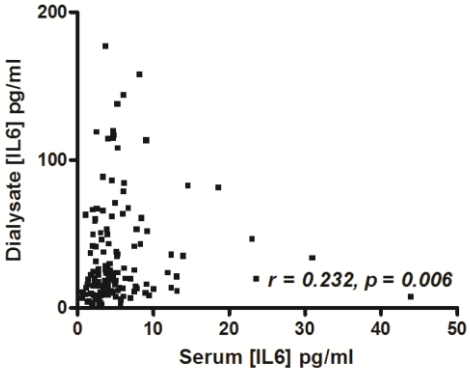


Figure 21: There is a weak correlation between serum and dialysate IL-6 levels

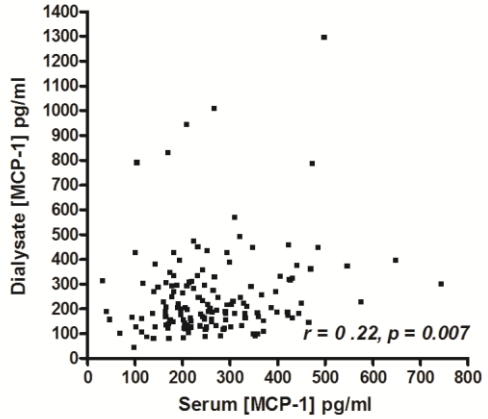


Figure 22: There is little correlation between serum and dialysate MCP-1 levels

There are only weak correlations between the levels of MCP-1, IL-6 and angiogenin in the dialysate and in the serum. In addition, dialysate MCP-1 levels are slightly higher than the serum levels (average dialysate to serum ratio 1.159). Dialysate IL-6 levels are considerable higher than serum levels (average dialysate to serum ratio 9.128). This also suggests that these cytokines are being produced within the peritoneal space rather than diffusing in from the circulation.

4.2(iii) Analysis of serum/dialysate protein concentration ratios by molecular weight: some dialysate cytokine levels are higher than expected

In order to further investigate the relationships between serum and dialysate cytokine levels, the concentrations of five proteins of varying molecular weights were measured in twenty paired serum and dialysate fluid samples. These proteins were selected as being readily measurable, covering a wide range of molecular weights and not being synthesised by peritoneal cells. The aim was to use this information to construct a plot of molecular weight versus dialysate to serum ratio. This approach was used by Mizutani et al (Mizutani *et al.*, 2010) to demonstrate an excess of connective tissue growth factor in the dialysate, which they argue indicates local production of the cytokine.

Molecule	Molecular weight	D/S ratio
Creatinine	0.113kDa	0.713
B2-microglobulin	11kDa	0.135111
Prolactin	24kDa	0.075714
Albumin	68kDa	0.013277
Immunoglobulin G	155kDa	0.006764

Table 6: measured dialysate to serum ratios of five marker proteins

Of note, there was no difference in dialysate to serum ratios of the marker proteins between patients with and without a history of peritonitis (although only three of twenty patients had ever experienced peritonitis).

GraphPad software was then used to model the relationship between molecular weight and dialysate to serum ratio:

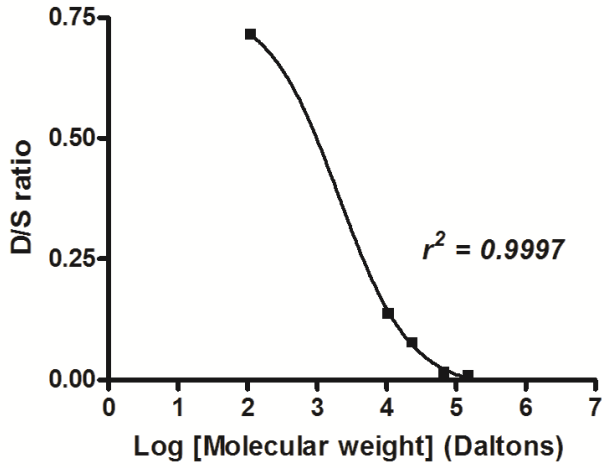


Figure 23: Non-linear fit of Dialysate to Serum ratio v. log (molecular weight) for marker proteins

The expected dialysate to serum ratio could then be calculated from the graph for each cytokine, dependent on its molecular weight:

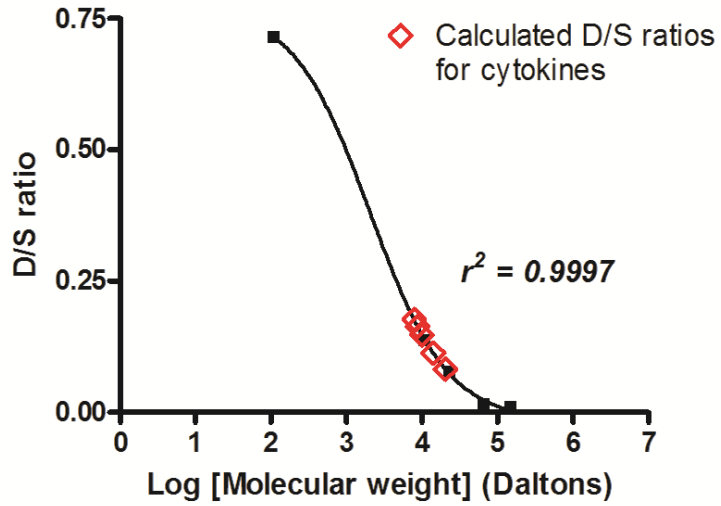


Figure 24: Dialysate to Serum ratio v. log (molecular weight) showing position of cytokines on the curve

This can then be compared with the measured dialysate to serum ratio for each cytokine:

Cytokine	Predicted D/S ratio	Measured D/S ratio
Angiogenin, MW 14000*	0.113	0.0802
CCL18	0.176	0.0288
CCL15	0.147	0.1012
IL-6	0.082	9.1277
MCP-1	0.163	1.1592
Angiogenin, MW 16500*	0.099	0.0802

Table 7:Predicted and measured dialysate to serum ratios for cytokines
**Two molecular weights for angiogenin are given*

It is notable that there appears to be less CCL18 within the dialysate than would be expected if CCL18 diffused in from the circulation, at least compared to other proteins of a similar molecular weight. One possibility is that CCL18 is protein-bound and therefore does not behave as a protein of 7.8kDalton molecular weight.

The values for angiogenin and CCL15 are roughly consistent with the possibility that dialysate levels of these cytokines are due to diffusion from the circulation. Estimates of molecular weight are variable and the differences between predicted and measured dialysate levels are relatively small. The results do not exclude the possibility of local production of these cytokines as it is possible that they are made by, and remain largely bound to, peritoneal mesothelial or other cell types within the peritoneum. This will be further investigated in the cell culture experiment section.

The values for both MCP-1 and IL-6 are much higher than would be expected if the presence of cytokines in dialysate fluid was only due to passive transfer from the circulation. This suggests that

there is significant local production within the peritoneum of these cytokines. This is consistent with a previous study by Zemel et al (Zemel *et al.*, 1992) which showed higher IL-6 levels in the overnight effluent dialysate of four patients than would be expected; in this study the restriction coefficients of α2 macroglobulin, β2 microglobulin and albumin transfer across the peritoneal membrane were used to establish the expected rate of diffusion of IL-6 from serum to dialysate.

4.2(iv) GFR and cytokine levels:

As small molecules (molecular weight smaller than albumin), cytokines such as MCP-1, CCL15, CCL18, angiogenin and IL-6 might be filtered at the glomerulus and the serum level would then be expected to show a negative correlation with GFR. The range of GFR (calculated as the mean of urea and creatinine clearance) is small in PD patients, with most patients having a GFR between 0 and 10mls/minute. For most cytokines the association between GFR and serum levels is weak or non-existent:

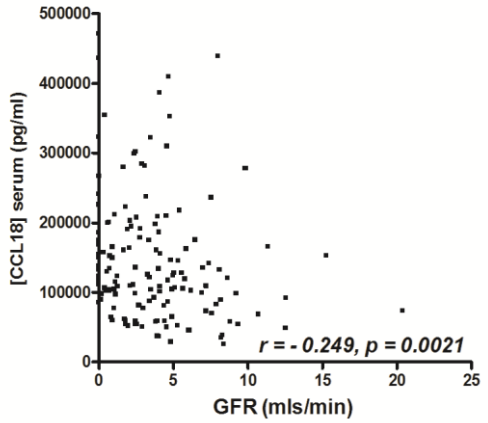


Figure 25: Serum concentrations of CCL18 correlate only weakly with GFR; CCL15 shows a similar pattern

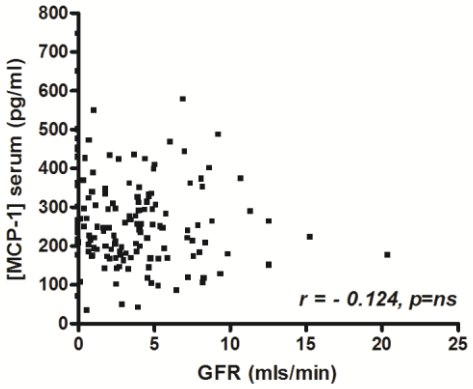


Figure 26: Serum concentrations of MCP-1 do not correlate with GFR; IL-6 serum concentrations show a similar pattern

This is consistent with work from our group (Dr Qureshi) showing no correlation between serum levels of CCL18 and GFR in over one hundred patients with CKD (GFR range 11-90mls/minute).

In contrast, there are stronger correlations observed between the appearance rate in the dialysate of MCP-1 and IL-6 and the GFR:

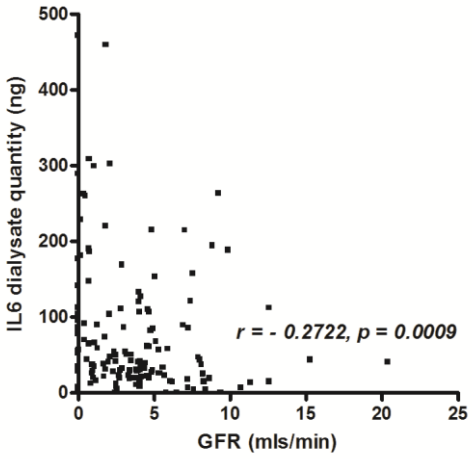


Figure 27: Unlike the serum levels, the quantity of IL-6 in the dialysate does correlate with the GFR

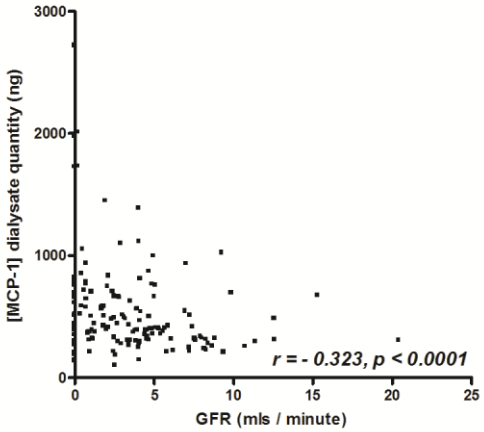


Figure 28: Similarly, the dialysate quantity of MCP-1 does correlate with GFR although the serum level does not

The relationships between dialysate cytokines and GFR do not exist only because the serum levels of cytokines are affected by the GFR. The correlations between serum levels of cytokine and GFR are weak or non-existent, and a closer relationship is usually seen between the dialysate quantity of a cytokine and GFR. The association between residual GFR and dialysate cytokine levels could be due to the increased levels of inflammation seen generally in patients with reduced renal function, or could be related to the increased exposure to dialysate that is necessary to achieve adequate clearances in patients with little residual GFR.

Summary

In this population of well-dialysed patients, it seems unlikely from this data that dialysate levels of cytokines depend only on the serum levels. The concentrations of IL-6 in the dialysate are higher than levels seen in the serum, making it likely that IL-6 is produced within the peritoneum. In some patients the same is true for MCP-1.

Analysis of the data describing transfer of proteins of variable molecular weight into the dialysate also suggests that there is production of IL-6 and MCP-1 within the peritoneum.

However, for CCL15 there is a close correlation between serum and dialysate levels. The dialysate to serum ratio is also consistent with the possibility that CCL15 might enter the dialysate by passive transfer from the circulation.

For CCL18 the quantities found in the dialysate are lower than would be expected given the molecular weight of this cytokine. A relationship between serum and dialysate concentrations is

seen, which might suggest that CCL18 enters the dialysate from the circulation. This data is consistent with the possibility that CCL18 in the peritoneum is bound to cells and therefore not found at the expected higher levels in the dialysate. Alternatively, CCL18 could be made in the peritoneum and the relationship with the serum levels might be seen because the same factors that determine serum levels also determine peritoneal levels.

Although angiogenin is found at approximately the expected levels in the dialysate to be consistent with the possibility that it enters passively from the circulation, the lack of a close correlation between serum and dialysate levels of angiogenin suggests that other factors must influence dialysate levels of angiogenin; perhaps properties of the peritoneal membrane or variable amounts of intraperitoneal production of angiogenin.

Residual renal function may be related to the dialysate levels of cytokines, but this is not because the serum levels of cytokines are affected by the level of residual renal function; as we have seen, dialysate cytokines frequently do not correlate with serum levels of cytokines, and in the GFR range of PD patients, serum levels of cytokines in turn do not correlate closely with GFR. It is possible that patients with little residual function had greater exposure to inflammation-inducing dialysate or another exposure associated with higher dialysate cytokine levels.

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CHAPTER 5

RESULTS: Cytokine levels - correlations with clinical factors

5.1 Dialysate cytokine levels correlate with clinically relevant factors

All quantities of cytokines in dialysate are the total amount in the dialysate effluent at the end of the four hour dwell period. This is a more useful result than the concentration of the cytokine in the dialysate, as the drained volume of dialysate at the end of the PET test will vary considerably between patients.

5.1(i) Duration of peritoneal dialysis

One of the most important risk factors for both ultrafiltration failure (Davies, 2004) and also for EPS (Kawanishi and Moriishi, 2005; Balasubramaniam *et al.*, 2009; Brown *et al.*, 2009b) after the cessation of peritoneal dialysis is the total duration of peritoneal dialysis.

Duration of PD did not differ significantly between the first, second and third group of fifty patients recruited for the study.

The peritoneal appearance rate of some cytokines increases with the duration of peritoneal dialysis at the time of sampling. This is most clearly seen when the correlations between duration of PD and IL-6 or MCP-1 are examined:

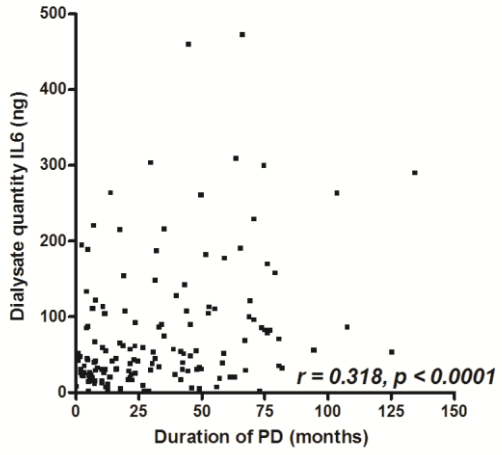


Figure 29: The dialysate quantity of IL-6 increases with the duration of PD

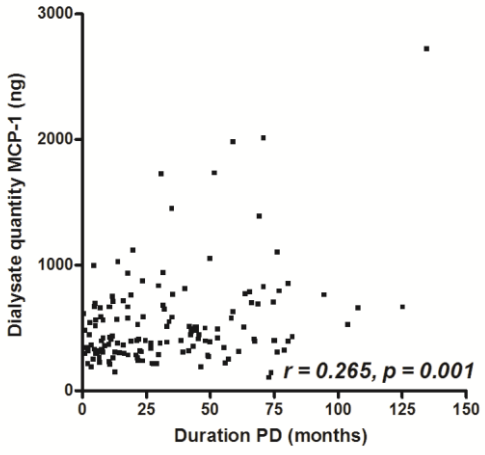


Figure 30: The dialysate quantity of MCP-1 increases with the duration of PD

The correlations of CCL15 with duration of PD, and CCL18 with duration of PD are less marked (although still significant):

CCL15: r = 0.1962, p = 0.0296

CCL18: r = 0.1708, p = 0.038

There was no correlation between the dialysate quantity of angiogenin and the duration of PD.

5.1(ii) Membrane transport status

The dialysate levels of all investigated cytokines correlate closely with the dialysate/plasma creatinine (the ratio of dialysate and plasma creatinine concentrations at the end of the PET test). This is not surprising in the case of CCL18 and CCL15, as we have seen that the dialysate and serum concentrations of these cytokines are closely correlated (section 3.2(ii), figures 17 and 18).

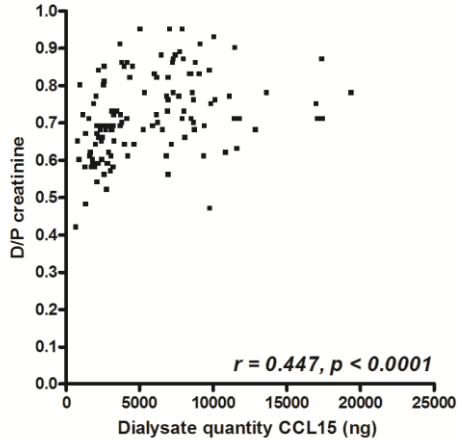


Figure 31: There is a significant correlation between the dialysate quantity of CCL15 and the D/P creatinine

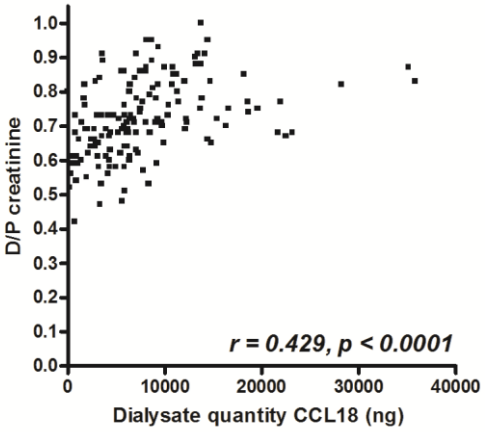


Figure 32: There is a significant correlation between the dialysate quantity of CCL18 and the D/P creatinine

Together, these data would suggest that CCL15 and CCL18 might enter the peritoneal space from the circulation at a rate which is related to the rate of transfer of creatinine into the dialysate, and that this might determine the amount of CCL15 and CCL18 found in the dialysate. This is consistent with the data from the study of serum:dialysate protein concentrations (section 3.2(iii)), in which the dialysate concentrations of CCL15 and CCL18 were the same or lower than that expected if the cytokine moved passively into the dialysate from the circulation.

The remaining three cytokines also show a close correlation between dialysate to plasma creatinine ratio and the quantity of cytokine found in the dialysate. However for these three cytokines, we know that there is no or a weak relationship between serum and dialysate levels.

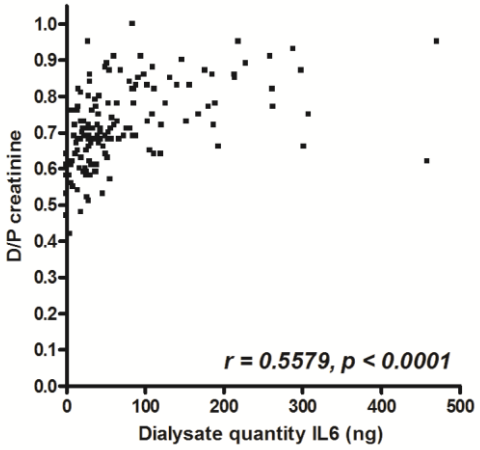


Figure 33: There is a significant correlation between the dialysate quantity of IL-6 and the D/P creatinine

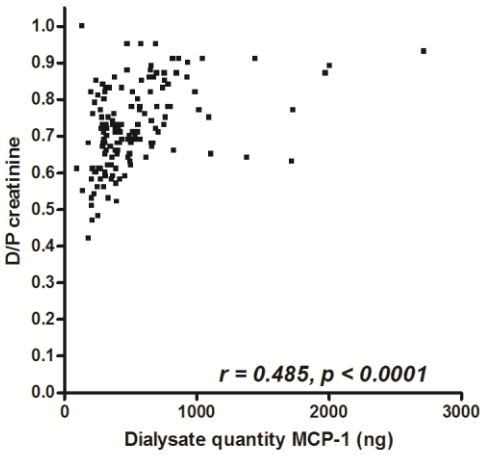


Figure 34: There is a significant correlation between the dialysate quantity of MCP-1 and the D/P creatinine

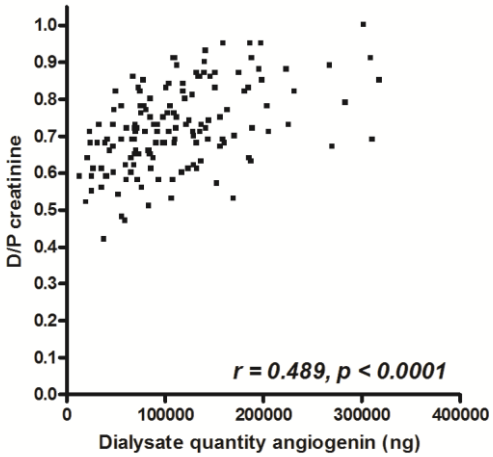


Figure 35: There is a significant correlation between the dialysate quantity of angiogenin and the D/P creatinine

Since the dialysate concentrations of these cytokines are not closely related to their respective serum concentrations, it seems unlikely that the observed correlation with the dialysate/plasma creatinine level has the same interpretation as suggested above for CCL18 and CCL15. One possible interpretation is that IL-6, MCP-1 and angiogenin produced in the peritoneum are important in determining the D/P creatinine. However it is also possible that a confounding factor – for example glucose exposure or duration of peritoneal dialysis – could affect both D/P creatinine and the quantity of IL-6, MCP-1 and angiogenin in the dialysate.

5.1(iii) Glucose Exposure

The glucose exposure was calculated as the amount of glucose (in mmol per twenty four hours) in the dialysate used by the patient according to their current prescription at the time of the PET and adequacy assessment. This is not an ideal measure of glucose exposure as it is only a point measure and in theory could differ considerably from previous prescriptions. However, a more

comprehensive measure of glucose exposure would require more detailed longitudinal records of the prescription than were available.

Glucose exposure is determined by the need to remove water from the dialysis patient. It is therefore related to:

- the salt and water intake of the patient
- the amount of residual urine output
- the ultrafiltration capacity of the peritoneal membrane

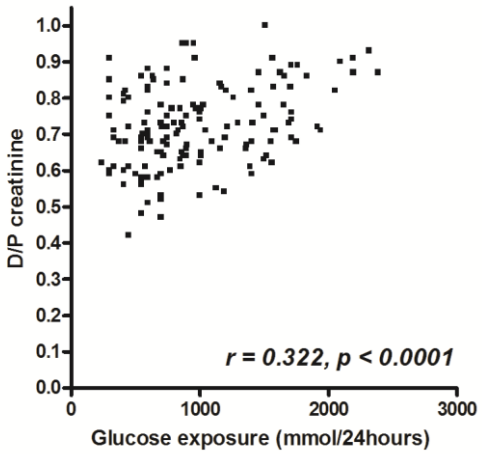


Figure 36: Glucose exposure is higher in patients who have a higher dialysate to plasma creatinine ratio

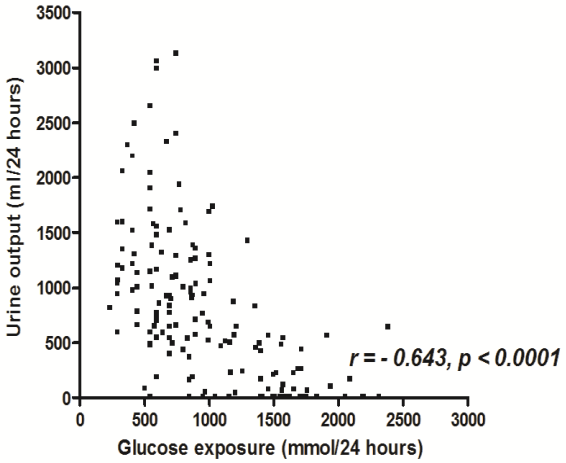


Figure 37: Glucose exposure is higher in patients who have less residual urine output

Interestingly, both IL-6 and MCP-1 levels in the dialysate correlate with glucose exposure. Data from comparing serum and dialysate levels of these cytokines suggests that they are both produced within the peritoneum, and that dialysate levels are not determined by serum levels or by the ability of the cytokines to enter the dialysate from the circulation. The observation that the dialysate levels of these cytokines correlates with glucose exposure (as well as D/P creatinine) suggests that these cytokines could be involved in the sequence of events from glucose exposure to increasing D/P creatinine seen in long term peritoneal dialysis patients. This could be because glucose exposure increases the level of MCP-1 and IL-6, and this in turn leads to changes in membrane transport properties. Alternatively, increased levels of MCP-1 and IL-6 could affect the peritoneal membrane such that greater glucose exposure was required to achieve ultrafiltration requirements.

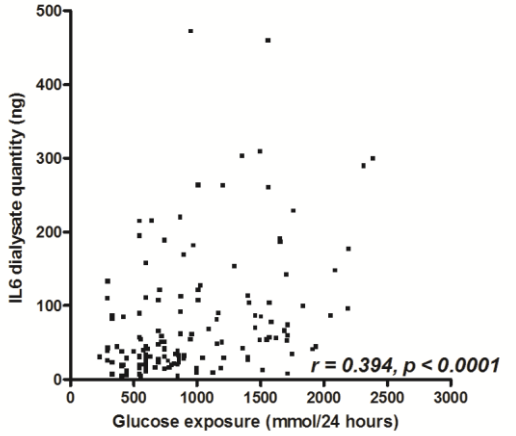


Figure 38: The level of IL-6 in the dialysate correlates with glucose exposure at the time of PET

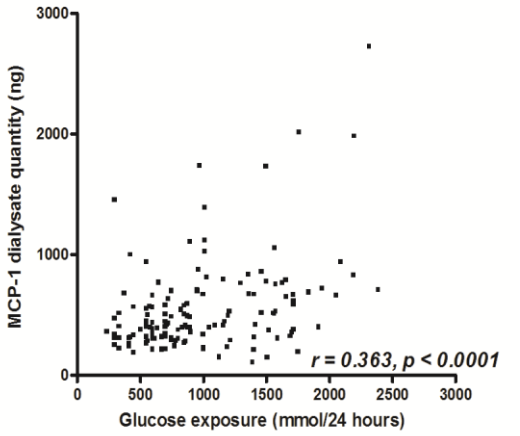


Figure 39: The level of MCP-1 in the dialysate correlates with glucose exposure at the time of PET

Dialysate levels of CCL15 and angiogenin also correlate with glucose exposure. However, the serum/dialysate ratios of these cytokines are consistent with the dialysate level being dependent on transfer of these cytokines across the peritoneal membrane from the circulation (section 3.2(iii)). It is possible that this observed correlation of CCL15 and angiogenin with glucose exposure is simply due to the correlation of glucose exposure with the characteristics of the peritoneal membrane, rather than being an indication that CCL15 and angiogenin mediate the effects of glucose exposure on the peritoneum. The dialysate quantity of CCL18 does not correlate significantly with glucose exposure.

5.1(iv) Dialysate volume

The volume of dialysate used by the patient over a twenty four hour period might show similar patterns to the glucose exposure. Patients with a high dialysate to plasma creatinine ratio - fast transporters – might be expected to need large volumes of dialysate per twenty four hours to achieve adequate ultrafiltration. A common dialysis prescription for a patient with fast membrane transport status would involve multiple fairly short duration exchanges on APD in order to maximise ultrafiltration. This means a larger volume of fluid is required by fast transporters on APD, and this is the result seen in figure 40:

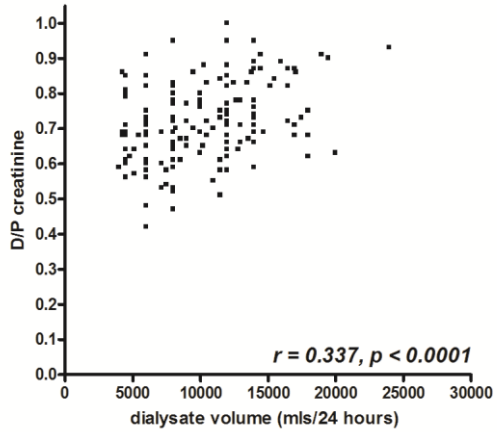


Figure 40: The volume of dialysate used per twenty four hours correlates with the D/P creatinine

The relationships of cytokine levels with dialysate volume must therefore be interpreted in the context of this relationship with dialysate to plasma creatinine ratio.

The significant correlations between both MCP-1 and IL-6 and dialysate volume may be interpreted in a similar manner to those with glucose exposure. Dialysate levels of IL-6 and MCP-1 are not determined by the membrane transport properties of the peritoneum, but do correlate with membrane transport properties (with the dialysate to plasma creatinine ratio). Therefore the observed relationship of IL-6 and MCP-1 with dialysate volume may be due to the co-correlation with the membrane transport status, or it may be that exposure to large volumes of dialysate increases both transport status and IL-6/MCP-1 (perhaps via the implied increase in exposure to glucose or other harmful constituents of dialysate).

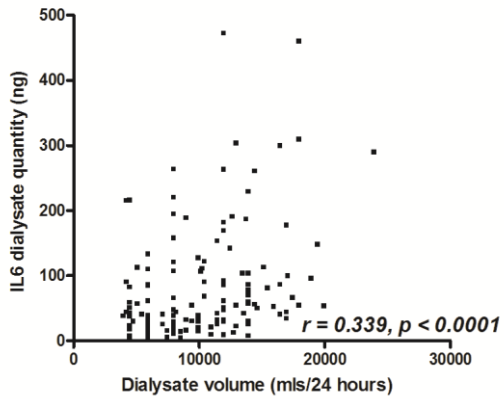


Figure 41: The volume of dialysate used per twenty four hours correlates with the quantity of IL-6 in the dialysate

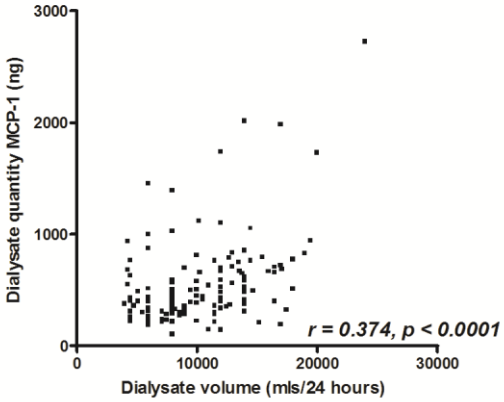


Figure 42: The volume of dialysate used per twenty four hours correlates with the quantity of MCP-1 in the dialysate

Angiogenin and CCL15 also correlate with dialysate volume, but clearly this might be due to the relationships between these cytokines and the membrane transport characteristics. However it is

worth noting that CCL18, which has a similar relationship with serum levels and D/P creatinine to angiogenin and CCL15, correlates with neither glucose exposure nor dialysate volume:

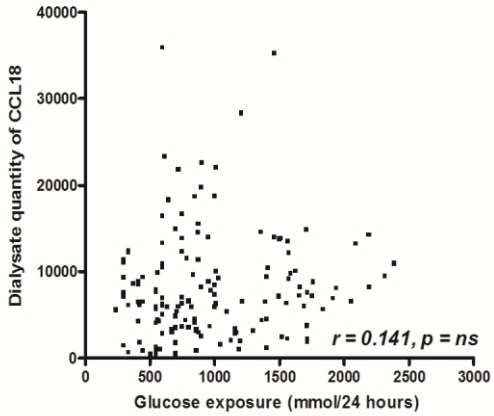


Figure 43: CCL18 levels in dialysate do not correlate with glucose exposure

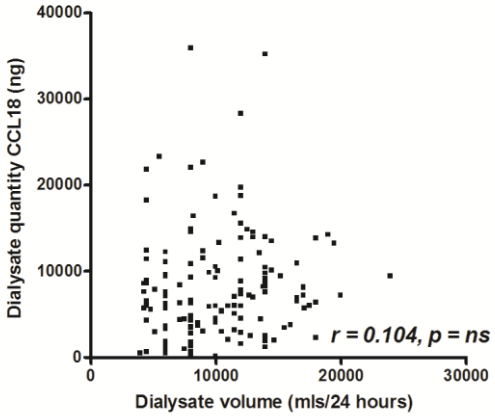


Figure 44: CCL18 levels in dialysate do not correlate with dialysate volume

Angiogenin and CCL15 are both of a similar size as CCL18, so it is interesting that the dialysate levels of angiogenin and CCL15 do correlate with dialysate volume and glucose exposure, and the levels of CCL18 do not. It implies that levels of these three molecules do not behave in the same manner, and this makes it less likely that passive transfer of these molecules from serum to dialysate is the only factor determining dialysate cytokine levels.

5.1(v) Peritonitis

PET tests were always performed at least six weeks after any episode of peritonitis. None of the measured cytokine levels were affected by the number of previous peritonitis episodes. There were no significant differences between cytokine levels in patients who had never experienced peritonitis and those who had. A trend towards a significant difference was seen for MCP-1 dialysate levels only; median MCP-1 production being 396ng in the group which had never experienced peritonitis and 478.6ng in the group which had experienced at least one episode of peritonitis. The p value for this comparison was 0.1, however. In a smaller group of patients (45 prevalent PD patients), Malik et al (Malik *et al.*, 2007) previously found a significant relationship between dialysate MCP-1 concentrations and past peritonitis; the difference in findings may be explained by the use of dialysate concentrations rather than total MCP-1 production in this earlier study.

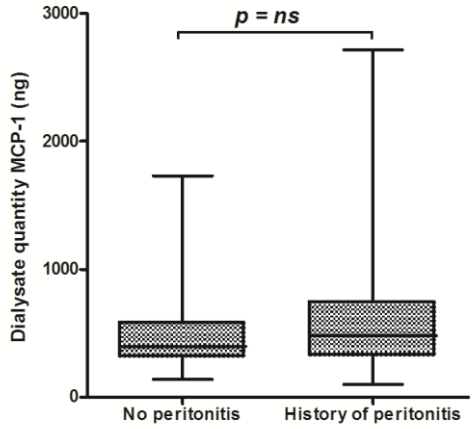


Figure 45: MCP-1 levels in dialysate are not higher in patients who have experienced peritonitis

5.1(vi) Dialysis modality

The levels of MCP-1, IL-6 and angiogenin are generally higher in the dialysate effluent from patients who are using automated nocturnal dialysis (APD) than from patients who are using manual exchanges (CAPD), although there is considerable overlap between the groups:

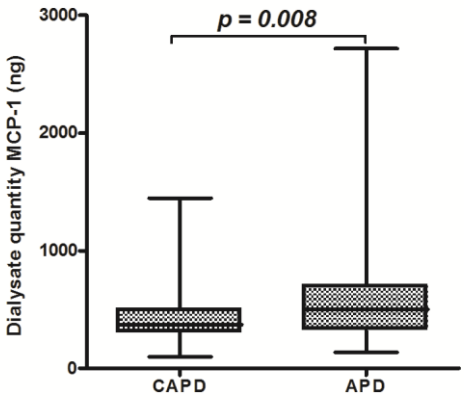


Figure 46: MCP-1 levels are higher in patients using APD

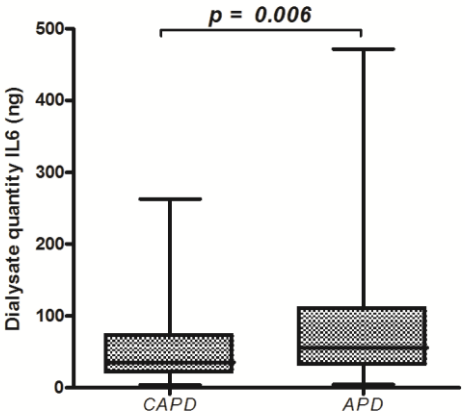


Figure 47: IL-6 levels are higher in patients using APD

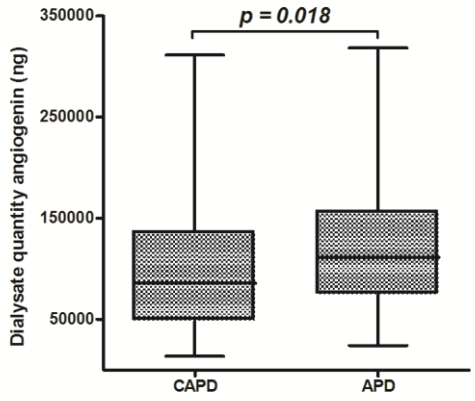


Figure 48: Angiogenin levels are higher in patients using APD; note this p value is not significant if corrected for multiple comparisons

It is likely that the increased levels of these cytokines in the dialysate of APD patients relate to the reasons why patients use a particular modality. While for some it is a lifestyle choice, for others who have a high dialysate to plasma creatinine ratio it is necessary to perform multiple short duration dwells, with a high dialysate volume and glucose exposure, to achieve adequate ultrafiltration. Anuric patients may require APD to achieve adequate creatinine clearances.

Since many cytokine levels correlate with dialysate volume used and glucose exposure it is not surprising that cytokine levels are higher in APD patients. Similarly the use of APD is more common in patients with low or no residual function ($p < 0.0001$) and these patients have higher cytokine levels. It is also possible that there is an effect directly related to increased fluid flows and associated physical stresses on the peritoneum.

5.1(vii) ACEi and ARB

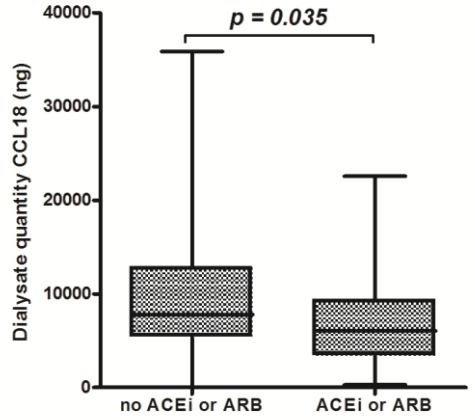


Figure 49: Dialysate levels of CCL18 were lower in patients who took an ACE inhibitor or angiotensin receptor blocker

The dialysate levels of CCL18 were lower in patients who took an ACEi or an angiotensin receptor blocker. As discussed in the introduction, the angiotensin system may be involved in the pathogenesis of peritoneal changes and particularly fibrosis. CCL18 is associated with fibrosis in other conditions. The fall in dialysate levels of CCL18 may be due to a fall in serum levels of CCL18 (the distribution of D/P creatinine is the same whether ACEi/ARBs are taken or not). This result should be treated as hypothesis-generating only as the p value would not be significant if corrected for multiple comparisons.

5.1(viii) Diabetes

Patients with diabetes were not found to have higher levels of cytokines in the dialysate effluent. This is interesting in that in the general population patients with diabetes have frequently been shown to have higher levels of MCP-1 (Piemonti *et al.*, 2009). It is possible that the high levels of glucose in the dialysate mimic the effect of diabetes on cells in the peritoneum, such that all PD patients have high MCP-1 production regardless of whether they have diabetes.

5.1(xi) Fluid type

One problem in assessing the effect of fluid type on cytokine levels in the sample population is that the use of Physioneal increased during the study, being 34% in the first 50 patients recruited, 24% in the second fifty patients recruited and 100% in the final fifty patients recruited. This is statistically significant ($p < 0.0001$, Krushal-Wallis test). This problem does not occur with other variables such as peritonitis rates or duration of PD at time of sampling, and no systematic differences are noted by recruitment time for serum and dialysate cytokine levels.

No significant differences were noted for dialysate cytokines between patients using Dianeal and those using Physioneal.

Summary

In this initial analysis, the levels of cytokines (apart from angiogenin) were seen to increase with duration of peritoneal dialysis. All cytokines correlated significantly with the D/P creatinine, but the mechanism by which this occurs is likely to differ between cytokines – while CCL18 and CCL15 levels in the dialysate might correlate closely with membrane transport properties because they enter the dialysate from the circulation, this is not likely to be the case for IL-6 and MCP-1. The relationship between these cytokines and the membrane transport of creatinine may be due to a confounding factor, such as glucose exposure, which affects both the peritoneal properties and cytokine levels, or might be due to an effect of these cytokines on the peritoneal membrane. Cytokines of similar molecular weight do not show identical relationships with dialysate volume and glucose exposure, suggesting that mechanisms may differ between one cytokine and another. The direction of effect is also not clear from this data.

No correlations were seen between cytokine level and peritonitis history, but interestingly CCL18 levels were seen to be lower in patients who were taking an ACEi or ARB, suggesting CCL18 may be a downstream mediator of the effects of angiotensin on the peritoneum. However, the groups of patients who did and did not take these medications may have differed in other respects such as duration of PD.

5.2 Multivariate analysis

Multivariate analysis was performed by Dr Bernard North of the Imperial College Statistics Advisory Service.

Multiple regression analysis was performed to identify the joint effect of the following candidate predictors:

- D/P creatinine
- Duration of PD
- Glucose exposure
- Dialysate volume
- Urine output
- Residual GFR
- Fluid type
- Number of peritonitis episodes

on the dialysate levels of each of the cytokines CCL18, MCP-1, CCL15, angiogenin and IL-6.

These candidate predictors were chosen as they are clinically important to the well-being, long term prognosis and dialysis management of a patient on peritoneal dialysis. Glucose exposure is known to have a long term effect on the characteristics of the peritoneal membrane (Davies *et al.*, 2001); residual function is a predictor of mortality in peritoneal dialysis patients (Wang and Lindholm, 2002). The D/P creatinine is an important determinant of solute clearance, as well as affecting achieved ultrafiltration, and might be expected to correlate with the rate of cytokine transfer from the circulation (although creatinine is a considerably smaller molecule than cytokines and might not pass through the peritoneum by the same route). Fluid type may also be important although the newer dialysis fluids containing fewer glucose degradation products and with a neutral pH have not so far been shown to have long term benefits on outcome. While single peritonitis episodes often do not have an impact on peritoneal membrane function there is evidence that a severe or recurrent series of peritonitis events can do so (Davies *et al.*, 1996). Duration of peritoneal dialysis is an important predictor to include since both membrane transport and ultrafiltration are known to change during the course of time (Davies, 2004).

All cytokines required log transformation to satisfy model assumptions (the non-transformed distribution of cytokines is non-normal); once transformed the distribution of cytokines is acceptably close to a normal distribution. Model fit was assessed by residual plots including Q-Q plots for normality of residuals and residuals vs fits plots to assess linearity of effect of the predictors on the log transformed cytokine level.

Log CCL18

(adj. R^2 = 0.243)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	2.447	0.193		12.675	< 0.001
D/P creatinine	1.838	0.265	0.498	6.946	< 0.001

Table 8: Linear regression for CCL18

Log CCL15

(adj. R^2 = 0.253)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	2.768	0.164		16.901	< 0.001
D/P creatinine	0.997	0.237	0.346	4.202	< 0.001
Glucose Exposure	0.000187	0.000	0.288	3.498	0.001

Table 9: Linear regression for CCL15

Log MCP-1

(adj. R^2 = 0.267)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	1.95	0.108		17.993	< 0.001
D/P creatinine	0.838	0.159	0.399	5.287	< 0.001
Dialysate volume	1.518E-5	0.000	0.267	3.583	< 0.001

Table 10: Linear regression for MCP-1

The effect of dialysate volume might be related to increased fluid flows and stress on the peritoneum but is more likely to be due to the increased glucose exposure; with dialysate volume excluded from the model the following coefficients are obtained:

(adj. R^2 = 0.267)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	1.95	0.108		17.993	< 0.001
D/P creatinine	0.838	0.159	0.399	5.287	< 0.001
Glucose Exposure	1.088E-4	0.000	0.230	3.051	0.003

Table 11: Linear regression for MCP-1

Log Angiogenin

(adj. R^2 = 0.249)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	4.121	0.124		33.269	< 0.001
D/P creatinine	1.197	0.170	0.504	7.051	< 0.001

Table 12: Linear regression for angiogenin

Log IL-6

(adj. R^2 = 0.342)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	0.380	0.178		2.135	0.034
D/P creatinine	1.585	0.266	0.442	5.951	< 0.001
Glucose Exposure	0.000145	0.000	0.183	2.425	0.017
Duration of PD	0.002	0.001	0.146	1.992	0.048

Table 13: Linear regression for IL-6

Summary

It is immediately clear that the strongest predictor for all cytokines in the multivariate analysis is the D/P creatinine. Although the other factors which remain significant in the multivariate model do so with very small unstandardised coefficients, the standardised coefficients indicate that the effects of factors other than D/P creatinine on the regression equation are relevant.

The outstanding question from this analysis would be the direction of this correlation with the D/P creatinine effect. One possibility is that cytokines have an effect on membrane transport properties and that this is why, for example, high levels of IL-6 are associated with a high D/P creatinine. This effect could occur via changes in the vasculature or the interstitium. The other possibility is that cytokine levels in the dialysate are largely dependent on the membrane transport properties because cytokines enter the dialysate from the circulation via the peritoneal membrane. This may well be the case for some cytokines, especially CCL15, but is not the case for IL-6 and MCP-1 because these molecules are found in dialysate at levels which are too high to have occurred by diffusion from the circulation.

Cytokine	*D/P creatinine*	*Glucose exposure*	*Duration of PD*
CCL18	$p < 0.001$		
Angiogenin	$p < 0.001$		
CCL15	$p < 0.001$	$p = 0.001$	
MCP-1	$p < 0.001$	$p = 0.003$	
IL-6	$p < 0.001$	$p = 0.017$	$p = 0.048$

Table 14: Summary of significant predictors of dialysate cytokine levels

5.3 Serum cytokine levels

The relationship between serum and dialysate levels of cytokines is discussed in section 3.2. In summary, for some cytokines (CCL15, CCL18 and angiogenin) there is a clear relationship between serum and dialysate cytokine levels and the amount of cytokine found in the dialysate appears to be related chiefly to serum cytokine level and the membrane transport properties of the patient. For MCP-1 and IL-6 the amount of cytokine found in the dialysate is much higher than would be expected if dialysate cytokine levels were determined only by serum cytokine level and transfer across the membrane into the peritoneal space, and it is likely that dialysate levels of these cytokines are determined by intra-peritoneal production.

In general, serum levels of cytokines are less closely correlated with peritoneal dialysis-related clinical factors than are dialysate levels. There is some evidence in the literature that serum levels of inflammatory biomarkers can show a very large variation at varying points in time in individual renal patients (LaClair *et al.*, 2008). Repeated measures of serum MCP-1, CCL18, angiogenin and CCL15, with samples taken over a period of several weeks or months, were performed in transplanted and haemodialysis patients as part of the study (repeated samples were only planned a year apart from peritoneal dialysis patients). The coefficient of variation was calculated as a measure of reliability for pairs of measurements made on a minimum of fifteen patients for each cytokine. Repeated measurement data is not available for IL-6.

Cytokine	*Number of repeats*	*Coefficient of variation*
MCP-1	15	26.7%
CCL18	17	26.9%
CCL15	19	12.5%
Angiogenin	19	14.3%

Table 15: Coefficient of variation for repeated cytokine measurement in serum

Some of the variation is related to inter-assay variation (between 7 and 8% for these cytokines) and the remainder is related to the changes in cytokine levels that occur from day to day and week to week in renal patients.

5.3(i) Duration of peritoneal dialysis

Serum levels of CCL18, MCP-1, IL-6 and angiogenin do not correlate with the duration of PD (all dialysate cytokine levels except angiogenin correlate with the duration of PD). The serum level of CCL15 shows a weak correlation with the duration of peritoneal dialysis (r = 0.217, p = 0.014). This lack of correlation between serum cytokines and dialysis duration is important in the light of the increase in dialysate cytokine levels with increased time on PD. Clearly the increase in dialysate cytokine concentrations with time is not due to an increase in serum levels. It is possible that the increase in CCL15 and CCL18 dialysate cytokine levels over time occurred due to a change in the membrane transfer properties with PD duration:

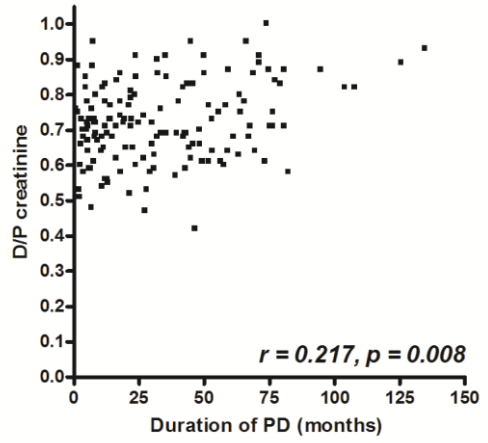


Figure 50: There is an increase in D/P creatinine over time

This increase in membrane transport rate for small molecules with time has been well documented in the literature (Struijk *et al.*, 1991; Davies *et al.*, 1996). In our study group, the D/P creatinine increases from 0.7 in patients who have used peritoneal dialysis for a year or less to 0.76 in patients who have been on peritoneal dialysis for more than four years; this is a selected group of stable long term patients, however.

The D/P creatinine describes small molecule transfer and transfer of larger molecules may not show the same relationship with dialysis duration. Struijk et al found that the peritoneal membrane permeability to macromolecules such as α2 macroglobulin actually fell with time on PD, perhaps because of increased connective tissue (Struijk *et al.*, 1991). So for larger cytokines such as IL-6 the increase in dialysate cytokine levels with duration of PD is due to neither an increase in serum levels, nor the observed change in membrane transport properties as this reflects small molecule transport.

5.3(ii) Membrane transport status

The serum levels of IL-6, MCP-1 and CCL18 correlate with the membrane transport rate. It is likely that many factors impact on the serum levels of cytokines in dialysis patients. In turn, cytokine mediators of inflammation in the serum could affect the peritoneal membrane, increasing transport of small molecules by inducing angiogenesis or vasodilatation.

Alternatively there could be another factor which correlates with both serum cytokine levels and membrane transport status. We have seen in section 3.2 that serum levels of IL-6 and MCP-1 do not correlate with the residual GFR, so this is not the potential link between serum levels of these cytokines and the peritoneal membrane transport rate. As discussed in section 5.3(i) the duration of PD also does not correlate with serum levels of most cytokines.

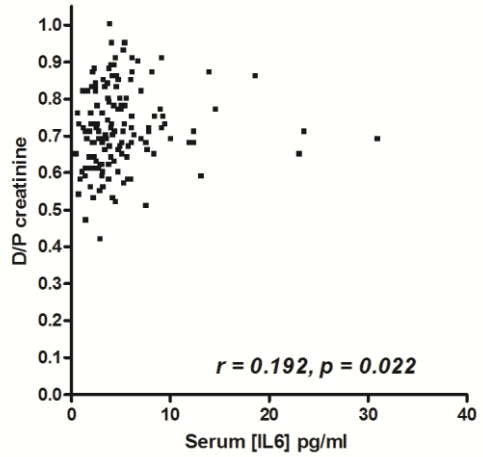


Figure 51: There is a small positive correlation between the serum IL-6 levels and the membrane transport rate

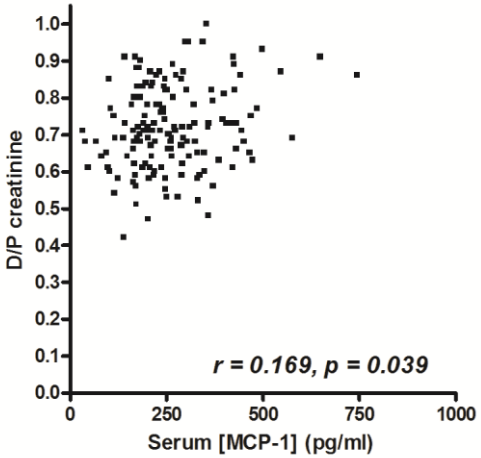


Figure 52: There is a positive correlation between the serum MCP-1 levels and the membrane transport rate

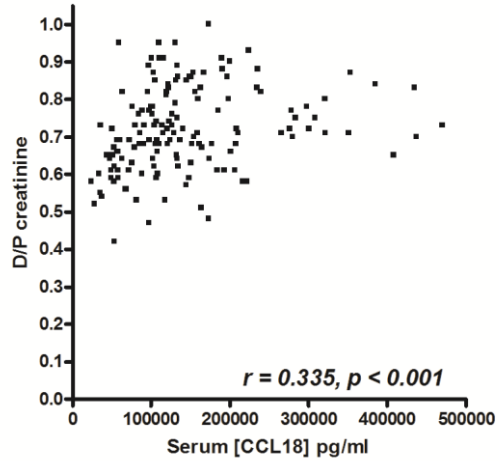


Figure 53: There is a positive correlation between the serum CCL18 levels and the membrane transport rate

Serum levels of CCL18 do correlate with the membrane transport rate and also with GFR (although weakly). It is possible that serum levels of CCL18 correlate with GFR and that this is the link between serum CCL18 levels and the peritoneal membrane transport rate. However, it is also possible that circulating CCL18 affects the peritoneal membrane and increases small solute permeability directly. This could occur via angiogenesis and an increase in the effective area for diffusion of small solutes.

5.3(iii) Glucose exposure

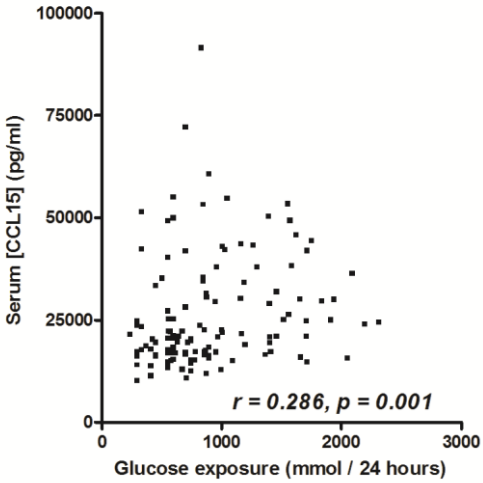


Figure 54: There is a correlation between the serum CCL15 levels and glucose exposure

The only cytokine for which the serum levels correlated with glucose exposure was CCL15. This is in contrast to the dialysate levels of cytokines, which we have seen in section 4.1(iii) largely correlated with exposure to glucose in the dialysate. In the case of serum CCL15 levels, it is possible that the correlation of serum CCL15 with residual function is responsible for the apparent correlation with glucose exposure, since residual function and glucose exposure are closely related.

5.3(iv) Dialysate volume

Similarly there are weak correlations between both CCL15 and CCL18 and the dialysate volume used per twenty four hours (CCL15: r =0 .274, p = 0.002; CCL18: r = 0.173, p = 0.035). Again it is difficult to disentangle whether this is due to any effects of CCL15 and CCL18 on the peritoneal membrane properties, or whether it is secondary to the fact that patients with poor residual function generally require larger volumes of dialysate.

5.3(v) Peritonitis

None of the study group samples were taken during or within 6 weeks of a peritonitis episode.

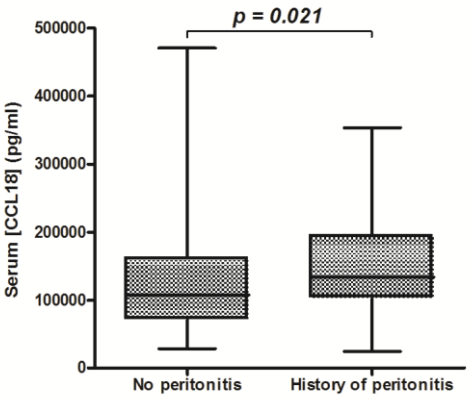


Figure 55: The serum level of CCL18 is higher in patients who have experienced peritonitis

The serum level of CCL18 was found at higher levels in patients with a history of peritonitis, although with considerable overlap between groups. When the data was analysed as zero, one or two or more episodes of peritonitis, then the Kruskal-Wallis statistic remained significant (p=0.0489). This suggests that there may be a persistent increase in serum CCL18 levels after an episode of peritonitis; however the difference is no longer significant if corrected for multiple comparisons.

5.3(vi) Dialysis modality

There was no difference in serum cytokine levels between patients using CAPD and APD. This is not unexpected in that the factors which tend to differ between APD and CAPD – dialysate volume and glucose exposure – have not been seen to correlate with most serum cytokine levels. In addition some patients will be using APD for lifestyle reasons rather than clinical necessity, and for these patients their cytokine levels might well differ from the group with high membrane transport rate who need to use APD to achieve adequate ultrafiltration.

One factor which might have been more prominent in APD, with more frequent fluid exchanges, was the role of fluid stress.

5.3(vii) ACEI

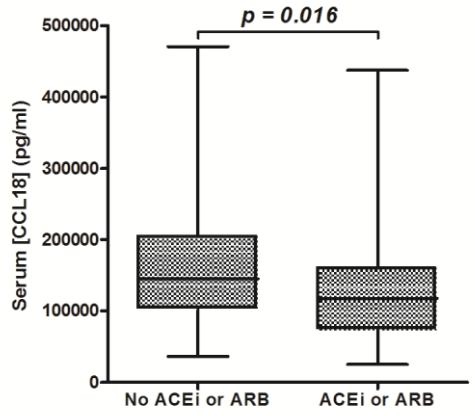


Figure 56: The serum level of CCL18 is higher in patients who are not taking an ACEi or ARB

Serum levels of CCL18 (as well as dialysate levels) were found to be higher in samples from patients who were not taking an angiotensin converting enzyme inhibitor or angiotensin receptor blocker than in samples from patients who took these medications. Activity of the renin-angiotensin system is associated with fibrosis in the peritoneum and elsewhere.

5.3(viii) Diabetes

None of the measured cytokines were found to be present at higher levels in PD patients with diabetes as compared to patients without diabetes. This is slightly surprising in the case of MCP-1, which is known to be found at higher levels in diabetic patients. One possibility is that the levels of MCP-1 are generally so high in peritoneal dialysis patients (perhaps because of the high levels of exposure to glucose in dialysate) that there is then no difference between diabetic and non-diabetic patients. In support of this, work from Dr Tam's group (A Qureshi) showed that the median level of MCP-1 in CKD patients not on dialysis is only 180pg/ml, whereas in the peritoneal dialysis population the median level is substantially higher at 245pg/ml. For most cytokines the levels were noted to be higher in haemodialysis patients than peritoneal dialysis patients (see section 8.1(i)), but this is not the case for MCP-1.

5.3(ix) Fluid type

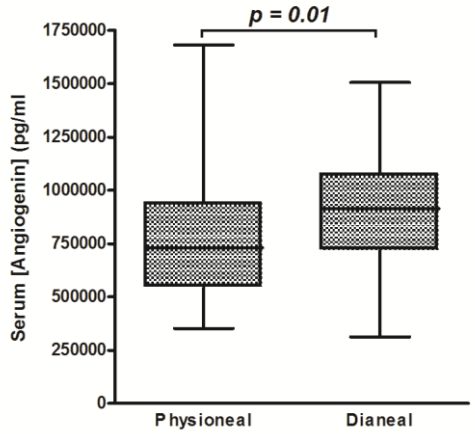


Figure 57: The serum level of angiogenin is higher in patients who are using Dianeal

There is a significant difference in median serum angiogenin levels only between the group of patients using Physioneal and those using Dianeal. This difference is quite small (median values 730257 v 914885 pg/ml) and the p value of 0.01 is only just significant if corrected for multiple comparisons.

Summary

The correlation of serum levels of cytokines with the membrane transport rate is interesting because most serum cytokine levels investigated did not correlate strongly, or at all, with other factors such as glucose exposure, residual renal function or duration of peritoneal dialysis. This makes it less likely that these factors, which are known to be associated with the membrane transport properties of peritoneal dialysis patients, are confounding the relationship between serum cytokine levels and the D/P creatinine. It may be the case that circulating cytokines affect peritoneal membrane properties by inducing angiogenesis or fibrosis. Serum levels of cytokines appear to be quite variable from one sample to another if measured repeatedly, however. Serum levels of CCL15 did correlate with duration of PD and glucose exposure; this means that it is more difficult to interpret the association with membrane transport properties.

The observation that CCL18 levels were lower in the dialysate of patients who took an ACEi or ARB was also observed in the serum levels of CCL18; lower serum levels of CCL18 were also seen in the group taking these medications and may be responsible for the lower dialysate levels.

5.4 Multivariate analysis of dialysate cytokine levels including serum levels

Having explored the relationships between clinical measures and both serum and dialysate cytokine levels, the respective serum cytokine level was then added to the multivariate analysis of each cytokine level in dialysate to establish whether serum cytokine levels were significant determinants of dialysate cytokine levels. As before, cytokine levels were log transformed to satisfy model

assumptions. Candidate predictors duration of PD and glucose exposure were also log transformed. Model fit was assessed by residual plots including QQ plots for normality of residuals and residuals versus fits plots for linearity of the effect of the predictors on the log transformed cytokine level. Analysis was performed using SPSS version 19 and the R project version R.13.

(adj R^2 = 0.382)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	2.453	0.172		14.223	< 0.001
D/P creatinine	1.449	0.245	0.394	5.922	< 0.001
CCL18 serum	0.000001903	< 0.0001	0.407	6.119	< 0.001

Table 16: Linear regression for dialysate CCL18

(adj R^2 = 0.713)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	- 2.502	0.367		- 6.809	< 0.001
Log CCL15 serum	1.098	0.081	0.687	13.521	< 0.001
D/P creatinine	1.218	0.147	0.425	8.312	< 0.001
Log glucose exp.	0.164	0.072	0.12	2.27	0.025

Table 17: Linear regression for dialysate CCL15

(adj R^2 = 0.277)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	4.066	0.135		30.148	< 0.001
D/P creatinine	1.238	0.171	0.522	7.253	< 0.001
Angiogenin serum	0.000000658	< 0.0001	0.19	2.66	0.009
Fluid type	-0.080	0.039	-0.15	-2.059	0.041

Table 18: Linear regression for dialysate angiogenin

As expected from the data suggesting that transfer of the cytokine from the circulation to the dialysate determines levels of these cytokines in dialysate effluent, the serum levels of CCL18, CCL15 and angiogenin are significant predictors of the levels of these cytokines in dialysate.

(adj R^2 = 0.281)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	1.43	0.216		6.607	< 0.001
D/P creatinine	0.843	0.156	0.404	5.402	< 0.001
Log glucose exp.	0.189	0.077	0.184	2.465	0.015
MCP-1 serum	0.00028	< 0.0001	0.14	1.915	0.057

Table 19: Linear regression for dialysate MCP-1

(adj R^2 = 0.331)	Unstandardised Coefficients		Standardised Coefficients	t	Significance
	B	Std. Error			
Constant	-0.59	0.355		-1.662	0.099
D/P creatinine	1.452	0.267	0.41	5.434	<0.001
Log duration PD	0.124	0.061	0.149	2.046	0.043
Log glucose exp.	0.331	0.126	0.198	2.625	0.01
Log IL-6 serum	0.189	0.101	0.132	1.875	0.063

Table 20: Linear regression for dialysate IL-6

Interestingly, the serum levels of MCP-1 and IL-6 are not significant (at $p < 0.05$) predictors of the dialysate levels of MCP-1 and IL-6 respectively. This finding was expected from the analysis suggesting that intra-peritoneal production of IL-6 and MCP-1 is likely to determine dialysate levels, and that concentrations of these cytokines in the dialysate effluent – a large volume of fluid only transiently exposed to the peritoneum – are often higher than serum concentrations. The near-significance of serum MCP-1 and IL-6 as predictors of the dialysate cytokine level may be because some movement of cytokines takes place from the serum to the dialysate early in the dwell period. Alternatively, it is possible that peritoneal production of these cytokines is great enough to influence systemic serum levels.

Another interesting finding is that the D/P creatinine remains a significant predictor of IL-6 and MCP-1 in the dialysate despite the finding that transport of cytokines across the peritoneal membrane is unlikely to determine these dialysate cytokine levels. It is possible that IL-6 and MCP-1 have an effect on the peritoneum such that high levels of IL-6 and MCP-1 are associated with a high membrane transport rate. Alternatively, both the membrane transport rate and the dialysate cytokine level may be affected by a common factor such as glucose exposure.

Cytokine	*D/P creatinine*	*Serum level*	*Glucose exposure*	*Duration of PD*	*Fluid type*
CCL18	p < 0.001	p < 0.001			
Angiogenin	p < 0.001	p = 0.009			p = 0.041
CCL15	p < 0.001	p < 0.001	p = 0.025		
MCP-1	p < 0.001	(p = 0.057)	p = 0.015		
IL-6	p < 0.001	(p = 0.063)	p = 0.01	p = 0.043	

Table 21: summary of significant predictors of dialysate cytokine levels including serum levels

Table 21 shows the significant predictors of dialysate cytokine levels by multivariate analysis. The p values for serum levels of MCP-1 and IL-6 are shown because these predictors had a borderline significance although not meeting the specified cut-off of 0.05.

Summary

Multivariate analysis provides some confirmation that dialysate levels of CCL15, angiogenin and CCL18 are likely to be determined by serum levels of the cytokine and the transport properties of the peritoneal membrane; thus levels of these cytokines measured in dialysate effluent are not determined by intra-peritoneal production (even if this may be important in the pathogenesis of membrane changes). Serum levels of MCP-1 and IL-6, however, were not significant predictors of dialysate levels of these cytokines. Overall, the relatively high dialysate levels of these cytokines and the lack of a close correlation with the respective serum level suggests that intra-peritoneal production is important in determining the dialysate level of IL-6 and MCP-1.

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CHAPTER 6

RESULTS: Follow-up levels at one year

6.1 Follow-up samples at one year

Follow up samples were obtained from 50 patients. Of the remaining 101 patients, 62 had discontinued peritoneal dialysis at the one year time point and had transferred to haemodialysis, died, recovered function, transferred to another unit or received a kidney transplant. The absence of samples from the remaining patients who were still on PD at one year after the original PET test and sample were taken reflects the fact that sometimes there was no opportunity for a sample to be collected when the patient attended for their one year PET test.

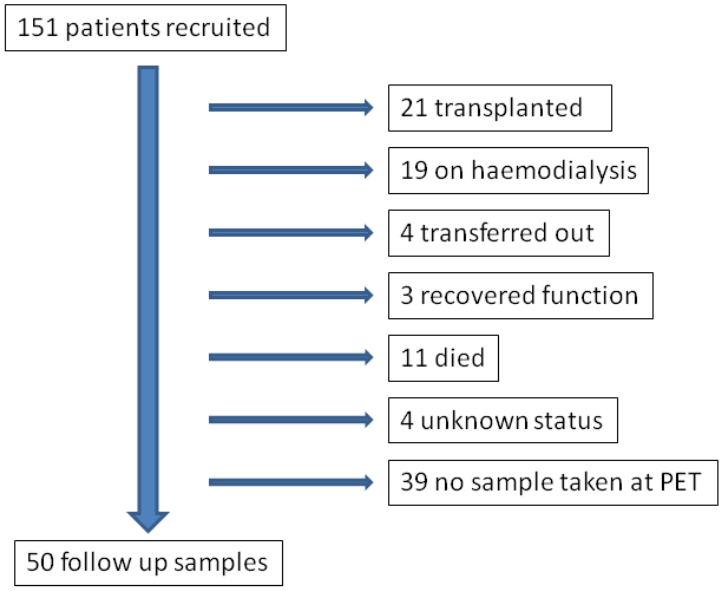


Figure 58: patient status at one year and follow-up sample collection

6.1(i) Changes in clinical data at one year

Follow up samples were collected a median of 14.2 months after the first sample collection. Examining only the patients with both baseline and follow up samples changes in clinical data were seen to be as expected given the known increase in D/P creatinine and decrease in residual renal function with time on PD:

More patients were now on APD (64.6% v.50% at baseline) and more used Physioneal rather than Dianeal (60.4% at one year used Physioneal v. 42% at baseline). The residual renal function of the patients had declined (from 4mls/minute to 3mls/minute, $p < 0.001$) and the dialysate/plasma

creatinine ratio had increased (from 0.71 to 0.75, p=0.006). The total creatinine clearance was slightly lower and the mean serum creatinine had increased (from 704 to 789µmol/ml, p < 0.001). These are all changes that might be anticipated given the well documented natural history of peritoneal membrane characteristics over time on peritoneal dialysis. Patients also required more dialysate (from a median of 8L per 24 hours, increased to 10.3L per 24 hours, p = 0.001) and therefore had a greater exposure to glucose (from a median of 769 to 821mmol/24 hours, p < 0.001).

6.1(ii) Changes in cytokine levels in follow up data

	Serum			**Dialysate**		
	Median difference (pg/ml)	***% change in medians***	***Significance (p value)***	***Median difference (ng)***	***% change in medians***	***Significance (p value)***
CCL18	+20176	+ 32%	0.002	+2398	+47%	0.002
Angiogenin	-342401	- 35%	< 0.001	+4343	+21%	ns
MCP-1	+12.8	+17%	ns	-75	-14%	ns
IL-6	+0.21	+0.5%	ns	+31.9	+151%	0.01
CCL15	-784	-2.3%	ns	+1976	+70%	< 0.001

Table 21: differences in cytokine levels at one year

In the group as a whole we have previously seen that there is little relationship between duration of peritoneal dialysis and serum levels of cytokines, while the dialysate levels of IL-6, MCP-1, CCL15 and CCL18 show a degree of correlation with duration of PD (section 5.1(i)). However, this may not reflect the findings in the fifty patients with follow up samples as they may be a selected group who are at a different point in their PD career.

For CCL18, there is an increase in the amount of cytokine in both serum and dialysate. The increase in dialysate CCL18 is proportionately more than the increase in serum CCL18. This suggests that either there has been an increase in the permeability of the membrane, so that more CCL18 enters the peritoneal space from the serum across the peritoneal membrane, or that there has been an increase in the intra-peritoneal production of CCL18. On examining the dialysate to serum cytokine ratios at one year (table 10), we see that there has been an increase in D/S ratio for most cytokines:

	Baseline D/S ratio	*One Year D/S ratio*	*Median / mean difference*	*% change in median /means*	*Significance (p value)*
CCL18	0.0185	0.0333	+0.0129	+80%	0.002
Angiogenin	0.0503	0.0767	+0.029	+52%	0.007
IL-6	5.601	9.932	+1.766	+77%	< 0.001
CCL15	0.085	0.1137	0.0519	+34%	<0.001
MCP-1	0.9252	0.7678	-0.218	-17%	0.001
Creatinine	0.708	0.75	0.042	+ 5.6%	0.006

Table 22: changes in dialysate/serum ratios of cytokines at one year. Creatinine is included for comparison

This might represent a generalised increase in membrane permeability to cytokines. In this case the increase in dialysate CCL18 levels would be due to the combination of an increase in serum levels and an increase in membrane permeability to CCL18. Of note, the increase in dialysate to serum ratio for CCL18 is much greater than the change seen for creatinine transfer. Creatinine is a considerably smaller molecule than CCL18 (molecular weight 0.113kDa versus 7.8kDa) and structural changes in the peritoneal membrane may have a different effect on the transfer of these two molecules.

Angiogenin levels have decreased in serum but remained constant in dialysate, again, a possible increase in membrane permeability to cytokines may explain why dialysate levels of angiogenin are maintained. Alternatively there may have been an increase in angiogenin production in the peritoneum.

Serum and dialysate levels of MCP-1 have not changed significantly over the course of the year.

For IL-6 and CCL15 the serum levels have remained constant but the dialysate content of these cytokines has increased. For IL-6 this is likely to reflect increased intra-peritoneal IL-6 production, since it is unlikely that the dialysate levels of IL-6 are determined by the serum level. The increase in dialysate CCL15, independently from an increase in serum CCL15, is interesting because dialysate CCL15 appears to be determined mainly by the serum level of CCL15 and the membrane permeability and CCL15 is not made by peritoneal cells (see Chapter 9). Thus if dialysate CCL15 has increased while the serum level has remained constant, it is likely that there has been a change in the permeability of the peritoneal membrane to CCL15. This makes it more likely that the increased dialysate levels of CCL18 and angiogenin relate to increased membrane permeability to all cytokines. The mechanism by which this occurs may be an increased vascular surface area secondary to neo-

angiogenesis within the peritoneal membrane. Alternatively there may be an increase in the number of small pores, which are thought to be clefts between the endothelial cells, without an increase in the number of blood vessels.

This data can be compared with the cross-sectional data relating to cytokine level and time on dialysis. It should be noted, however, that the group who have stayed on peritoneal dialysis and had a repeat PET may be a selected group who show differing cytokine patterns. In addition, the cross-sectional data for duration of dialysis includes a very wide range of values. However, as discussed below, the cross-sectional data relating to dialysis duration and cytokine levels is broadly similar to that expected from the changes seen after an additional one year on dialysis in those patients who had a follow-up sample available.

For CCL18 an increase in both serum and dialysate levels was seen in the one year group. Both serum and dialysate levels correlate with duration of dialysis in the cross-sectional survey. However the dialysate/serum ratio of CCL18 was found to have increased in the one year samples whilst there was no significant correlation of D/S CCL18 ratio with duration of dialysis in the cross-sectional data.

For angiogenin a fall in serum levels was noted in the one year follow up samples, with an increase in D/S angiogenin ratio. In the cross-sectional data no correlations were seen between duration of dialysis and angiogenin levels in serum or dialysate, or between duration of dialysis and D/S angiogenin ratio. It is possible that this is an effect of selective sampling at one year follow up.

For IL-6 the dialysate levels, but not the serum levels, increased in the one year samples. In keeping with this the D/S ratio of IL-6 also increased. In the cross-sectional data the same pattern was seen, with dialysate IL-6 levels and D/S IL-6 ratios correlating with dialysis duration. Serum IL-6 levels did not correlate with dialysis duration.

For CCL15 the dialysate levels and D/S ratio also increased in the one year samples. However, in the cross-sectional data, a positive correlation between CCL15 levels in serum and duration of peritoneal dialysis was noted as well as between CCL15 levels in the dialysate, with therefore no significant correlation between D/S ratio CCL15 and duration of dialysis.

For MCP-1, there were no significant changes in the dialysate and serum levels in the one year samples; the direction of the trend in these parameters meant that the D/S ratio fell slightly at one year. In the cross-sectional data a positive correlation between both serum and dialysate levels of MCP-1 and duration of dialysis was noted. These differences probably relate to the different patient populations. It is possible that changes in MCP-1 production rate vary with the duration of PD; this is

not the same in the complete baseline population and the population with one-year follow-up samples (median 27 v. 34 months, p = 0.008).

6.1(iii) High baseline serum angiogenin levels are associated with a greater increase in D/P creatinine

The increase in D/P creatinine was examined to investigate whether those patients in whom the D/P creatinine had increased by more than 0.05 over the course of one year (n=23) showed any differences in cytokine profile from the group in which D/P creatinine was stable (n=27).

Serum levels of angiogenin at baseline were higher in the group in which D/P rose (1004549pg/ml v. 731643pg/ml, p=0.033).

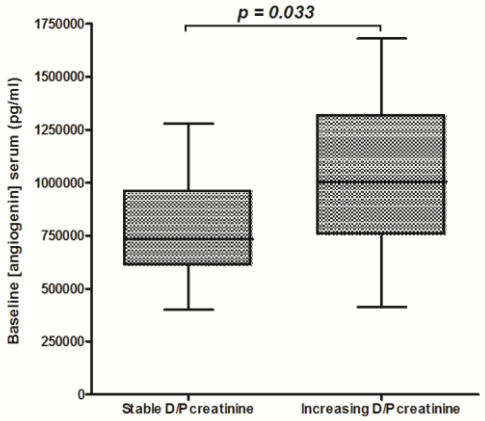


Figure 59: The baseline serum level of angiogenin is higher in patients who have an increasing D/P creatinine

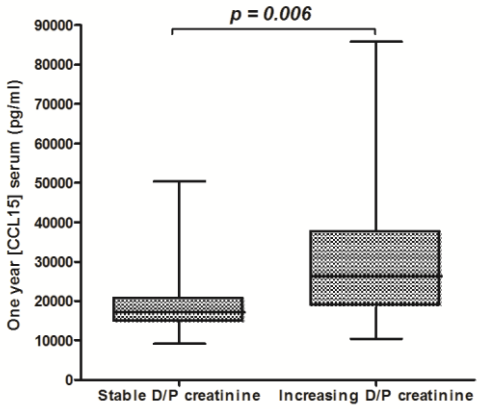


Figure 60: The one year serum level of CCL15 is higher in patients who have had an increase in D/P creatinine

Serum levels of CCL15 were higher at one year in those patients who had experienced an increase in D/P creatinine (26348 v. 17175 pg/ml, p=0.006). Angiogenin may have an effect on the D/P creatinine because neovascularisation would increase the transfer of creatinine across the peritoneal membrane by increasing the surface area over which movement of creatinine may occur. The reasons for the higher serum levels of CCL15 are not clear; it is possible that there is a systemic inflammatory response in patients who have an increasing D/P creatinine, although there was no increase in systemic IL-6 levels.

Summary

The data suggests that there is an increase in membrane permeability to the cytokines CCL15, CCL18 and angiogenin over the course of the one year follow-up period. This increase in permeability to the 7-14kDa cytokines is greater than the increase in permeability to creatinine. The greater increase in cytokine permeability than small molecule permeability may perhaps be due to the difference in the sizes of these molecules. Perhaps changes in blood vessel number or structure have a disproportionate effect on cytokine transport. High levels of angiogenin in the serum are associated with a greater increase in the D/P creatinine ratio. This is interesting because neovascularisation related to angiogenin could be responsible for the increased membrane permeability. However, the association is relatively weak ($p = 0.033$) and could be a statistical artefact due to multiple comparisons.

The levels of MCP-1 are stable in the group of patients with follow-up samples. Levels of IL-6 increased in the dialysate. Serum IL-6 levels are stable and dialysate levels of IL-6 are likely to be determined by intra-peritoneal IL-6 production. An overall increase in membrane permeability to cytokines is not likely to be responsible for the increased levels of IL-6 in the dialysate as dialysate levels of IL-6 are higher than serum levels. Additionally IL-6 is a larger molecule and transit across the peritoneum of IL-6 may be affected in a different way to the transit of smaller cytokines.

Comparison with the cross-sectional data looking at correlations between cytokine levels and duration of PD shows that a very similar picture is seen for IL-6; the patterns for other cytokines differ slightly and this may be due to differences in the patient populations and variation in dialysis duration.

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CHAPTER 7

RESULTS: Outcome data - EPS and UFF

7.1 Patient outcomes

Patient outcomes at one year from sample collection

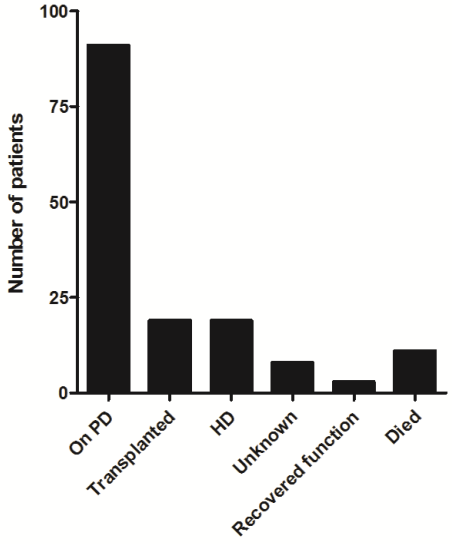


Figure 61: Patient outcomes at one year from sample collection. A small number of patients had transferred out and their outcome is unknown. A substantial proportion – nearly half – of all patients had stopped PD one year after sample collection.

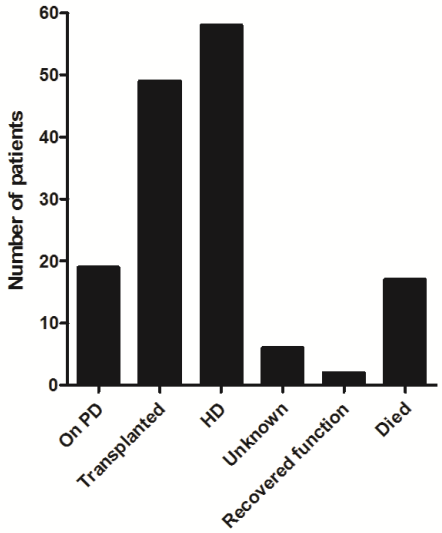


Figure 62: Patient outcomes at November 2011. The first samples were collected in 2003 and the last in 2010.

7.2 Outcomes: EPS

7.2(i) Clinical predictors for future EPS

EPS developed in 17 patients in the study cohort. The time from peritoneal dialysis cessation (either transplantation or transfer to haemodialysis) until the development of EPS was short in this cohort, with median value of 3.12 months (range: 0.6 – 7.7 months); one patient was diagnosed with EPS

while still on peritoneal dialysis and the other 16 developed EPS after stopping PD. The median time from baseline sampling to the development of EPS was 13.6 months (range: 4.8 – 46 months); all but one of the EPS patients developed EPS within twenty six months of sample collection. The median time since diagnosis of the study group patients is now 68 months (range: 35-97 months). Ten of the patients with EPS have now died (eight of these patients died of EPS or complications of EPS treatment such as TPN-related line sepsis). Of the remaining patients, most are usually well and require no nutritional supplements, but still have occasional admissions to hospital with abdominal pain, abdominal distension or vomiting.

	Age	**Renal diagnosis**	**Duration PD (months)**	**Peritonitis**	**D/P Cre**	**GFR (mls/ min)**	**RRT at EPS**	**Outcome**	**Months to RIP or follow-up since EPS**
1	70	Hypertension	110	1	0.82	0.35	HD	Died - not EPS	16
2	48	Membranous	87	2	0.91	0	Tx	Alive symptomatic	86
3	49	GN	146	1	0.93	0	Tx	Alive symptomatic	69
4	48	APKD	74	1	0.87	0	HD	Died of EPS	14
5	61	DM	83	1	0.71	0	Tx	Died of EPS	10
6	51	IgA	84	0	0.64	3.51	HD	Alive asymptomatic	61
7	34	ATN	109	1	0.82	0	HD	Died of EPS	84
8	52	Unknown	51	2	0.83	1.26	Tx	Alive symptomatic	79
9	67	GN	137	0	0.89	0	HD	Died of EPS	2
10	65	DM	63	1	0.90	0.71	Tx	Died of EPS	22
11	55	DM	70	0	0.77	9.27	Tx	Died of EPS	5
12	76	Unknown	56	2	0.86	0.75	HD	Died - not EPS	3
13	52	IgA	104	1	0.87	0	HD	Alive symptomatic	72
14	62	Unknown	67	1	0.80	0.62	HD	Alive symptomatic	77
15	70	DM	51	1	0.66	2.12	Tx	Alive asymptomatic	64
16	49	Allport's	76	0	0.95	0	Tx	Died of EPS	43
17	53	APKD	55	2	0.7	3.13	Tx	Died of EPS	1

Table 23: characteristics of the EPS patients: GFR and D/P creatinine refer to final PET test result on PD

Compared to the remainder of the cohort, patients who developed EPS had been on peritoneal dialysis for a longer period of time (total duration of PD 76 versus 40 months). Glucose exposure at time of sampling was higher and both urine output and residual GFR lower.

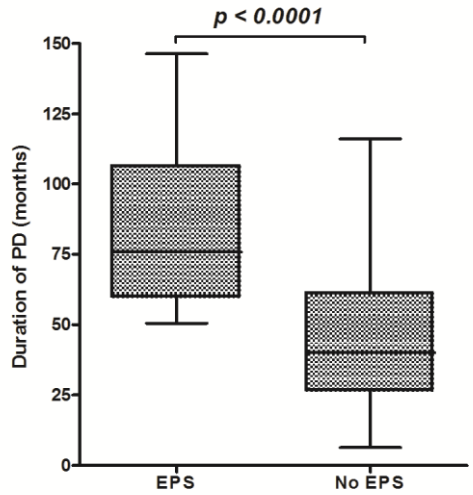


Figure 63: Total duration of peritoneal dialysis was higher in patients who developed EPS

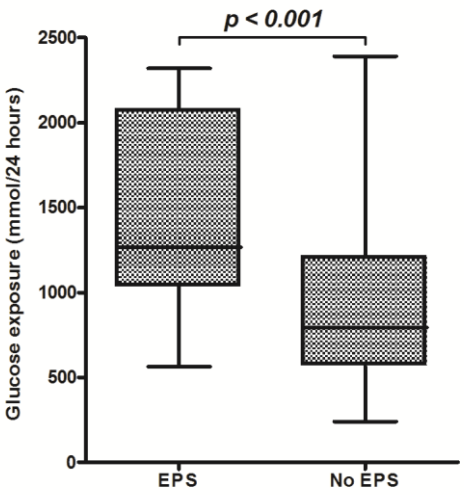


Figure 64: Glucose exposure was higher in patients who developed EPS

Glucose exposure at time of sampling was 1266 v.796mmols/24 hours (higher in the EPS group, $p < 0.001$). Urine output was lower (median 40mls/24 hours in the EPS group v. 764 mls/24 hours in the non-EPS group). Similarly residual GFR was 0.35 mls/minute in the EPS group and 3.77 mls/minute in the non-EPS group.

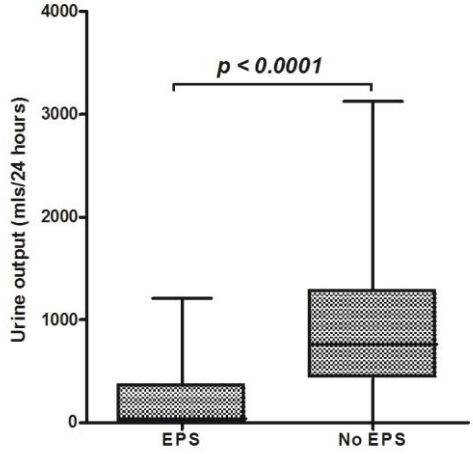


Figure 65: The urine output was lower in patients who later developed EPS

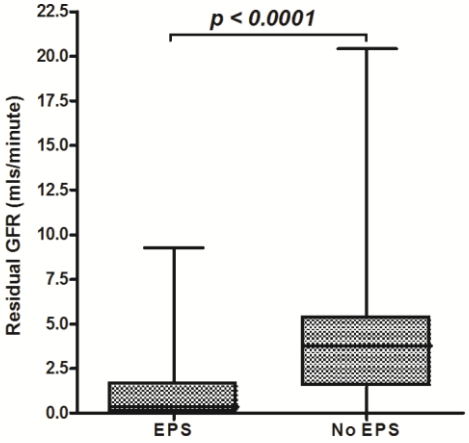


Figure 66: The residual GFR was lower in patients who later developed EPS

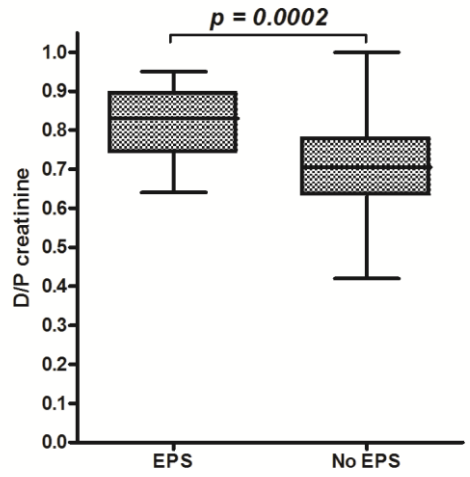


Figure 67: The D/P creatinine ratio was higher in patients who subsequently developed EPS

All of these findings are similar to those described in other published series and are as expected in a group of patients who have spent a long period of time on PD. A history of peritonitis was more common in the EPS group – 76.5% of EPS patients (CI 56-97%) v 38% of non-EPS patients (CI 30-46%), p = 0.004, had had at least one episode of peritonitis. Again this may simply reflect more months of exposure to the risk of peritonitis.

A history of ultrafiltration failure was also more common in the EPS group – 47% of EPS patients (27-71%) v. 8% of non-EPS patients (CI 4-13%), $p < 0.001$; the incidence of ultrafiltration failure increases with the duration of PD.

7.2(ii) Chemokine levels are higher in patients who later develop EPS

Dialysate levels of IL-6, CCL15 and MCP-1 were higher in patients who later developed EPS.

	EPS	No EPS
MCP-1	658ng (range 390-2718ng)	395ng (range 100-2009ng)
IL-6	92ng (range 28-471ng)	40ng (range 4-459ng)
CCL15	8017ng (range 2060-17414ng)	4014ng (range 726-19394ng)

Table 24: Median dialysate levels of cytokines in EPS and non-EPS patients

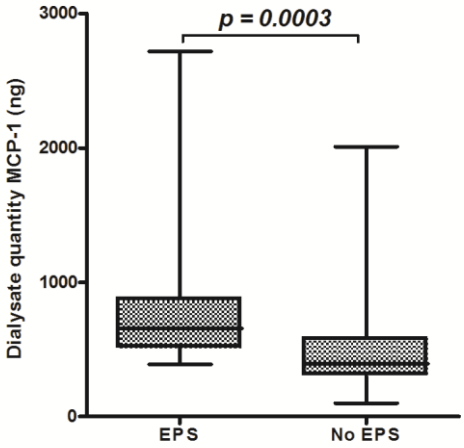


Figure 68: The dialysate appearance of MCP-1 was higher in patients who subsequently developed EPS

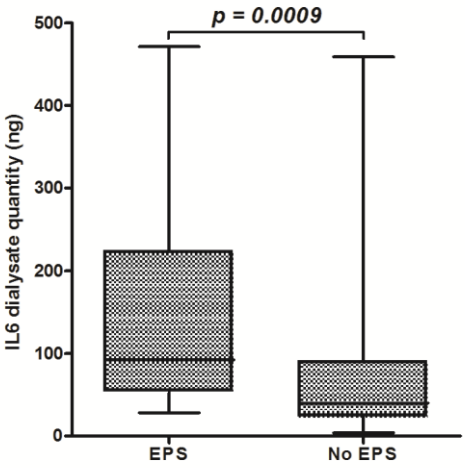


Figure 69: The dialysate appearance of IL-6 was higher in patients who subsequently developed EPS

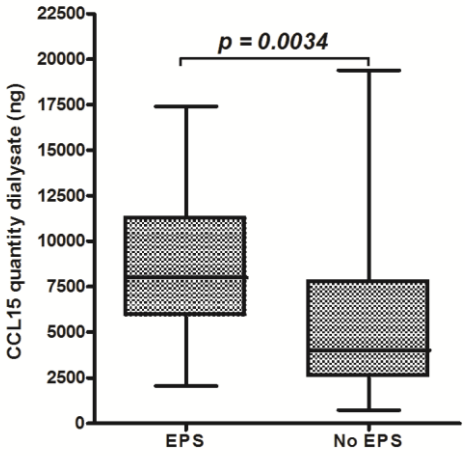


Figure 70: The dialysate appearance of CCL15 was higher in patients who subsequently developed EPS

One patient was first sampled nearly four years before the onset of EPS. This patient had had a follow-up sample and a later sample taken four months before EPS was diagnosed; the changes in his data during the course of the four years before the cessation of PD were generally not large (table 25). An exception was a lower level of MCP-1 in the dialysate at the later date; using this value made minimal difference to the median MCP-1 level for the EPS group and dialysate MCP-1 remained a significant predictor of future EPS.

The other difference in later samples from this patient was that serum CCL18 was higher at later time points. Using this later value and comparing between the EPS and non-EPS groups, serum CCL18 becomes significantly different between the two groups (median 151590 v. 119959 pg/ml); however the p value is borderline at 0.039 and this finding may therefore be due to chance. For consistency the sample taken at recruitment is used in the analysis, as with all other patients. The remaining 16 EPS patients were sampled much closer in time to EPS onset.

	46 months pre-EPS D/P cre = 0.77		32 months pre-EPS D/P cre = 0.88		4 months pre-EPS D/P cre = 0.83	
	Serum	**Dialysate**	**Serum**	**Dialysate**	**Serum**	**Dialysate**
CCL18	98034	21979	209266	26642	492016	23421
MCP-1	486	1022	411	640	362	574
IL-6	4.8	263	-	686	6.9	272
Angiogenin	838515	163895	798314	131652	891801	167530
CCL15	42840	11134	37665	7523	26359	15527

Table 25: serial changes in cytokine levels in a patient who developed EPS

When only the group of patients who had been on PD for more than 46 months at time of sampling is considered (33 non-EPS and 14 EPS patients), the duration of PD at sampling is no longer significantly different between the two groups (67 months in the EPS patients v. 64 months in the non-EPS group); the total duration of PD is also not significantly different (85 v. 73 months).

Once duration of PD is eliminated as a confounding factor, patients who would later develop EPS continue to have significantly lower urine outputs (median 0 v. 491mls/24 hours, p = 0.011) and less residual function (median GFR of 0 v. 1.82mls/minute). The glucose exposure remains higher in this group (1238mmol v. 900mmol/24hours, p = 0.017) and the D/P creatinine remains higher (0.83 v. 0.71, p = 0.026) – see table 26.

This suggests that while the duration of PD is undoubtedly a significant risk factor for the development of EPS, glucose exposure, residual function and membrane transport properties are all risk markers for EPS development independently of their association with duration of PD. Cytokine levels are no longer significant predictors of EPS in this group of long term PD patients only. However this data does not prove which (if any) of these factors are part of the pathogenesis of EPS, especially as these variables correlate closely with each other.

	EPS	Non-EPS	p value
Duration PD at sampling (months)	67 (46-135)	64 (47-82)	0.383
Final duration PD (months)	83 (51-146)	73 (6-116)	0.199
Glucose exp. (mmol/24 hours)	1238 (563-2319)	900 (300-2391)	0.02
Residual GFR (mls/min)	0 (0-3.51)	1.82 (0-12.58)	0.008
Urine output (mls/24 hours)	0 (0-1085)	491 (0-2985)	0.009
D/P creatinine	0.83 (0.64-0.95)	0.71 (0.42-1)	0.019
Dialysate CCL18 (ng)	8112 (1472-28265)	6803 (578-35856)	0.92
Dialysate MCP-1 (ng)	655 (390-2718)	413 (100-2009)	0.062
Dialysate IL-6 (ng)	86 (28-471)	80.5 (4-308)	0.403
Dialysate angiogenin (ng)	140290 (21795-309993)	108495 (27800-302629)	0.616
Dialysate CCL15 (ng)	7927 (2060-17414)	6851 (726-17456)	0.227

Table 26: Long term PD patients (> 46 months at time of sampling). Median and range are shown

7.3 Historical EPS group

7.3(i) Description of cohort

Before the prospective collection of data and samples was initiated at our centre, a number of patients had developed EPS. The first case was diagnosed in 1999. The total number of cases which were documented was 29, in addition to the study patients for whom a full set of data and clinical samples were collected. A complete set of data is not available on all of these historical patients, but some basic information could be collected. It is not possible to find matched control patients (separate from the study group) for these EPS patients; many ceased peritoneal dialysis some years ago and the PET/adequacy data for this cohort of peritoneal dialysis patients is no longer in existence.

7.3(ii) Comparison with study group

The median duration of PD in this historical group of EPS patients was 77.1 months and their age at diagnosis of PD was a median of 54.4 years. This compares to 75.86 months on PD and a median age of 53.2 years in the study group patients. The median number of peritonitis episodes was 1 in both groups and the rate of peritonitis per month was constant at 0.012 episodes / month in both groups. The median D/P creatinine was 0.81 in the historical cohort and 0.83 in the study group (at the last PET test). The median GFR was 0.35 in the study group and 0 in the historical group and this difference was not statistically significant. 15 of the patients were still alive at the end of the study;

none are still TPN dependent but some of the patients still have occasional short admissions with abdominal pain and distension. The time since diagnosis in this group of patients is a median of 80 months (range 32-146 months).

This data is interesting because it suggests that the study group patients are similar in terms of membrane function and duration of dialysis to other EPS patients managed at our institution in the past, as well as showing similar clinical risk factors for EPS to those described in the literature at other institutions. It is therefore more likely that findings in the study group population will apply to other peritoneal dialysis and EPS patients.

7.4 Outcomes: Ultrafiltration failure

7.4(i) Clinical predictors of future UFF

The definition of UFF used for this study is mainly clinical, since adequate data on ultrafiltration capacity were not available for all patients. Ultrafiltration failure was defined as either:

a) less than 750mls/24 hours of total fluid removed (urine and dialysis ultrafiltration combined) or
b) patient stopping PD because of refractory fluid overload

Clearly this definition will include some patients who could potentially have increased their ultrafiltration volumes by a change in dialysis prescription, or who might have remained on peritoneal dialysis if they had limited their salt and water intake further.

Unsurprisingly, patients who went on to develop ultrafiltration failure had a lower urine output and residual GFR and used a greater volume of dialysate with a higher glucose exposure. In line with the literature, patients who had ultrafiltration failure had been on peritoneal dialysis for longer (32 v. 23 months, $p = 0.037$), and had a higher D/P creatinine (0.78 v. 0.71, $p = 0.022$).

	UFF	No UFF	p value (Mann Whitney test)
Duration of PD (months)	32	23	0.037
Glucose exposure (mmol/24 hours)	1028	750	0.021
Dialysate volume (L/day)	12900	8214	0.007
Urine output (mls/24 hours)	199	917	<0.001
GFR (ml/minute)	0.8	4.04	<0.001
CCL15 dialysate (ng)	7327	4014	0.041
D/P creatinine	0.78	0.71	0.022

Table 27: Factors differing between patients who did and did not develop ultrafiltration failure

7.4(ii) CCL15 levels are higher in patients who later develop UFF

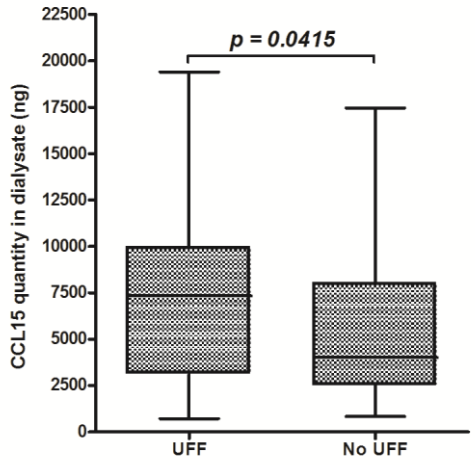


Figure 71: The dialysate appearance of CCL15 was slightly higher in patients who subsequently developed UFF

Although the level of CCL15 was higher in patients who went on to develop ultrafiltration failure, the difference is fairly small and barely statistically significant. It is probable that it relates to the higher D/P creatinine in this group.

None of the other cytokines were found at higher levels in the group of patients who developed ultrafiltration failure, suggesting that it is the alteration in membrane transport properties which affects the CCL15 level in the dialysate.

7.5 Can chemokine levels help predict future EPS or UFF?

With regard to ultrafiltration failure, there is only a very small difference in CCL15 levels between the groups who did and not develop ultrafiltration failure. It is likely that this increase in CCL15 level in the dialysate is explained by the higher D/P creatinine in this group and cytokines will not add anything to available clinical data in terms of the prediction of ultrafiltration failure.

With regard to EPS, there are three cytokines which were found at a higher level in patients who went on to develop EPS. These were MCP-1, IL-6 and CCL15 in the dialysate. When building a predictive model to investigate whether these cytokines can predict EPS with any more accuracy than the clinical risk factors can, it is necessary to bear in mind the relatively small number of EPS patients in our cohort. Peduzzi et al (Peduzzi *et al.*, 1996) demonstrated that a logistic regression analysis requires a minimum of about 10 events for each variable investigated; another formulation by Hosmer and Lemeshow gives the maximum number of parameters which can be estimated as (m/10)-1, where m is the smallest group (in this case, the number of EPS patients) (Hosmer and Lemeshow, 2000). Although a more flexible approach is argued by Vittinghoff, permitting perhaps 5-9 events per variable (Vittinghoff and McCulloch, 2007), as there are only 17 EPS cases it is still not possible to investigate a large number of potential predictive variables. Dr North (Imperial College statistics advisory service) constructed two logistic regression models, one which uses final duration of PD and D/P creatinine as clinical predictors of EPS, and the other using MCP-1, IL-6 and CCL15. The clinical predictors were chosen as duration of PD is the most commonly-cited risk factor for EPS in the literature and has been demonstrated in multiple dialysis units worldwide. The D/P creatinine was chosen as an objective, measurable factor which is supported by other studies in the literature in which high D/P creatinine was a risk factor for EPS (Habib *et al.*, 2010). It is noted that in some patients with severe membrane damage the D/P creatinine can fall slightly just before the onset of EPS (after an early rapid rise) (Sampimon *et al.*, 2010a). This fall in D/P creatinine was associated with other late onset features such as loss of ultrafiltration capacity and may be a marker of early EPS or at least very significant peritoneal fibrosis. In a study by Lambie et al the D/P creatinine was seen to rise only at the last PET test before the discontinuation of PD (Lambie *et al.*, 2010). These differences in the timing of increase in D/P creatinine may reflect differences in local practice; the Dutch EPS cohort, for example, contains a group of patients who continued on PD for some time after a diagnosis of ultrafiltration failure had been made.

Other possible discriminating features such as high glucose exposure are dependent on the clinician's prescription and therefore may be specific to a particular dialysis centre. Residual renal function is difficult to quantify in peritoneal dialysis patients, where it will typically be about 0-10mls/minute. Creatinine clearance is notoriously unreliable as a measure of renal function at this

level of GFR. The usual practice is to take the average value of the creatinine and urea clearances, as the measurement errors are in opposite directions for these two substances and the average value becomes a reasonable approximation of function. Ultrafiltration capacity might also be a discriminating factor, but to some extent the D/P creatinine result covers this measure, since patients with a high D/P creatinine have poor ultrafiltration.

Using logistic regression analysis, a model using the readily available clinical data – duration of PD and D/P creatinine - was constructed first. The duration of PD data required log transformation to obtain a normal distribution.

	B	S.E	Wald	df	Significance	Exp(B)
Log Final duration PD	7.682	2.218	11.992	1	0.001	2169.167
D/P creatinine	8.23	3.016	7.448	1	0.006	3750.026
Constant	-22.165	4.894	20.509	1	<0.001	0

Table 28: Logistic regression analysis of EPS predictors

The Hosmer and Lemeshow test for this regression has a p value of 0.973, and chi squared value of 2.228, indicating a good model fit.

In order to assess the usefulness of this analysis in the prediction of EPS, a ROC curve was constructed for the regression:

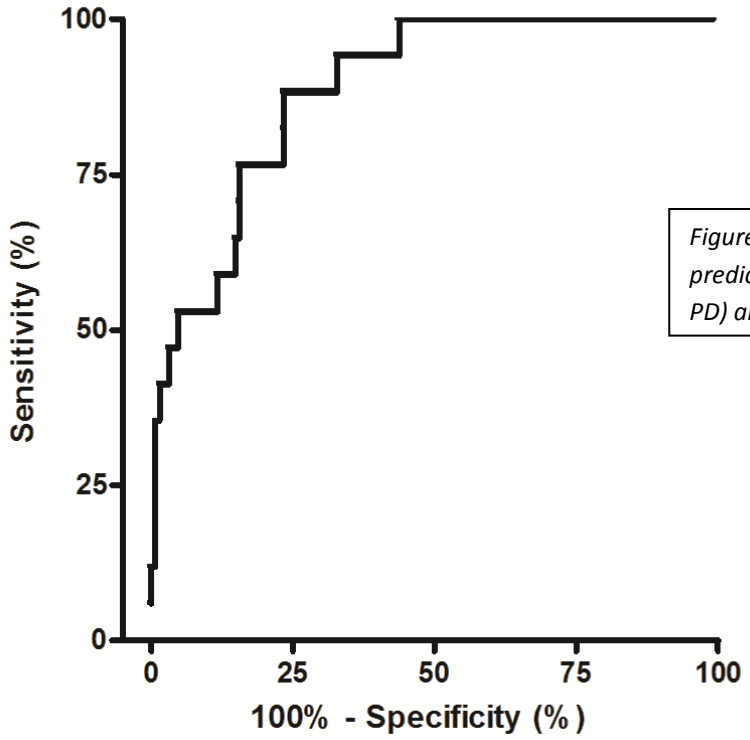


Figure 72: ROC curve of logistic regression; predictors of EPS are log(final duration of PD) and D/P creatinine

The determination of the "best" cut point for such a curve is a matter of debate, and depends on the clinical importance of sensitivity versus specificity. In this case, it could be argued that the risk of mortality associated with EPS is significant, whereas the risks of changing dialysis modality are less. On this basis sensitivity should be optimised. A perhaps more objective method of determining the optimal cut point is to choose the coordinates on the ROC curve that have the minimum distance from the perfect situation where sensitivity and specificity are both 100%; thus the cut point is chosen as that point where $(100\%\text{-sensitivity})^2+(100\%\text{-specificity})^2$ is at a minimum (this is the square of the length of the line from the observed regression ROC curve to where "perfect" curve would lie by Pythagoras). For the above ROC this occurs where sensitivity is 88.2% (95% CI 64 - 99%) and specificity is 76.6% (95% CI 68 – 84%) at a cut point of > 0.1239. The classification table using this cut point would be as follows:

Observed		**Predicted**		
		No EPS	**EPS**	**% correct**
EPS ever	**No EPS**	98	30	76.6%
	EPS	2	15	88.2%
Overall percentage				77.9%

Table 29: Classification table with cut point of 0.1239

The choice of cut point is therefore dependent on the weight placed upon not "missing" a possible future case of EPS. It is clear that this is a far from perfect tool for predicting the likelihood of future EPS, being insufficiently specific at this cut-point.

Of note, although the predictors used are universally assessed in peritoneal dialysis patients, the utility of the predictive equation would be expected to vary with the incidence of EPS in other peritoneal dialysis populations.

The next question asked was whether cytokine levels could be used to produce a more useful predictive model.

Using the three cytokines that were found by univariate analysis to be significantly different between EPS and non-EPS patients - IL-6, CCL15 and MCP-1 - (all log transformed) the following model is obtained:

	B	S.E	Wald	df	Significance	Exp(B)
Log MCP-1	3.123	1.778	3.085	1	0.079	22.703
Log CCL15	1.993	1.318	2.288	1	0.13	7.340
Log IL-6	1.183	1.064	1.237	1	0.266	3.265
Constant	-20.311	5.883	11.918	1	0.001	0.000

Table 30: Logistic regression analysis using cytokines to predict EPS

It can be seen that none of the p values in this model are significant for the individual variables, although the overall model is significant at $p < 0.001$. There is some debate regarding the necessity for each term to be significant in a logistic regression model, since the model is dealing with risk estimation rather than hypothesis testing (Steyerberg, 2009; Pencina *et al.*, 2008). However the situations in which a predictor with a non-significant p value might be retained in a logistic regression model are those where there is a rare but strong predictor and a small data set; this is not the case in this study. Although using more predictors may slightly increase the area under the ROC curve, this does not necessarily mean that the predictive ability of the model is improved. Assessing risk prediction models is complex; to demonstrate a benefit with the inclusion of the extra predictors further testing such as reclassification (Cook, 2008; Cook, 2007) would be required, although this in itself remains a matter of debate (Pepe and Janes, 2011). For these reasons, the model using three cytokines was not examined further.

Using a conditional forward selection model, the only cytokine of the original three predictors (MCP-1, IL-6 and CCL15) which remains in the model is MCP-1:

	B	S.E	Wald	df	Significance	Exp(B)
Log MCP-1	5.112	1.493	11.722	1	0.001	166.039
Constant	-16.041	4.194	14.632	1	<0.001	0.000

Table 31: Logistic regression analysis using MCP-1 levels to predict EPS

A ROC curve is shown to illustrate the regression:

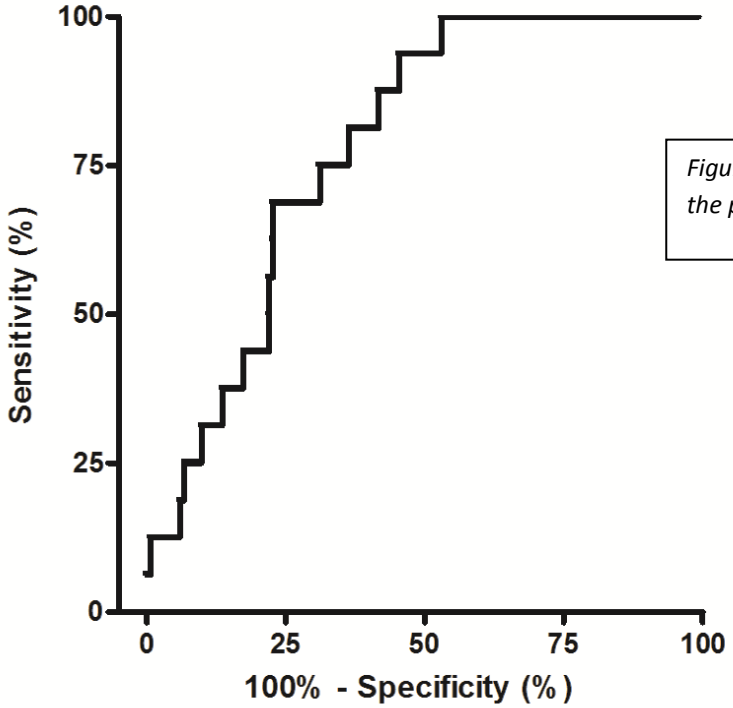


Figure 73: ROC curve of logistic regression; the predictor of EPS is log(MCP-1)

Inspection of this curve suggests that this is no improvement on the model constructed using clinical predictors only, and lies closer to the line of identity. In particular, if the sensitivity is required to be reasonably high, the specificity is low. The cut point of > 0.1473 is the closest point on the observed ROC curve to the perfect curve (calculated as the point with the least sum of squares). At this point sensitivity is 69% (95% CI 41 - 89%) and specificity is 77% (95% CI 69 - 84%)- by no means adequate for use in clinical practice. If sensitivity is improved, specificity is poor: a cut point of > 0.1062 gives a sensitivity of 75% (95% CI 48 - 93%) and specificity of only 69% (95% CI 60 – 77%).

Combining the duration of peritoneal dialysis with PET and MCP-1 is not an appropriate analysis to perform because PET result and MCP-1 are closely correlated. Also this would be equivalent to trying to find a predictive subset out of five candidate markers and as such would break the 10-events-per-variable rule.

Summary

Using dialysate cytokine levels as predictors of EPS does not have any benefit beyond that of a model using the basic clinical parameters, duration of PD and D/P creatinine. Although the model using these clinical parameters appeared to have some potential to predict EPS, it would be necessary to investigate the performance of the model across different dialysis populations to

establish whether it has any utility. Performance of the model in other populations may be expected to be less good than in the sample population; there is likely to be optimism due to over-fitting in a model that was not pre-defined before the study commenced. Data from our patient population suggests that, in a patient who is approaching four years on peritoneal dialysis, clinical features associated with EPS after the cessation of PD are lack of residual renal function, high glucose requirements and high D/P creatinine. Raised levels of cytokines in the dialysate will at the most confirm a clinical impression that the patient is at higher risk for EPS post transfer to haemodialysis or transplantation; they are not sufficient to use alone as a predictive tool.

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CHAPTER 8

RESULTS: Serum cytokine levels in EPS and non-EPS patients

8.1 Serum cytokine levels in PD, HD and transplanted patients

8.1(i) Serum cytokine levels vary between HD, PD and transplanted patients and this is not due solely to GFR

Samples were collected from patients who were currently on PD, patients on HD with no history of PD (n=9) and transplanted patients with no history of PD (n=11). Levels of cytokines were found to vary significantly between modalities, being in general highest in the HD group and lowest in the transplanted group.

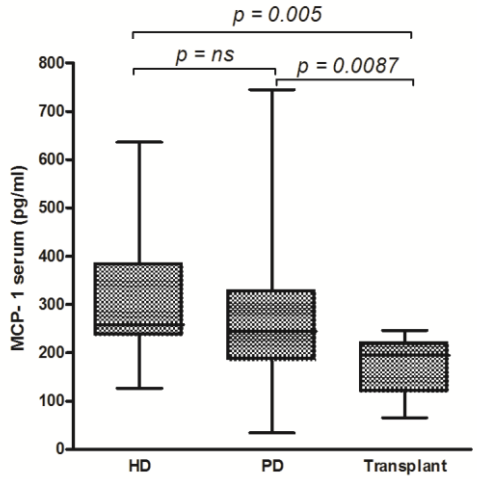


Figure 74: Levels of MCP-1 in patients with differing forms of renal replacement modality

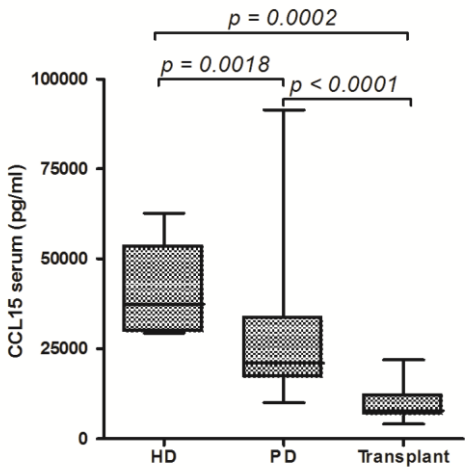


Figure 75: Levels of CCL15 in patients with differing forms of renal replacement modality

The levels of the cytokines IL-6 and MCP-1 in serum do not correlate with GFR within the GFR range of PD and HD patients (usually 0-10mls/minute) and the levels of CCL18 and CCL15 correlated only weakly with GFR in this range. Residual GFR is not regularly measured in our haemodialysis population but this was a group of very long duration ESRD patients and little or no residual function would be expected. GFR differences cannot completely explain the observed patterns in the peritoneal and haemodialysis groups since some cytokines (angiogenin and MCP-1) show no difference between peritoneal dialysis and haemodialysis patients and in contrast CCL18 and CCL15 are found at higher levels in haemodialysis patients.

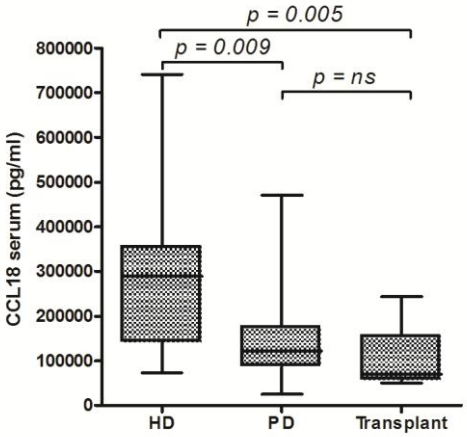


Figure 76: Levels of CCL18 in patients with differing forms of renal replacement modality

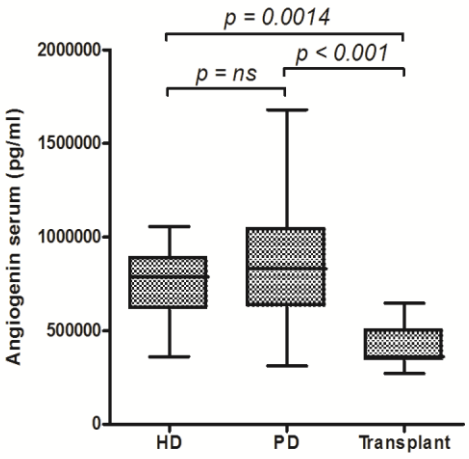


Figure 77: Levels of angiogenin in patients with differing forms of renal replacement modality

In the transplant population the range of GFR is much higher and this might result in lower serum levels of cytokines in this group. A negative correlation between GFR and cytokine levels was noted for MCP-1, CCL18, angiogenin and CCL15 across the range of GFR values found from PD to transplant patients. This is only an approximation of any true relationship with renal function since the calculated GFR used for the transplant population and that used for the PD population (the mean of creatinine clearance and urea clearance) are both estimations. HD patient data was not included as residual GFR is not routinely measured in the HD population.

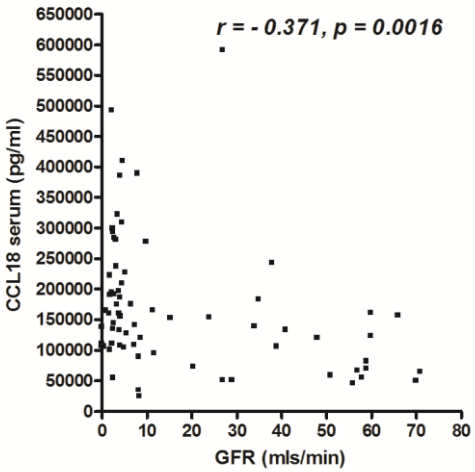


Figure 78: CCL18 serum levels correlate with GFR across the range seen in PD and transplant patients

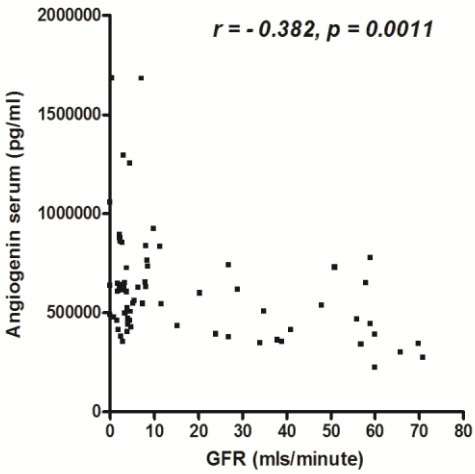


Figure 79: Angiogenin serum levels correlate with GFR across the range seen in PD and transplant patients

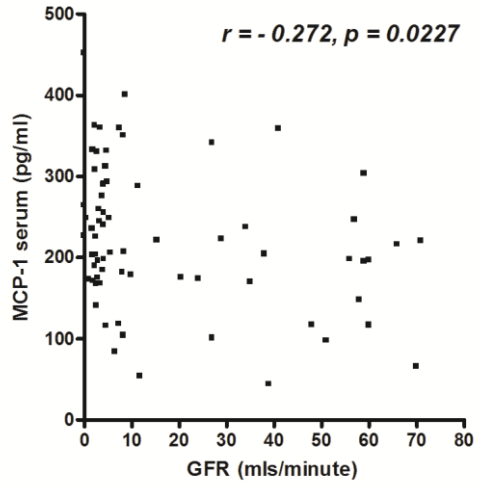


Figure 80: MCP-1 serum levels correlate more weakly with GFR across the range seen in PD and transplant patients

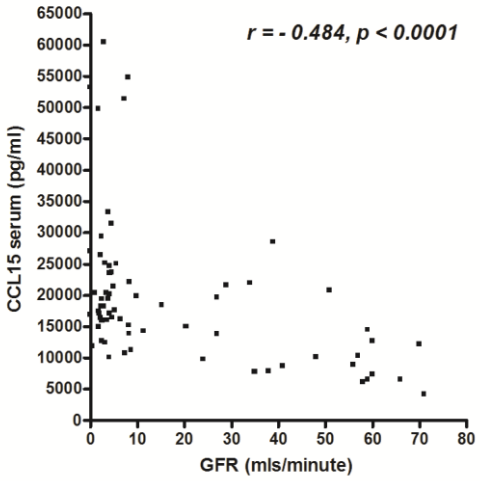


Figure 81: CCL15 serum levels correlate with GFR across the range seen in PD and transplant patients

There are many potential confounders, such as the immunosuppressants taken by transplanted patients, which may underlie this correlation. Interestingly, Qureshi (work from Dr Tam's group) looked at the relationship between serum CCL18 and GFR in over a hundred CKD patients (GFR range 11 to 90) and found no correlation between serum CCL18 and GFR. This suggests that the differences in CCL18 levels between different modality groups are likely to be at least partly secondary to factors such as increased inflammation in the dialysis population and immunosuppression in the transplanted population rather than variation in residual or transplant function.

8.2 Serum cytokine levels in EPS patients after cessation of PD

8.2(i) Serum cytokine levels in transplanted patients do not differ between EPS and non-EPS patients

Samples were collected from patients with a diagnosis of EPS post-transplantation. These patients were then matched by age, sex and duration of ESRD to a control group of patients who had never used PD.

No significant differences were found between the levels of CCL18, MCP-1, CCL15 and angiogenin in the EPS and non-EPS transplant patients.

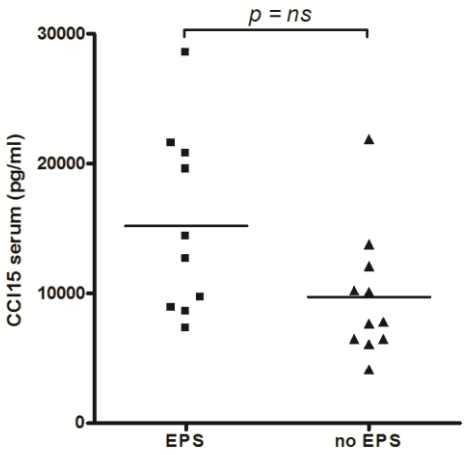


Figure 82: There is no difference between CCL15 levels in transplanted patients who have and have not been diagnosed with EPS

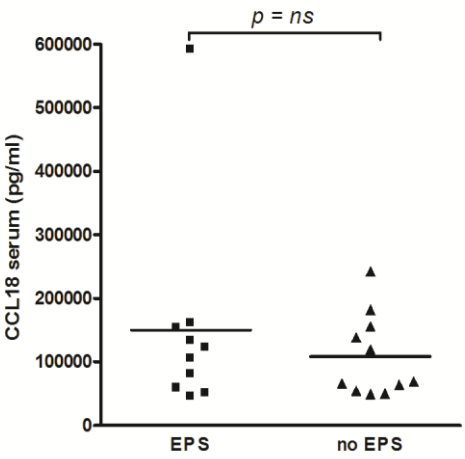


Figure 83: There is no difference between CCL18 levels in transplanted patients who have and have not been diagnosed with EPS

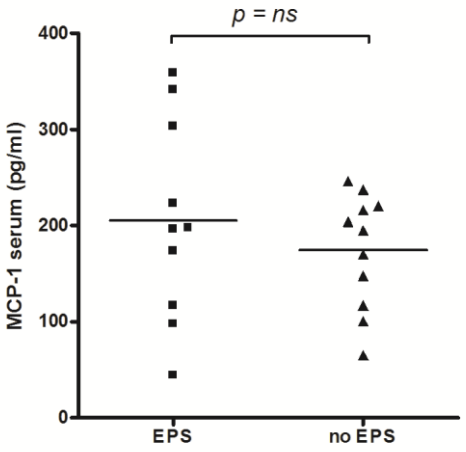


Figure 84: There is no difference between MCP-1 levels in transplanted patients who have and have not been diagnosed with EPS

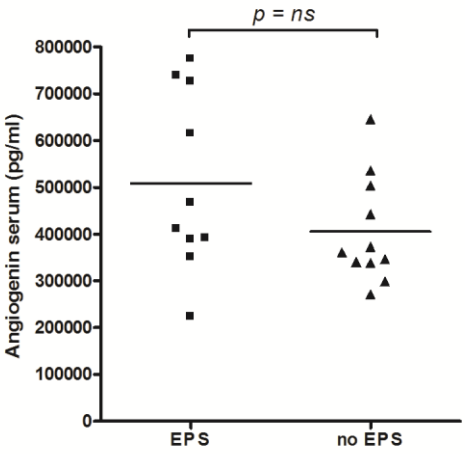


Figure 85: There is no difference between angiogenin levels in transplanted patients who have and have not been diagnosed with EPS

It is likely that there are many complicating factors affecting cytokine levels in transplant patients such as immunosuppressant use.

8.2(ii) Serum CCL18 levels are higher in haemodialysis patients with EPS than in non-EPS HD patients

Samples were also collected from EPS patients now on haemodialysis, and haemodialysis patients matched for age, sex, dialysis unit and duration of ESRF who had never been on PD. No difference was found between EPS and non-EPS patients for MCP-1, CCL15 and angiogenin, but levels of CCL18

were significantly different between the EPS and non-EPS groups. There was considerable overlap between the groups:

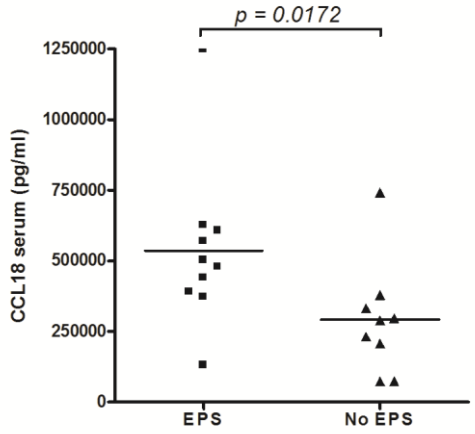


Figure 86: There is a significant difference between CCL18 levels in HD patients who have and have not been diagnosed with EPS

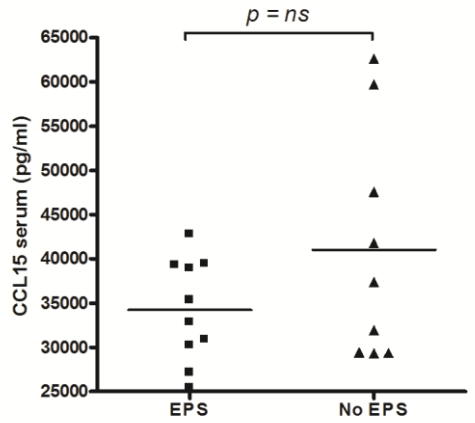


Figure 87: There is no significant difference between CCL15 levels in HD patients who have and have not been diagnosed with EPS

It should be note that this is only a very small group of patients – there are ten samples from EPS patients who remain alive and are maintained on haemodialysis. However it is interesting that it is CCL18 which appears to become high after some time from diagnosis of EPS. These samples were collected at an average of 32 months after the diagnosis of EPS was made in these patients. These patients were not acutely unwell with inflammatory features of EPS such as ascites, fever and weight loss. Instead they were experiencing recurrent episodes of bowel obstruction, with symptoms relating to fibrosis around the bowel. As discussed in the introduction, CCL18 is known to be involved in the pathogenesis of fibrosis.

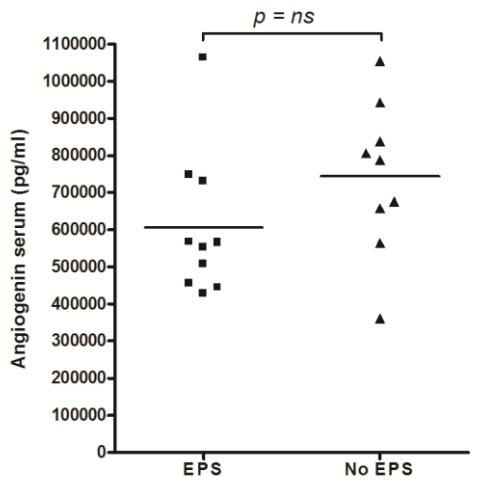


Figure 88: There is no significant difference between angiogenin levels in HD patients who have and have not been diagnosed with EPS

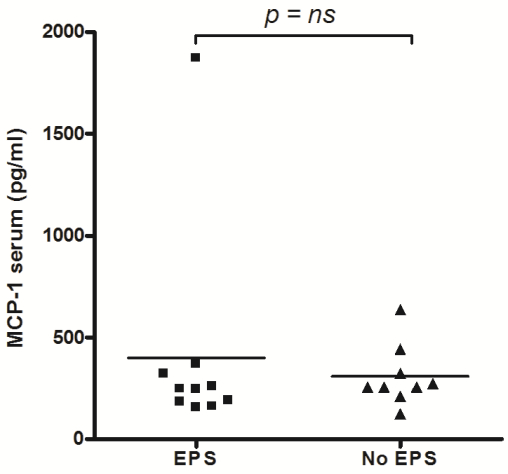


Figure 89: There is no significant difference between MCP-1 levels in HD patients who have and have not been diagnosed with EPS

Summary

In summary, there is a significantly higher level of CCL18 in haemodialysis patients with established EPS. This is not seen in transplanted patients with EPS, perhaps because of the effects of immunosuppression on the production of cytokines. It is less likely that the renal function of transplant patients obscures any differences in CCL18. CCL18 might be involved in ongoing fibrosis in these HD patients with symptoms of repeated obstruction due to EPS. All cytokines are found at higher levels in dialysis patients than in transplanted patients; the mechanism for this is not clear but may reflect both the use of immunosuppression in the transplanted population and perhaps the almost complete absence of renal clearance in the dialysis population.

CHAPTER 9: RESULTS – Production of cytokines by mesothelial cells in culture

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CHAPTER 9

RESULTS: Cytokine production by mesothelial cells in culture

9.1 Peritoneal mesothelial cells in culture

9.1(i) Peritoneal mesothelial cells in culture show varying morphology

In order to establish whether mesothelial cells can produce cytokines, dialysate effluent samples from overnight bags were used as a source of mesothelial cells for culture. Mesothelial cells were cultured as described in section 3.4(i). Photographs of mesothelial cells in culture show varying morphology:

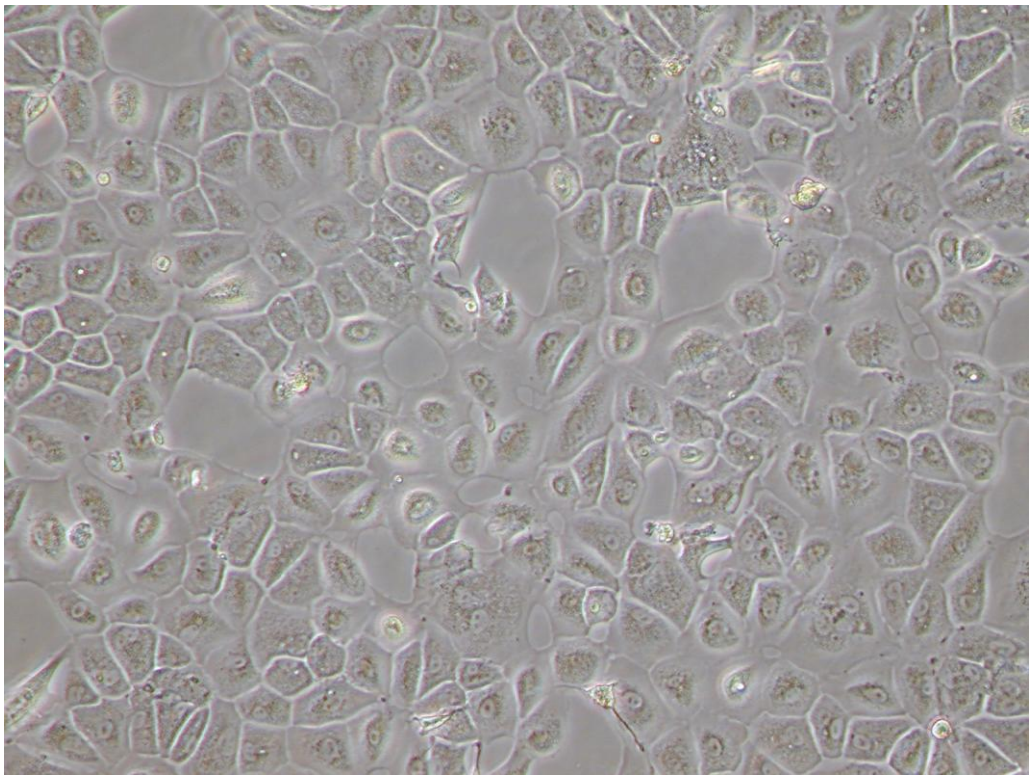


Figure 90: Human peritoneal mesothelial cells in culture with a cuboidal morphology. These cells were taken from a patient who had only very recently started peritoneal dialysis. The picture was taken after 37 days in culture before the first passage; these are slow growing cells

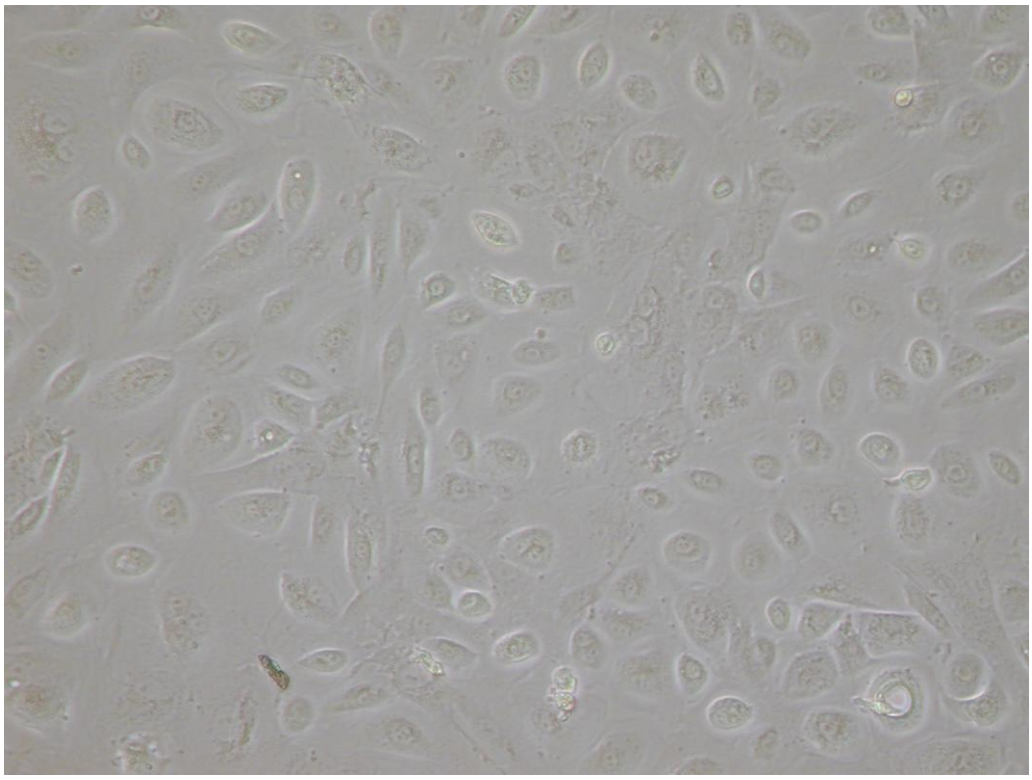


Figure 91: Human peritoneal mesothelial cells in culture. Some of these cells are rounded but some have a more elongated shape. Day 22 passage 1

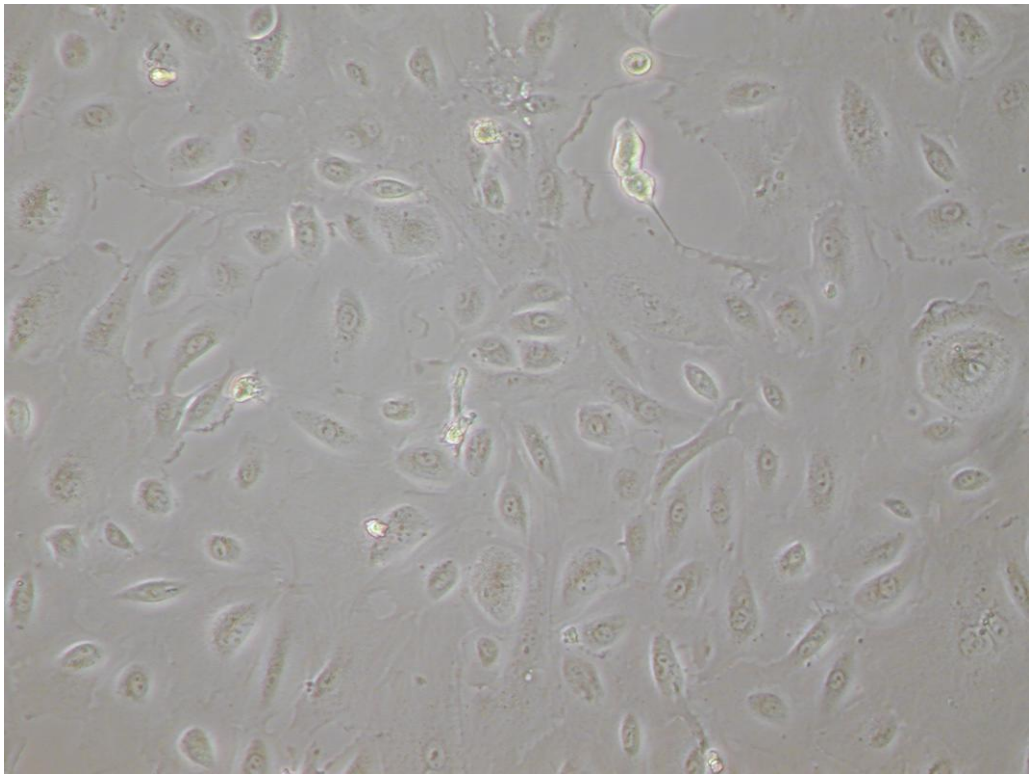


Figure 92: Human peritoneal mesothelial cells in culture. More of these cells have an elongated shape. This patient had been on PD for 27 months. Day 15 passage 1

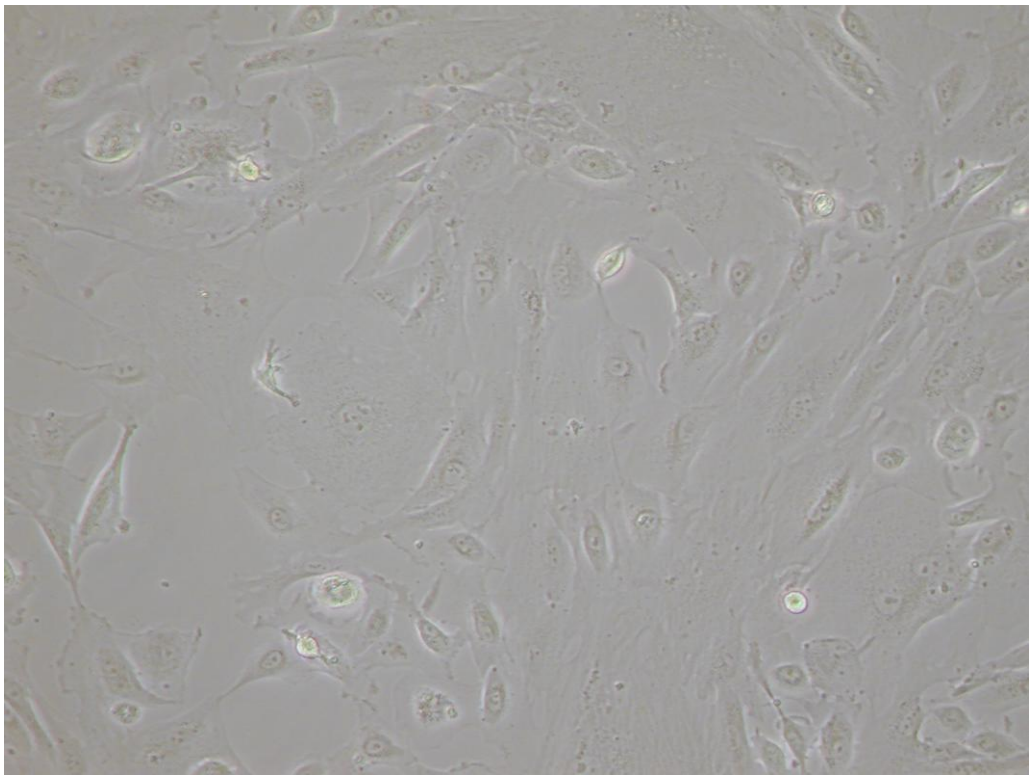


Figure 93: Human peritoneal mesothelial cells in culture. These cells have a more fibroblast-like appearance. Day 14 passage 1

9.1(ii) Peritoneal mesothelial cells in culture stain for epithelial and mesenchymal markers

Mesothelial cells from were stained for the epithelial marker cytokeratin 18 and the mesenchymal markers alpha smooth muscle actin and vimentin. The epithelial marker cytokeratin would be expected to be positive in mesothelial cells. The alpha smooth muscle and vimentin staining would be expected to be variable; these are mesenchymal markers and will be positive in cells during or after epithelial to mesenchymal transition. It was not possible to culture sufficient numbers of cells from any single patient to both perform staining and carry out the stimulation experiment (see section 9.2) so these pictures are from further patient samples cultured under the same conditions as those used for the stimulation experiments.

While the cells were in culture, samples of the culture medium were taken and cytokine production measured by ELISA.

Cytokeratin (epithelial marker)

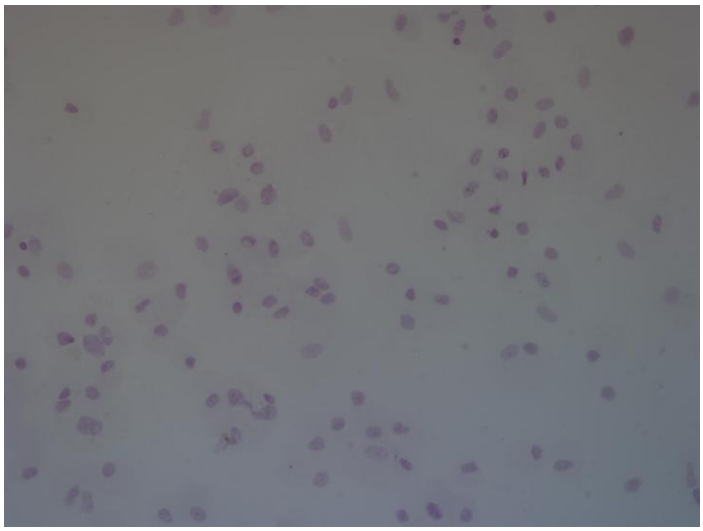


Figure 94a: Human peritoneal mesothelial cells in culture x20. Typical negative control for cytokeratin staining, using Marvel block

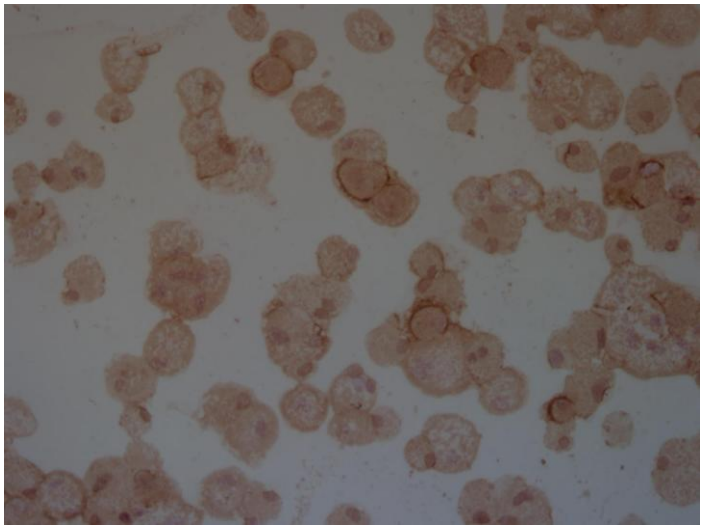


Figure 94b: Human peritoneal mesothelial cells in culture x20. Positive staining for cytokeratin (seen in all samples)

Vimentin:

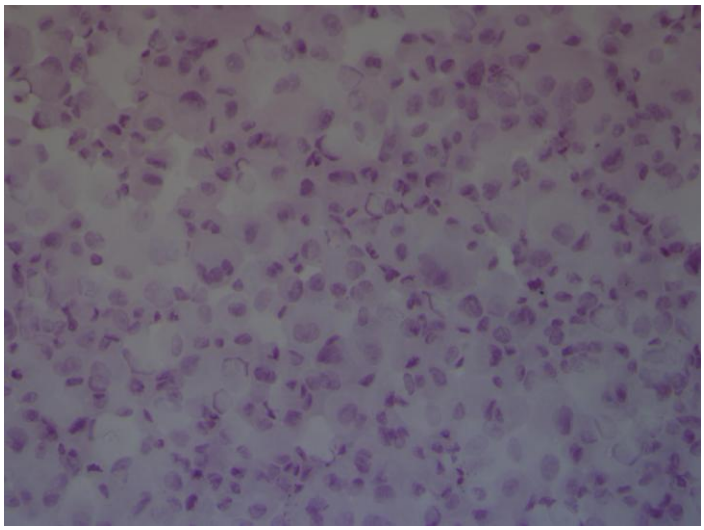


Figure 95a: Human peritoneal mesothelial cells in culture x20. Typical negative control for vimentin staining

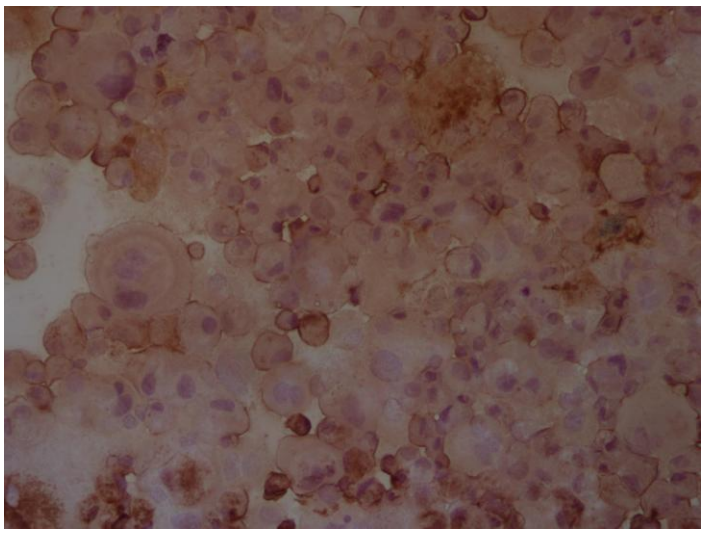


Figure 95b: Human peritoneal mesothelial cells in culture x20. The cells from this patient show strongly positive staining for vimentin

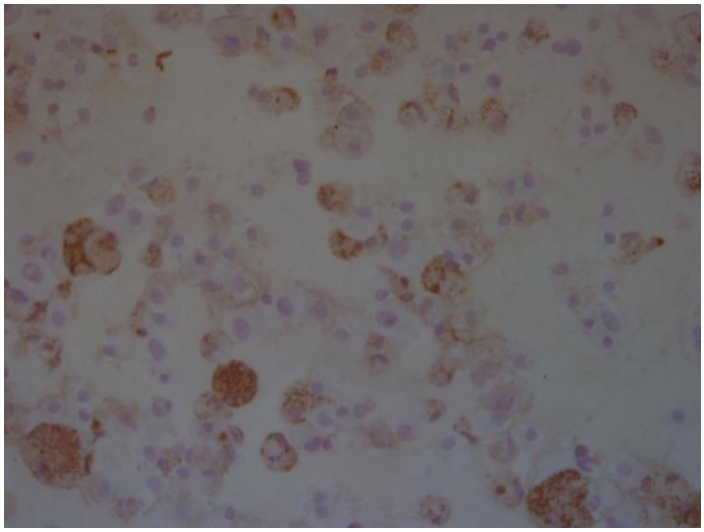


Figure 95c: Human peritoneal mesothelial cells in culture x20. Fewer of the cells from this patient show positive staining for vimentin

Alpha SMA:

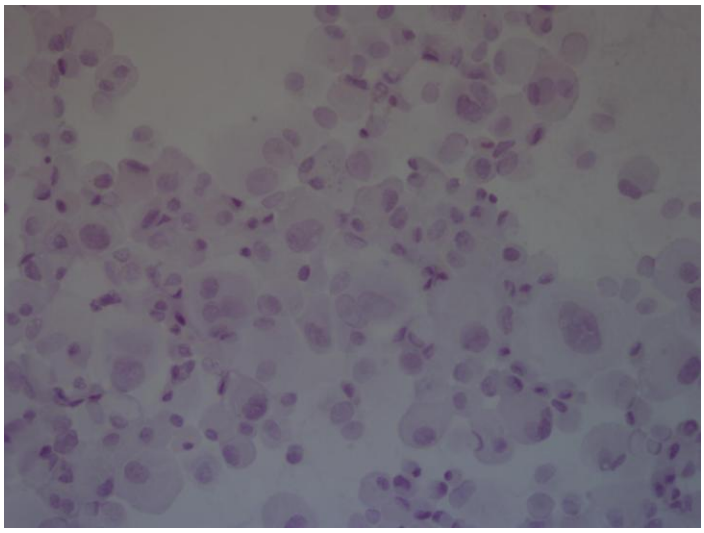


Figure 96a: Human peritoneal mesothelial cells in culture x20. Typical negative control for alpha SMA

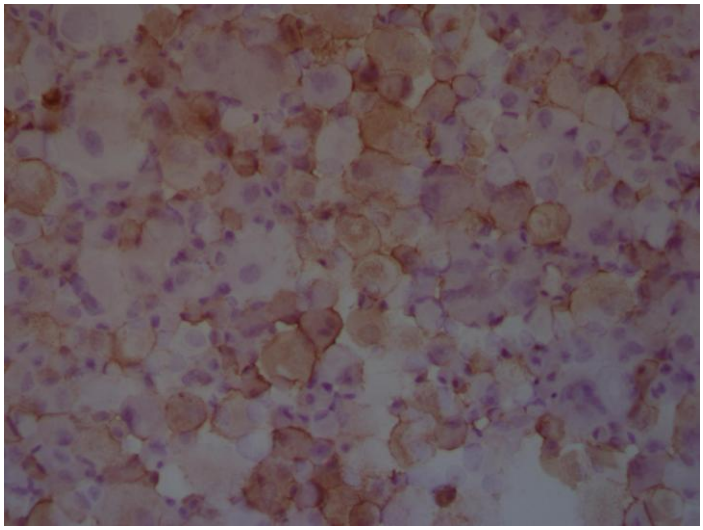


Figure 96b: Human peritoneal mesothelial cells in culture x20. The cells from this patient are strongly positive for alpha SMA (and were also strongly positive for vimentin)

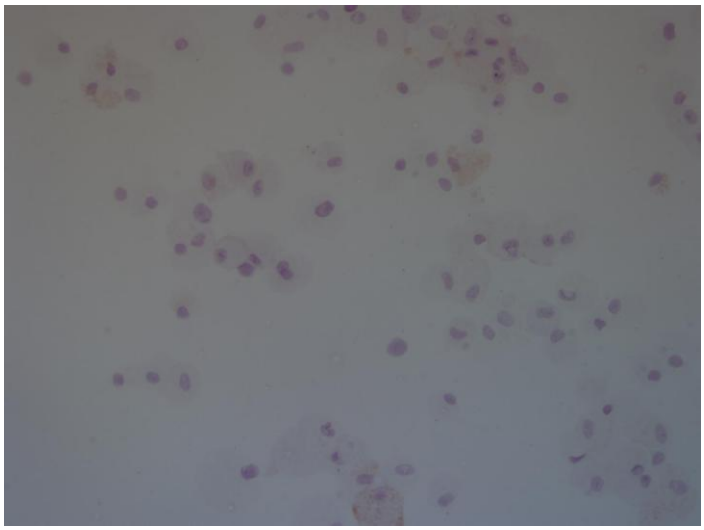


Figure 96c: Human peritoneal mesothelial cells in culture x20. Fewer of the cells from this patient, as with the vimentin stain on the same patient's cells, show positive staining for alpha SMA

9.1(iii) Peritoneal mesothelial cells in culture produce CCL18

Samples were taken each time the tissue culture medium was changed and levels of CCL18 in the culture medium were measured by ELISA. The results are displayed as pg/ml/day, since the interval between changes of culture medium varied. It is not possible to count the cells at each change of medium as they are adherent to the culture flask.

Patient	Renal diagnosis	Duration PD (months)	D/P creatinine	Residual GFR (mls/minute)	Glucose exp. (mmol/24hr)	Peritonitis episodes
1	Unknown	24	0.85	0.4	878	0
2	Hypertension	22	0.81	8.7	414	2
3	Diabetes	5	0.65	11.7	150	0
4	Diabetes	1	0.88	3.2	750	0
5	Diabetes	2	0.51	1.0	638	0
6	Obstruction	16	0.62	4.9	240	0
7	Unknown	31	0.77	4.1	750	0

Table 32: Clinical data of patients who gave overnight dialysate samples for cell culture experiments

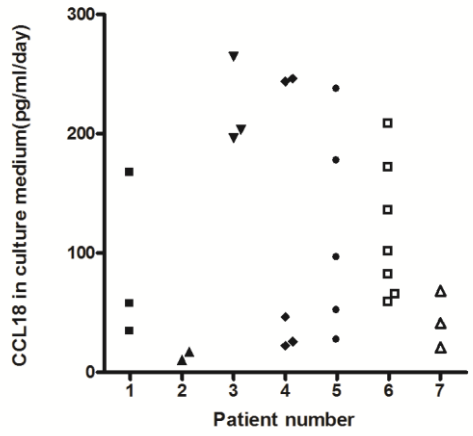


Figure 97: CCL18 production by human peritoneal mesothelial cells in culture. Each column shows the CCL18 levels measured in tissue culture medium for one patient. Each point represents a sample of tissue culture medium taken at the time of changing the medium, corrected for number of days since last change of medium. Variation in levels is expected as these results are not corrected for cell number. CCL18 was not detected in tissue culture medium that had not been exposed to mesothelial cells.

The source of CCL18 in the culture medium could be the peritoneal mesothelial cells. Alternatively it is possible that there were also other cell types cultured along with the mesothelial cells, despite the culture conditions being most suitable for mesothelial cell growth. The production of CCL18 by alternatively activated macrophages in the peritoneum is well documented (Bellón *et al.*, 2011); macrophage-like cells were however not observed in the culture. Dendritic cells have also been shown to produce CCL18 (Schutyser *et al.*, 2005) and other tissue-specific cells have been seen to produce CCL18 in our laboratory (unpublished data). CCL18 may be actively secreted by mesothelial or other cell types into the culture medium. Because the medium was immediately centrifuged at

4°C after collection (to reduce cell lysis) and only the supernatant was analysed for cytokines it is not likely that the detected CCL18 was released from damaged cells after sample collection. However it is possible that CCL18 in the culture medium was present because some cells that had been damaged or died prior to collection of the sample had released intra-cellular stores of CCL18, rather than the release of CCL18 being due to active secretion.

The possibility of local production of CCL18 within the peritoneum is interesting because we have seen in section 5.2 that levels of CCL18 measured in the dialysate effluent are dependent on the membrane transport properties. Levels of CCL18 in the dialysate are lower than would be expected by passive transfer of a 14kDa protein from the circulation (section 4.2(iii)), which suggests CCL18 may be protein bound either within the circulation or to the peritoneum. We could speculate that locally produced CCL18 is not released into the dialysate (hence the low levels found in effluent dialysate) but may still have a local effect to promote fibrosis.

9.1(iv) Peritoneal mesothelial cells in culture produce angiogenin

Levels of angiogenin were also measured in samples of culture medium which were taken during culture of the mesothelial cells. Angiogenin was not detectable in the unused tissue culture medium (lower level of detection 20pg/ml).

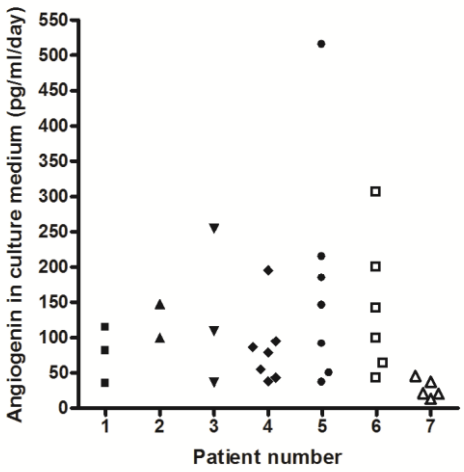


Figure 98: Angiogenin production by human peritoneal mesothelial cells in culture. Each column shows the angiogenin levels measured in tissue culture medium for one patient during the period the cells were in culture. Each point represents a sample of tissue culture medium taken at the time of changing the medium, corrected for number of days since last change of medium. Variation in levels is expected as these results are not corrected for cell number. Angiogenin was not detected in tissue culture medium that had not been exposed to mesothelial cells.

Angiogenin is known to be produced by a variety of cell types including cancer cells (stromal tissue) (Etoh *et al.*, 2000), fibroblasts (Lioté *et al.*, 2003) and renal proximal tubular cells (Nakamura *et al.*, 2006) – the mRNA is found is a wide variety of cells (Rybak *et al.*, 1987). It is likely, therefore, that the peritoneal mesothelial cells can produce angiogenin. In vivo angiogenin production is increased by hypoxia and also by IL-6 (Verselis *et al.*, 1999). It is possible that within a thickened and fibrotic peritoneal membrane there is hypoxia and angiogenin release which drives neovascularisation. Alternatively high levels of inflammation and IL-6 might drive angiogenin production in the

peritoneum. Levels of angiogenin in the dialysate were chiefly related to serum levels of angiogenin and the transport characteristics of the peritoneal membrane. However, this does not exclude the possibility of local production of angiogenin by the peritoneal cells. This local production might not greatly elevate dialysate angiogenin levels but could still have an important paracrine effect.

9.1(v) Peritoneal mesothelial cells in culture produce MCP-1

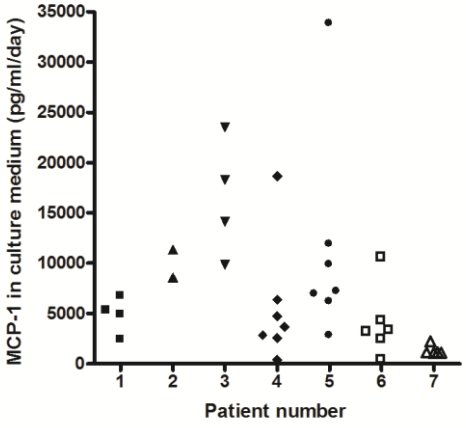


Figure 99: MCP-1 production by human peritoneal mesothelial cells in culture. Each column shows the MCP-1 levels measured in tissue culture medium for one patient during the period the cells were in culture. Each point represents a sample of tissue culture medium taken at the time of changing the medium, corrected for number of days since last change of medium. Variation in levels is expected as these results are not corrected for cell number. MCP-1 was not detected in tissue culture medium that had not been exposed to mesothelial cells.

Peritoneal mesothelial cells have been previously reported to produce MCP-1 (Lee *et al.*, 2001; Wong *et al.*, 2003). Production of MCP-1 is seen here by the peritoneal mesothelial cells in culture. This appears to be occurring at higher levels (in terms of pg/ml/day) than that of other cytokines, and might well be contributing substantially to the levels of MCP-1 observed in the dialysate effluent.

9.1 (vi) Peritoneal mesothelial cells in culture do not produce CCL15

No CCL15 was detected in the tissue culture fluid from the mesothelial cell culture, suggesting that these cells do not produce CCL15. CCL15 is known to be produced in the gut (Pardigol *et al.*, 1998), and may well enter the peritoneal cavity via the peritoneal membrane. This would be consistent with the observations that CCL15 levels in the dialysate correlate strongly with serum levels and that membrane transport characteristics are a strong predictor of dialysate CCL15 levels.

9.2 Stimulation of peritoneal mesothelial cells

9.2(i) Peritoneal mesothelial cell cytokine production is not affected by addition of CCL15

CCL15 was found in the dialysate effluent, and levels of CCL15 in the dialysate effluent correlate with those of other cytokines, but CCL15 does not appear to be made by the mesothelial cells. However CCL15 might still have a role to affect the nature of the inflammatory response in the peritoneum. CCL15 was used to stimulate the mesothelial cells in culture to investigate whether addition of CCL15 to the culture fluid had any effect on levels of cytokine production. This experiment was performed twice with three patient samples on each occasion. The higher level of CCL15 stimulation was used on the repeat experiment only.

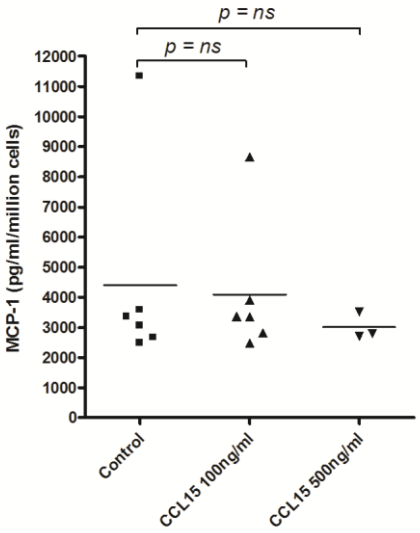


Figure 100: There is no significant difference in MCP-1 production by mesothelial cells after CCL15 stimulation

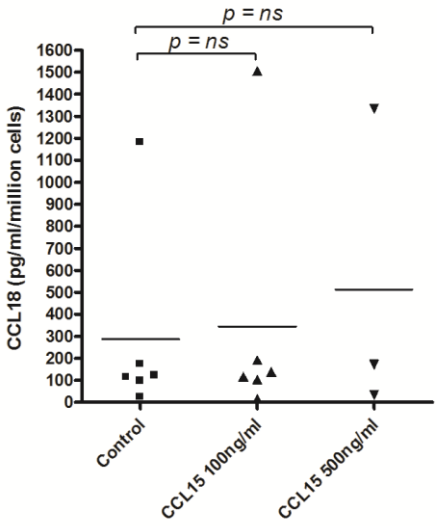


Figure 101: There is no significant difference in CCL18 production by mesothelial cells after CCL15 stimulation

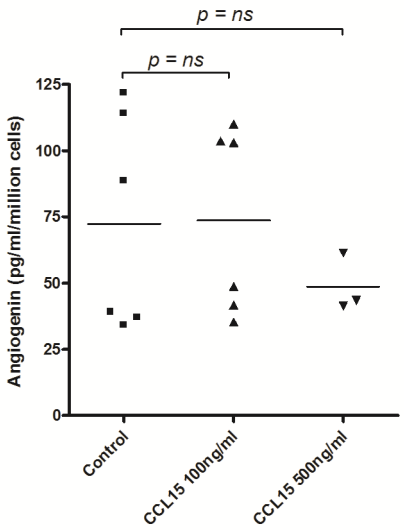


Figure 102: There is no significant difference in angiogenin production by mesothelial cells after CCL15 stimulation

The lack of response to stimulation by CCL15 may mean that this cytokine does not have an important role in the pathogenesis of peritoneal changes and is only a marker of increased peritoneal permeability. It is also possible that this culture system was not suitable for demonstrating an effect of CCL15 and that in vivo, where monocytes, macrophages and leukocytes are also present, CCL15 might have a relevant biological effect. Finally, changing to serum-free medium before the stimulation experiment had not resulted in a low baseline level of cytokine production prior to stimulation and it may be that cytokine production was already maximal prior to stimulation.

9.2(ii) Peritoneal mesothelial cell cytokine production is not affected by addition of IL-6

IL-6 also had no effect on the production of cytokines by the peritoneal mesothelial cells. This is probably explained by the observation by Hurst et al (Hurst *et al.*, 2001) that unlike leukocytes, mesothelial cells may not have an IL-6 receptor and responses to IL-6 then require the presence of the soluble IL-6 receptor.

9.3 Cytokine production by U937 cells

In order to assess whether CCL15 might have any effect on monocytes within the peritoneum, the effect of CCL15 stimulation on cytokine production by U937 cells was assessed.

U937 cells are a monocyte cell line. Increased production of MCP-1 from monocytes after stimulation with CCL15 was shown by Lee et al (Lee *et al.*, 2002). Baseline production of CCL18 and CCL15 was low or non-existent by the U937 cell line and no significant changes were seen post stimulation. In addition, no effect on MCP-1 production was seen after stimulation of U937 cells with CCL15 in these culture conditions:

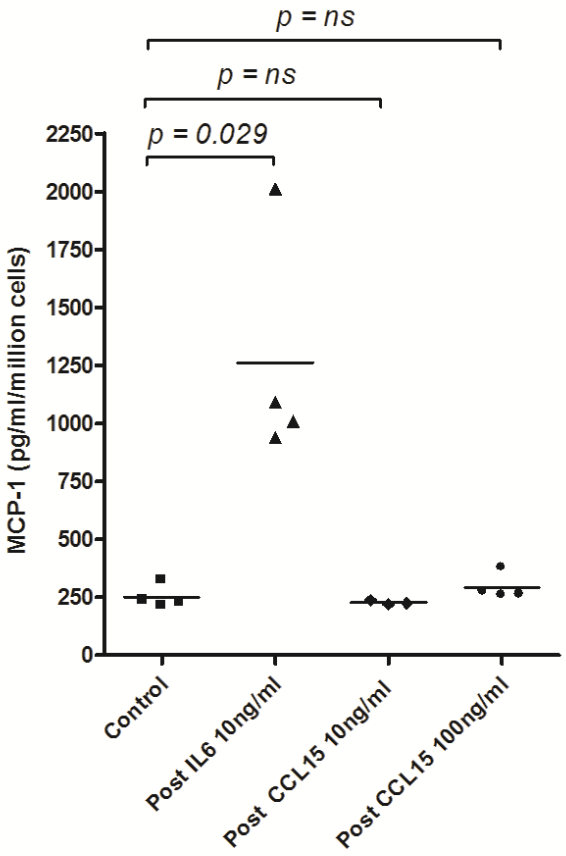


Figure 103: CCL15 stimulation did not affect the levels of MCP-1 production

However, a marked increase in MCP-1 production was seen after stimulation of the U937 cells with IL-6. This suggests that experimental factors such as time from stimulation of the cells before collecting samples of tissue fluid were unlikely to be the reason why no response to CCL15 was seen.

Summary

Cells which have been cultured from the overnight bag of dialysate effluent fluid under conditions expected to promote the growth of mesothelial cells show varying morphologies. Cells stain for the epithelial marker cytokeratin and variably for the mesenchymal markers vimentin and alpha SMA, in keeping with the literature describing EMT in PD patient mesothelial cells.

MCP-1, CCL18 and angiogenin are detected in the tissue culture medium after exposure to these cell cultures. CCL15 is not detected. This suggests that there is local production of these cytokines, but not of CCL15. Despite the low levels of CCL18 and angiogenin these cytokines could have a local effect on the peritoneal membrane.

No effect on cytokine production was seen after CCL15 stimulation of either the peritoneal cell cultures or a monocyte culture. Further work is required to define the role of CCL15; it may be that the effects of CCL15 cannot be seen in a cell culture system because interactions with other molecules or cells of the immune system are required.

CHAPTER 10: CT as a screening modality for EPS.....*135*

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Summary..... *141*

Chapter 10

Radiology: CT as a screening method for EPS

This part of the study was published in Nephrology Dialysis Transplantation (Nephrol Dial Transplant 2011 Apr;26(4):1374-9 "Screening for Encapsulating Sclerosing Peritonitis: Role of CT Scanning") - see Appendix C.

10.1 Methods

The aim of this part of the study was to investigate the validity of CT scanning of peritoneal dialysis patients as a screening method for future EPS. It is known that CT scanning is useful in the symptomatic patient to help confirm the diagnosis of EPS (Tarzi *et al.*, 2008; Vlijm *et al.*, 2009), but many units also use CT as a form of screening.

This was a retrospective study. Sufficient and appropriately timed CT scans were available for twenty patients who had developed EPS. For each patient a CT scan taken at least three months prior to the development of symptoms of EPS was selected (pre-diagnostic CT scan), together with a CT scan taken when EPS was clinically apparent (post-diagnostic CT scan). The notes, discharge summaries and electronic records, including the indication for CT scanning, were reviewed to determine when the patient first became symptomatic and to determine whether there were even minor bowel symptoms at the time the pre-diagnostic CT was performed.

CT scans from twenty control patients were also scored. These were peritoneal dialysis patients who had CT scans for indications such as screening, investigation of possible malignancy or as part of a pre-transplant work-up. None of these patients have developed EPS after over 2.5 years of follow-up; the majority have changed dialysis modality to haemodialysis or received a transplant. The median duration of PD at the time of scanning in the control group was 3.2 years.

CT scans were performed at the Hammersmith Hospital using a GE Lightspeed 8 slice scanner (GE Milwaukee, WI) or at Charing Cross Hospital using a Siemens Somatron Sensation 16 slice scanner (Siemens, Erlangen, Germany). Intravenous contrast was given (100mls of Ultravist 300 was the standard during the period within which the scans were obtained); one pre-diagnostic scan had intra-peritoneal contrast. Contrast was injected peripherally at 3mls/second and the abdominal and pelvic images were acquired at 60 seconds post-contrast in the venous portal phase.

CT scans were scored by three radiologists using the system described by Tarzi et al (Tarzi *et al.*, 2008). Each scan was scored blindly and a mean score obtained.

Score	Peritoneal thickening	Peritoneal calcification	Loculations
0	Not identified	Not identified	Not present
1	Localised area < 20%	Localised area < 20%	< 3 locules
2	> 20%	>20%	3-6 locules
3	> 50%	>50%	> 6 locules
4	> 80% of peritoneum	>80% of peritoneum	
	Bowel Wall Thickening	**Bowel Dilatation**	**Bowel Tethering**
0	Not identified	Not identified	Not present
1	Localised bowel	Localised bowel	Mild tethering
2	> 20% of bowel	> 20% of bowel	Moderate tethering
3	> 50% of bowel	> 50% of bowel	Marked tethering
4	> 80% of bowel	> 80% of bowel	

Table 33: CT scoring system. The highest score is 22 and scores above 2.5 are abnormal

This system of scoring CT scans for the changes characteristic of EPS was shown by Tarzi et al (Tarzi *et al.*, 2008) to be reproducible, with kappa values for inter-observer variability showing good agreement between radiologists. A score is determined for each of the CT features of EPS – peritoneal thickening, peritoneal calcification, fluid loculations, bowel wall thickening, bowel dilatation and bowel tethering and these scores are added to obtain a final total score.

10.2 Results

10.2(i) CT is not a suitable screening method for EPS in stable PD patients

The median CT scores were 1 in the control group, 2 in the pre-diagnostic group and 9 in scans performed at the time of diagnosis of EPS. The difference between the scores of the control group scans and the scores of scans taken from patients with clinical symptoms of EPS was highly significant ($p < 0.001$), as found previously (Tarzi *et al.*, 2008). There was a statistically significant difference between the scores of the pre-diagnostic group and the scores of the control group ($p = 0.003$); however, inspection of the results shows that there is considerable overlap between the two groups and in particular a score of 1 or 2 was common in both groups.

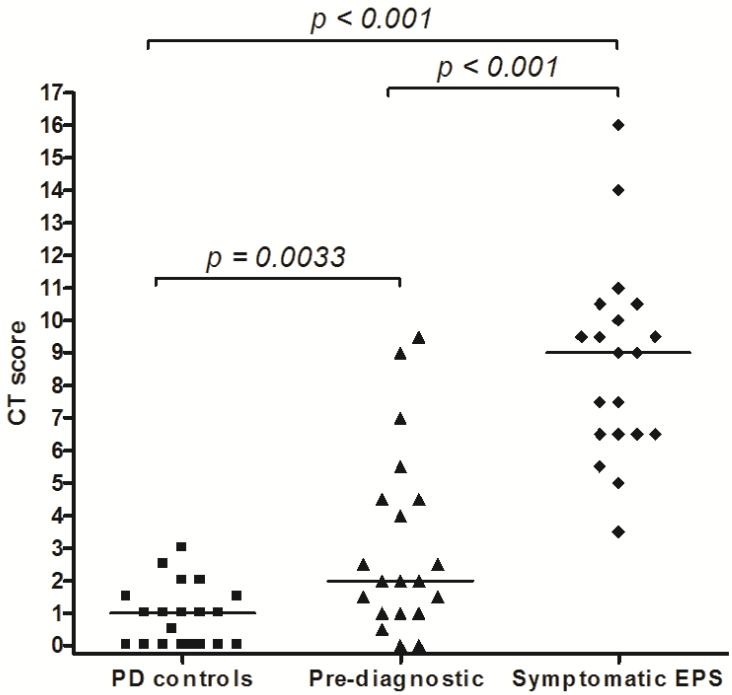


Figure 104: CT scan scores are significantly higher in the pre-diagnostic group than the control group; however, half of the pre-diagnostic scans scored in the same range as the control scans making CT scanning a poor choice of screening test

This means that there were ten patients who had a CT scan score which was considered normal but then developed symptomatic EPS (with an abnormal scan) within a period of time which could be as little as three or four months (range 3.7 – 37 months). There was no correlation between the pre-diagnostic CT scan score and the time period before the development of EPS. This may well be because all the patients in this group developed EPS after the cessation of PD. The timing of EPS onset was therefore determined by the reasons for stopping PD – transplantation, severe peritonitis etc.

This data strongly suggests that in PD patients who undergo a "screening" CT, the possibility of EPS on cessation of peritoneal dialysis is not excluded by a normal scan.

The lack of usefulness of CT scanning as a screening test for EPS can be illustrated by the ROC curve:

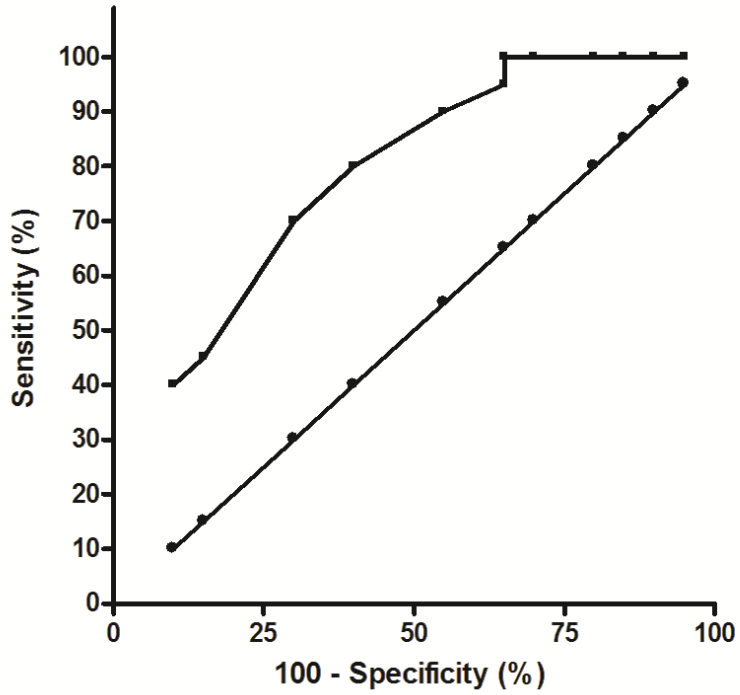


Figure 105: ROC curve for CT scan scores as a predictor of EPS. The area under the curve is only 0.772 and the line lies close to the line of identity. CT has a sensitivity and specificity of 70% with a cut off of a score of 1.25, which is clearly not adequate for clinical purposes

10.2(ii) Minor abdominal symptoms and an abnormal CT may indicate a higher risk of EPS

When the pre-diagnostic group is separated into those who had had a minor episode of symptoms and those who were documented as asymptomatic, a significant difference in scan score between the symptomatic and control groups is seen (no difference was seen between the asymptomatic and the control groups). In total 12 patients were documented to be completely asymptomatic at the time of the pre-diagnostic scan, which was performed for reasons such as screening prior to transplantation or retroperitoneal haematoma. EPS was diagnosed a median of 7.9 months after the pre-diagnostic scan in these patients (range 3.4 – 32 months). The other 8 patients had had a brief (one or two days) hospital admission with transient symptoms such as constipation or abdominal pain, which had prompted the CT scan. These symptoms had then resolved (persistent symptoms would have suggested that the diagnosis of EPS should have been made earlier). In fact symptoms of EPS appeared at a median of 11.6 months after the pre-diagnostic scans were performed in this group (range 5 – 37 months), slightly later than was seen in the asymptomatic group.

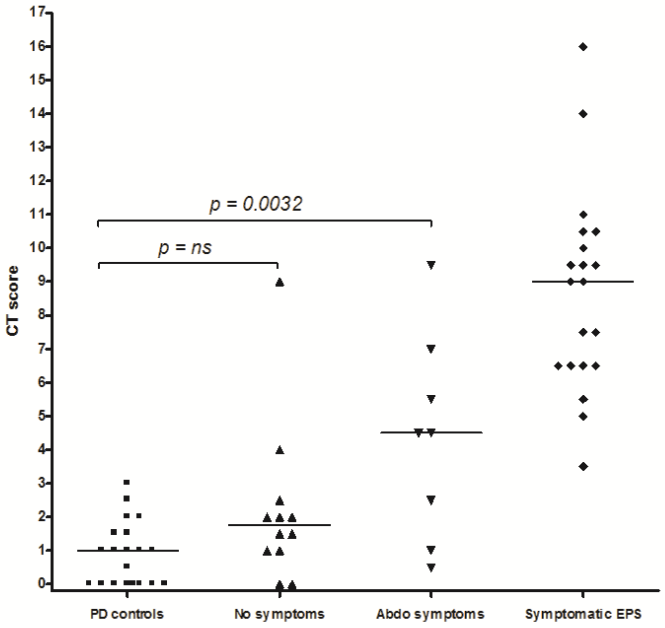


Figure 106: A significant difference is seen between the group of PD patients who had experienced a transient episode of abdominal symptoms and the control group. A smaller proportion of briefly symptomatic patients had a normal scan score than was seen in the pre-diagnostic group as a whole

The median score of the PD controls was 1; the median score of the group with no symptoms was 1.75 (not significantly higher). The median score in the group who had had an episode of abdominal symptoms was 4.5, which was significantly higher than the control group.

10.2 (iii) Does CT scanning help to predict EPS in patients with transient abdominal symptoms?

A group of 8 peritoneal dialysis patients were identified who had had a CT scan for abdominal symptoms such as abdominal pain, change in bowel habit and weight loss, who had not then developed EPS. The CT scan scores in this group were significantly different from the scores in the group of symptomatic patients who had gone on to develop EPS; the median score in the group who did not develop EPS was 1, and in the group who did develop EPS the CT scan score was 4.5 (p = 0.02).

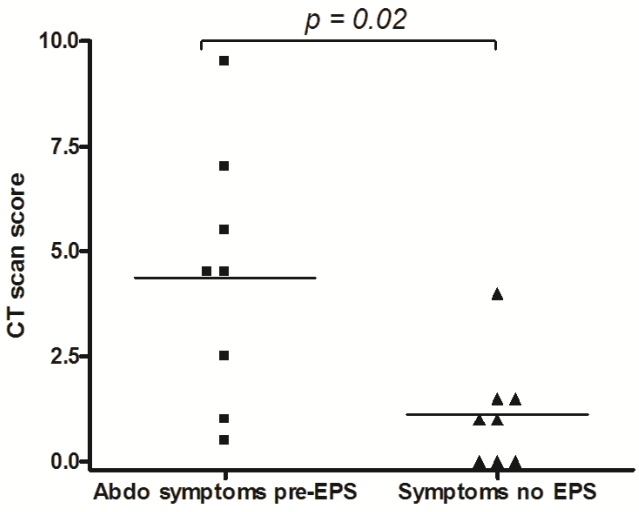


Figure 107: Looking at PD patients who had experienced a transient episode of abdominal symptoms, CT scan scores were higher in the group who went on to develop EPS than in the group that did not

This data suggests that a CT scan may be of use if a PD patient develops a transient episode of abdominal symptoms. Although a CT score >2 appears to be highly predictive of EPS in this data (85% of symptomatic patients with a CT score greater than 2 developed EPS), this is not a randomly selected sample of PD patients with abdominal symptoms and in practice the low prevalence of EPS means that the positive predictive value of CT scanning in symptomatic patients would not be this high. It does appear from this data that a high CT score may imply a risk of future EPS which is greater than the risk conferred by an episode of abdominal symptoms with a normal CT; this requires confirmation in a prospective study.

Summary

CT scanning is not suitable as a screening test for future EPS in stable peritoneal dialysis patients since many patients developed EPS within months of a normal CT scan. The presence of even transient abdominal symptoms and an abnormal CT scan did appear to be associated with a higher risk of EPS after the cessation of PD. Although data from our survey of symptoms in peritoneal dialysis patients (see Appendix B) suggests that many patients experience abdominal symptoms, few rate them as severe (the median severity score was 0 for all symptoms except loss of appetite). Perhaps CT scanning is indicated in PD patients who complain of any episodes of more severe symptoms, even if those symptoms have quickly resolved. CT scanning would then provide information which could be combined with other clinical data such as duration of PD and membrane transport status to improve the overall risk assessment of the patient.

CHAPTER 11:

Final discussion and conclusions

As a mode of renal replacement therapy, peritoneal dialysis provides outcomes for patients which are broadly similar to haemodialysis (Quinn *et al.*, 2011), (Chiu *et al.*, 2011) and for some patients there are particular advantages to peritoneal dialysis. For example the life-style advantages of a home-based therapy may be considerable for some; for others difficulties with vascular access may make haemodialysis problematic. However, considerable evidence suggests that the performance of peritoneal dialysis in the long term is limited by changes in the peritoneal membrane structure (Williams *et al.*, 2002) and function (Davies *et al.*, 2001).

The pathogenesis of the epithelial to mesenchymal transition and sub-mesothelial fibrosis seen in long term peritoneal dialysis patients is not yet fully defined. There are multiple factors associated with peritoneal dialysis which may potentially contribute to membrane damage. Uraemia (Williams *et al.*, 2002), glucose (Davies *et al.*, 2005) and its degradation products (Hirahara *et al.*, 2009), advanced glycosylation end-products (De Vriese *et al.*, 2006), fluid flow stresses (Aoki *et al.*, 2011b), activation of the renin-angiotensin system (Chang *et al.*, 2011) and peritoneal infection (Davies *et al.*, 1996) are all implicated.

In keeping with the many possible causes of peritoneal damage, there may also be multiple mediators of inflammation and fibrosis involved, with more than one pathway combining to result in a common histological and functional outcome. This is the case with fibrosis and EMT in other systems such as renal fibrosis (Liu, 2010), where more than one interacting pathway leads to the development of EMT. The pathways from acute inflammation to fibrosis in the peritoneum are not well-defined, but it is likely that similar multiple signalling pathways exist.

We could consider the changes in the peritoneum as being related to acute and chronic inflammation, vascular changes and fibrosis. Within the peritoneum, IL-6 is well established in the literature to be involved in acute inflammation and the switch from acute to chronic inflammation (Hurst *et al.*, 2001) and also is associated with functional changes in the peritoneal membrane (Pecoits-Filho *et al.*, 2002a). Similarly, VEGF is known to induce angiogenesis in multiple systems and has been demonstrated to be involved in the genesis of peritoneal blood vessel changes (De Vriese *et al.*, 2001). TGF-β is perhaps the best established mediator of peritoneal fibrosis; there is evidence for TGF-β causing both induction of EMT (Loureiro *et al.*, 2011) and production of matrix by fibroblasts (Medcalf *et al.*, 2001) .

However, it is likely that other mediators of peritoneal damage exist given the many factors observed in clinical studies to be associated with peritoneal membrane damage. In addition other molecules will be involved in the same pathway as the better-known mediators of peritoneal damage and could be potential therapeutic targets in the future. For example, CTGF is likely to be a downstream mediator of TGF-β activity (Leung *et al.*, 2009).

The cytokines investigated in this study were chosen as both being present in a multiplex array assay of dialysate effluent and having a biologically plausible role to play in the pathogenesis of peritoneal changes. As CCL15 initially attracts an influx of neutrophils but becomes a chemoattractant for monocytes after local processing by neutrophil cathespin or matrix metalloproteases (Richter *et al.*, 2005; Starr *et al.*, 2011), it may be involved with IL-6 in a change from acute to chronic inflammation as well as having a potential role in angiogenesis (Hwang *et al.*, 2004). MCP-1 is associated with chronic inflammation, especially related to hyperglycaemia (Dragomir and Simionescu, 2006), making it a good candidate mediator in the peritoneum where glucose is implicated as a cause of pathology. CCL18 is associated with fibrosis in humans (Schutyser *et al.*, 2005; Luzina *et al.*, 2006a) in a TGF-β independent manner, suggesting it may act downstream of TGF-β or by a separate pathway. Angiogenin has been shown to be essential for the action of VEGF (Kishimoto *et al.*, 2005), and therefore might well be required for peritoneal vascular changes to occur.

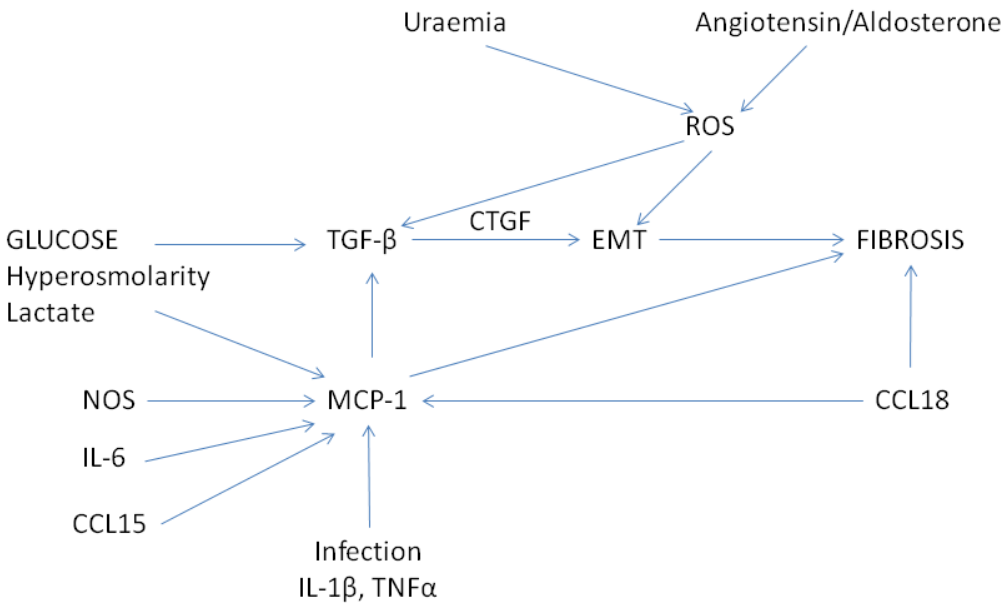


Figure 108: Some possible pathways resulting in peritoneal fibrosis. Other pathways and mediators are likely to exist. ROS=reactive oxygen species, EMT=epithelial to mesenchymal transition, CTGF=connective tissue growth factor, TGF-β=transforming growth factor-β, MCP-1=monocyte chemotactic protein-1, NOS=nitric oxide synthase, IL-6=interleukin-6, IL-1β=interleukin-1β, TNFα=tumour necrosis factor α, CCL15=leukotactin, and CCL18=PARC, pulmonary and activation-regulated chemokine

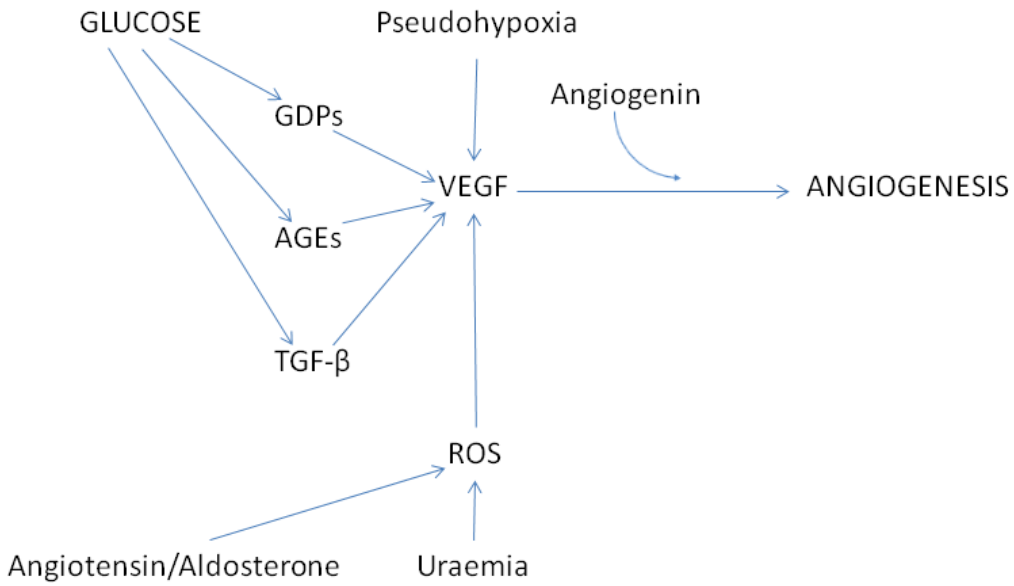


Figure 109: Some possible pathways resulting in peritoneal angiogenesis. Other pathways and mediators are likely to exist. ROS=reactive oxygen species, TGF-β=transforming growth factor-β, AGE=advanced glycosylation end product, GDP=glucose degradation product and VEGF=vascular endothelial growth factor

This study is based on data collected from patients at their usual peritoneal equilibration tests. Patients were not selected for the study – samples were collected whenever the researcher was available to attend the PET. No patients were excluded from the study. The data is therefore representative of patients encountered in normal clinical practice. Both clinical data and cytokine levels were studied in order to obtain as complete a picture as possible. Levels of cytokines were investigated in both the dialysate effluent and serum, as it was expected that there might be a relationship between cytokine levels in the two compartments. In order to assess the relative contributions of the multiple clinical factors found to correlate with cytokine levels, multivariate analysis was performed. Cytokine production by mesothelial cells was studied, and the potential actions of the novel cytokine CCL15 on mesothelial cells were investigated in a cell culture model.

Cytokines are present in the dialysate and levels correlate with clinical markers associated with peritoneal damage.

The results of this study indicate that these cytokines are present in the effluent dialysate of peritoneal dialysis patients. The presence of CCL15 in dialysate effluent has not previously been reported. In addition, the levels of cytokines within the dialysate correlate with confirmed clinical risk factors for peritoneal damage such as duration of peritoneal dialysis, glucose exposure and lack of residual renal function. On multivariate analysis the most significant clinical feature which predicted dialysate cytokine levels was the D/P creatinine. D/P creatinine increases with duration of

PD and glucose exposure; it describes the rate of transfer of the small molecule creatinine across the peritoneal membrane. The correlation of cytokine levels with D/P creatinine has a number of potential underlying mechanisms; the mechanism need not be the same for each cytokine.

Dialysate CCL15 levels depend on transfer across the peritoneal membrane from the circulation

Several different explanations for the observation that dialysate cytokines correlate with D/P creatinine can be suggested. One is that cytokine levels in the dialysate are determined by serum dialysate levels and membrane permeability; that is, intra-peritoneal production of cytokine is not important in determining dialysate cytokine levels. This appears true for CCL15, which is produced in the gut (Pardigol *et al.*, 1998) but was not made by peritoneal cells in culture. It is found in dialysate at levels roughly expected from its molecular weight and serum concentration when compared to other proteins that enter from the circulation. It is likely that this cytokine does enter the dialysate across the peritoneal membrane; thus the correlation between CCL15 levels in dialysate and membrane transport rate for creatinine is not unexpected and does not imply that CCL15 determines the membrane transfer properties for small solutes.

Dialysate levels of MCP-1 and IL-6 are not determined by transfer from the circulation

Another potential explanation for the correlation of dialysate cytokine levels with the D/P creatinine is that the transport properties of the membrane are influenced by the cytokines. This might be true, for example, of MCP-1 and IL-6. MCP-1 (Lee *et al.*, 2001) is produced by peritoneal cells, as is IL-6 (Topley *et al.*, 1993). MCP-1 was found in tissue culture medium from peritoneal mesothelial cells at considerably higher levels than was seen for other cytokines. In addition, the study investigating transfer across the peritoneal membrane of proteins of varied molecular weight demonstrates that levels of both MCP-1 and IL-6 in the dialysate effluent fluid are higher than would be expected if dialysate levels were determined chiefly by passive transfer of cytokine from the circulation. It is therefore likely that local production of MCP-1 and IL-6 has a significant impact on the levels found in dialysate. In addition MCP-1 and IL-6 levels in dialysate only show a weak correlation with serum levels. Therefore the existence of a correlation between both MCP-1 and IL-6 and the D/P creatinine result suggests that these cytokines may affect the membrane transport properties. Alternatively, both MCP-1 and IL-6 levels in dialysate and the peritoneal membrane transport rate could be increased by another factor (such as glucose exposure) and MCP-1/IL-6 might have no direct role in mediating membrane changes.

Although there is local production of CCL18 and angiogenin, levels in dialysate effluent are correlated with serum levels and membrane properties.

For CCL18 and angiogenin the data suggests a different interpretation. The ELISAs performed on culture medium from peritoneal cell cultures suggest that these cytokines are also produced by peritoneal cells; however the amounts detected are considerably lower than the amounts of MCP-1 that were detected in culture medium. There is no evidence from the experiment investigating transfer of proteins by molecular weight to suggest that there is excess angiogenin in the dialysate compared to the quantity that might be expected to enter from the circulation during the four hour dwell, and there was even less CCL18 than expected. It may be that although there is local production of these cytokines, which could have important paracrine effects, concentrations found in the dialysate effluent are determined by cytokine transfer from the circulation across the peritoneal membrane rather than by this level of local production. This does not mean that CCL18 and angiogenin do not have any effects on the peritoneal membrane, but there is no clear evidence from this study that they affect small solute transfer. The correlations observed between dialysate levels of CCL18, angiogenin, CCL15 and clinical data such as glucose exposure and duration of dialysis might well be due to the relationships between these clinical factors and the membrane transport properties of the patient, which in turn determines the dialysate cytokine quantity.

Interestingly serum levels of MCP-1, IL-6 and CCL18 also correlate with the membrane transport factor. Perhaps circulating cytokines could also affect the peritoneal membrane, as well as those produced locally.

After one year of follow-up an increase in membrane permeability to cytokines increases the dialysate levels of angiogenin, CCL15 and CCl18.

The changes in cytokine levels seen in the one year follow-up samples are consistent with an increase in membrane permeability to the cytokines CCL15, angiogenin and CCL18. This is not unexpected given an increase in the D/P creatinine with time is well-recognised in PD patients; it may represent an increase in the number of blood vessels in the peritoneum with time and hence an increase in the available area for solute transfer. In this respect it is interesting that patients who had an increase in D/P creatinine ratio at one year had higher serum angiogenin levels – although the membrane change is a localised process. It may not be a statistically robust finding and further study with a larger group of follow-up samples would be needed to confirm or refute this possibility.

The levels of MCP-1 in serum and dialysate are stable at one year, despite the increase in D/P creatinine; again, this is consistent with the levels of MCP-1 in dialysate being chiefly due to

peritoneal production of MCP-1. Increased levels of IL-6 in dialysate samples at one year are likely to reflect increased intra-peritoneal IL-6 production.

The action of CCL15 within the peritoneum is not established

Although the finding of CCL15 in the effluent dialysate fluid is interesting and novel, the role of CCL15 in the peritoneum remains unclear. No effect on cytokine production was seen when CCL15 was used to stimulate either peritoneal cells or monocyte cells in culture, although others have shown response in monocytes to CCL15 stimulation (Lee *et al.*, 2002). Clearly CCL15 might have effects on other cytokines or molecules that were not measured. In addition it may be that other factors are required for CCL15 activity to occur – other cell types or soluble factors may be present in the peritoneum which are absent from the tissue culture situation.

There is some evidence that CCL18 is involved in ongoing EPS after cessation of PD

In support of a possible role of CCL18 in the development of EPS, CCL18 is found at higher levels in the serum of haemodialysis patients with EPS than haemodialysis patients of similar age, sex and dialysis duration who had never undergone PD. The number of patients available for this part of the study was small, however. Bellón et al found CCL18 levels were higher in the dialysate of 6 peritoneal dialysis patients who later developed EPS (Bellón *et al.*, 2011), and in an analysis of 106 patients our group previously noted higher levels of dialysate and serum CCL18 in 7 patients who subsequently developed EPS (Ahmad *et al.*, 2010) during 12 months of follow-up. In the larger group of patients investigated in this study levels of dialysate and serum CCL18 were not markers for later EPS; however in this analysis patients were included who developed EPS more than one year after sampling (median 14 months, range 3 – 46 months) and this may explain the lack of association between CCL18 levels and later EPS. However, the findings of raised CCL18 levels in such a small group of haemodialysis patients (*n*=10 EPS patients on haemodialysis were sampled) should be considered as preliminary only.

Radiological screening for EPS using CT scanning is not useful in asymptomatic PD patients

The demonstration that severe symptomatic EPS can develop within months of stopping peritoneal dialysis even in patients with a recently normal CT scan shows that CT scanning of asymptomatic PD patients is not an appropriate screening method. This is an important finding, as patients contemplating transplantation or transfer to haemodialysis require counselling as to their risk of EPS after modality change and the information they are given should be as accurate as possible.

However an abnormal CT scan, performed in the context of a history of even transient abdominal symptoms, should raise concerns that the patient is vulnerable to the development of EPS after the cessation of PD.

Dialysate cytokine levels in this study are not suitable as biomarkers for the prediction of EPS

Using these dialysate cytokine levels as predictors of EPS does not have any benefit beyond that of a simple model using the duration of PD and D/P creatinine, which had some potential to predict EPS in our population. Whether this clinically-based model would be useful and reproducible in other populations would require further investigation, as it is likely to perform less well in a new population with potentially a different incidence of EPS. At the best it will help to quantify a risk associated with long duration of PD and fast transporter status which is already appreciated by clinicians. Assessment of cytokines as potential biomarkers combined with clinical markers in this study was to some extent limited by the small number of EPS patients (17), which limits the number of predictors that can be analysed.

The use of cytokines – or other small proteins - in the dialysate fluid as a biomarker which could help to define the risk of future EPS in a patient who is currently stable on peritoneal dialysis is an exciting concept. Dialysate is easy to collect and cytokines can be measured with reasonable inter-assay variability using standard commercially available ELISA kits. Given the current lack of radiological methods to assess for later EPS risk in stable PD patients, and the difficulties inherent in the collection and interpretation of peritoneal biopsies, a small molecule marker which could be measured in effluent dialysate would be highly useful to clinicians and patients. When considering dialysate cytokines as possible biomarkers for EPS it is worth considering that a cytokine might function well as a biomarker even if it is not involved in the pathogenesis of EPS or produced by peritoneal cells; for this application it is only the strength of the association of the cytokine level with EPS which is relevant. Conversely, a cytokine which is causally involved in the pathogenesis of EPS might not show dialysate levels that can be used as a biomarker because it is produced in the peritoneum in small quantities, is largely bound to cells and acts in a paracrine fashion, while overall dialysate levels are determined by influx of the cytokine from the circulation. CCL18, for example, might be in this category; it is seen at high levels in the serum of EPS patients but in dialysate levels are lower than predicted from its molecular weight and are not associated with later EPS.

Cytokine	***Predictors of dialysate levels***			***Peritoneal production in cell culture?***	***Dialysate level determined by intra-peritoneal production?***	***Higher levels in pre-EPS patients?***
	D/P creatinine	***Serum level***	***Other factor***			
CCL18	p < 0.001	p < 0.001		Yes	No	No
Angiogenin	p < 0.001	p = 0.009	Fluid type p = 0.041	Yes	No	No
CCL15	p < 0.001	p < 0.001	Glucose exp p = 0.025	No	No	Yes
MCP-1	p < 0.001	(p = 0.057)	Glucose exp p = 0.015	Yes	Yes	Yes
IL-6	p < 0.001	(p = 0.063)	Glucose exp p = 0.01 Duration PD p =0.043	Yes (shown by other groups)	Yes	Yes

Table 34: Summary of findings relating to dialysate cytokine levels

Areas for further study

The potential role of CCL15 in the peritoneum is not clear and this could be further studied by studying the mechanism of action of CCL15 in a different cell culture system, examining more broadly for changes in cytokine production profiles. It would be interesting to investigate the effect of CCL15 on peritoneal macrophages or a fibroblast culture.

The ability of cytokines such as MCP-1 and CCL18 to worsen peritoneal fibrosis could be investigated in an animal model of chronic peritoneal dialysis, as could the ability of angiogenin to promote vascular changes in the peritoneal membrane.

The production of matrix elements by the cells of the peritoneum has not been investigated extensively in the literature and could be an interesting avenue to pursue, since this is an important part of the pathology. There is some evidence implicating matrix-metalloprotease (MMP) levels in the pathogenesis of peritoneal sclerosis, and intra-peritoneal production of MMPs has been demonstrated (Hirahara *et al.*, 2011). However it may be the balance between the activity of the MMPs and tissue inhibitors of MMPs which is more important in fibrosis. MMPs may also be relevant as an early step in neo-angiogenesis (downstream of angiogenin). It would be interesting to investigate the response of peritoneal cells in culture conditions to the application of cytokines in terms of the production of matrix components such as collagen, fibronectin and proteoglycans.

These components are generally derived from fibroblasts, so may also be produced by mesothelial cells that have undergone EMT.

It would be useful to examine the expression of cytokines in peritoneal biopsy material; this might also help to define which cell types produce the cytokines detected in dialysate.

Final remarks

Cytokines are produced by peritoneal cells, and are present in the effluent dialysate of peritoneal dialysis patients at levels which correlate with important clinical features. There is evidence that dialysate levels of MCP-1 and IL-6 are determined by intraperitoneal production, while transfer from the circulation could determine dialysate levels of CCL18, CCL15 and angiogenin. The relatively low levels of CCL18 in dialysate relative to serum levels suggest it may be bound to protein in the peritoneum. Although dialysate cytokine levels are very accessible and convenient, measuring the levels in dialysate effluent does not necessarily reflect any local, paracrine activity of the cytokine within the peritoneum.

Cytokines – CCL15, MCP-1 and IL-6 - were found at higher levels in the dialysate of patients who later developed EPS; in addition CCL18 is found at higher levels in EPS patients maintained on HD. It may be that cytokines are involved in the pathogenesis of EPS but it is also possible that cytokine levels are just acting as markers of other mediators more directly involved in the development of membrane damage. Although in this study cytokine levels could not be used alone to predict the later onset of EPS, raised levels of cytokines in the dialysate might help to confirm a clinical impression (based on duration of PD, lack of residual renal function, high glucose requirements and high D/P creatinine) that the patient is at relatively high risk of EPS after cessation of PD. Further investigation of this possibility requires a large, multi-centre prospective study, with adequate numbers of EPS patients. The clinical assessment of risk would be further increased if an episode of even transient abdominal symptoms had occurred; in these patients a CT scan which detected abnormalities similar in nature to those found in patients with EPS would be a further marker of higher risk of symptomatic EPS after the cessation of PD.

Until a more reliable method of quantifying the risk of EPS is available to clinicians and patients, the clinician must continue to use all the available information in the context of the patient's general condition and wishes to guide decisions, especially regarding duration of peritoneal dialysis. The search for a reliable and readily measured biomarker of EPS risk should continue to be a priority for PD researchers.

Appendix A:

Symptoms Questionnaire

Study No:			**Date:**	
For each symptom below, could you please place an X against the most appropriate box for severity and frequency				
Symptom	**Severity**		**Frequency**	
Pain in joints	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Blurred vision	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Weakness of limbs	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Headaches	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Light-headed or Dizziness	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Cold hands and feet	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Cramps in legs	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Shortness of breath	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	

Study No:			Date:	
For each symptom below, could you please place an X against the most appropriate box for severity and frequency				
Symptom	**Severity**		**Frequency**	
Unsteadiness	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Dry mouth	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Sleep problems	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Swelling of legs	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Itching	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Lack of energy	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	

Study No:			Date:	
For each symptom below, could you please place an X against the most appropriate box for severity and frequency				
Symptom	**Severity**		**Frequency**	
Vomiting	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Abdominal Pain	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Abdominal distension (bloating)	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Constipation	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Diarrhoea	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Loss of appetite	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Nausea	None		Never	
	Mild		Once a week or less	
	Moderate		Most days but not all	
	Severe		Every day	
Thank you very much for completing the questionnaire				

Appendix B

Symptoms in stable PD patients (data presented at the EuroPD meeting in Strasbourg, October 2009)

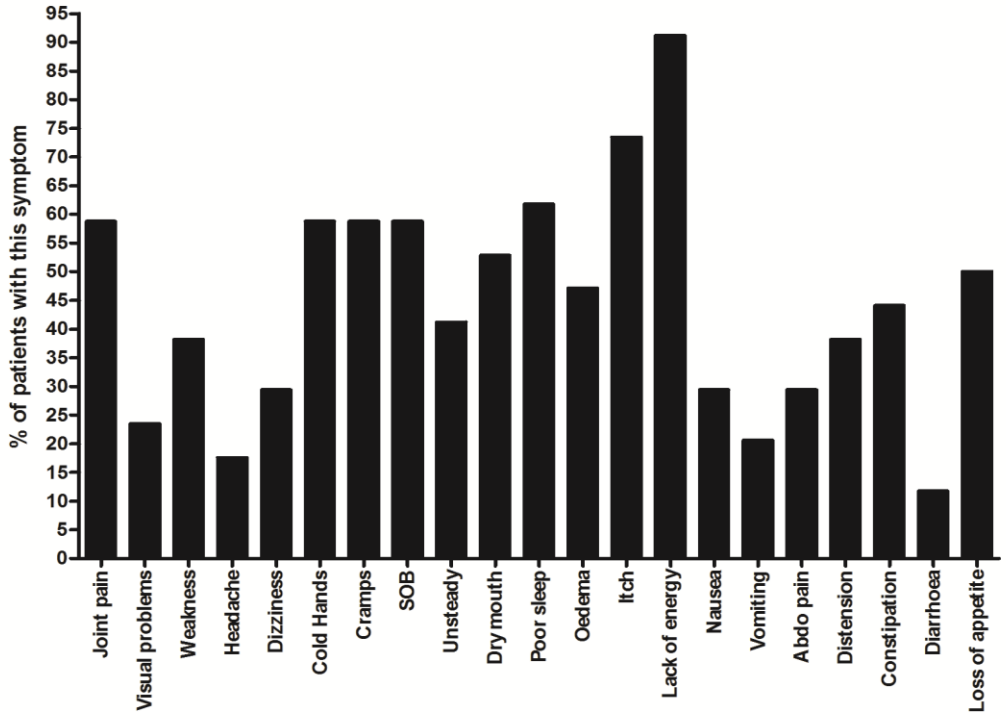


Figure 107: Percentage of patients experiencing each symptom

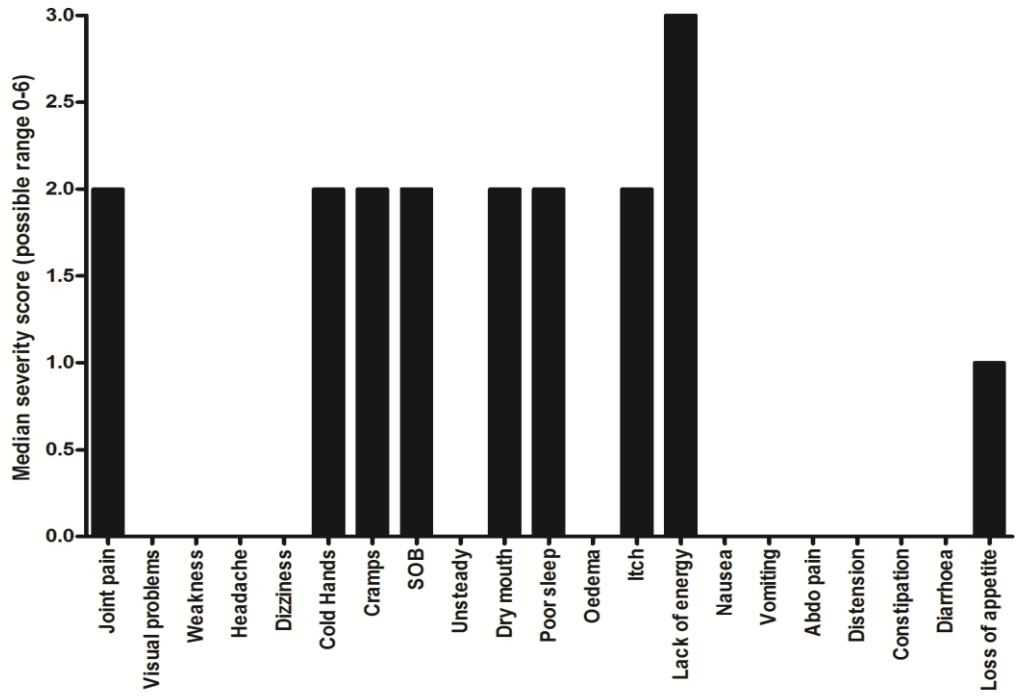


Figure 108: Median severity score for each symptom

Appendix C

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Screening for encapsulating peritoneal sclerosis in patients on peritoneal dialysis: role of CT scanning

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Abstract

Background. We previously validated a scoring system for abdominal/pelvic CT scans in patients with symptomatic encapsulating peritoneal sclerosis (EPS). CT scans of patients with symptomatic EPS were significantly different from control peritoneal dialysis (PD) or haemodialysis patient scans; scans performed before EPS was clinically evident were near normal in 9 of 13 patients. We have now investigated CT scanning as a screening modality in a larger group of patients on long-term PD.

Methods. Pre-diagnostic CT scans performed in 20 patients for routine screening or other indications at least 3 months before EPS developed, and later diagnostic scans when EPS was clinically evident, were scored by three radiologists. The control group included CT scans of 20 PD patients who had not developed EPS (median follow-up 2.25 years). Analysis was by non-parametric tests. CT scores ranged from 0 to 22; >2.5 was considered abnormal.

Results. Clinical EPS only developed after transplantation or transfer to HD. Diagnostic scans scored significantly higher than pre-diagnostic or control scans (median scores 9, 2 and 1; $P < 0.001$), confirming previous work. The pre-EPS diagnosis of 12 asymptomatic patients had a median CT score = 1.75, similar to the control group. Eight patients

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had had a limited episode of abdominal symptoms (seven required hospitalization), but did not have the clinical picture of EPS; their median CT score was 4.5 (P = 0.0016 cf control group). The time from pre-diagnostic scan to clinical EPS (median 0.82 years) and duration of PD at time of pre-diagnostic scan (median 7.1 years) did not differ significantly between the symptomatic and asymptomatic groups. **Conclusions.** CT screening of asymptomatic PD patients is not indicated; EPS may occur within a year or less of a normal CT scan. Abdominal symptoms in long-term PD patients can be associated with CT scan abnormalities; these patients are at increased risk of EPS after stopping PD.

Keywords: CT scan; encapsulating peritoneal sclerosis; peritoneal dialysis; screening

Introduction

Encapsulating peritoneal sclerosis (EPS) is a devastating complication of long-term peritoneal dialysis. The incidence is known to increase with the duration of peritoneal dialysis. The early Japanese registry data found a very low incidence of EPS, with 0.7% at 5 years on peritoneal dialysis, 2.1% at 8 years and 5.9% after 10 years [1]. The recent Scottish EPS registry data following a cohort of 1238 peritoneal dialysis patients found an incidence of 8.1% after 4–5 years on peritoneal dialysis [2]. A diagnosis of EPS carries a substantial mortality—53% of 111 patients died of the condition in the Pan-Thames EPS study [3]—as well as morbidity related to malnutrition and sepsis.

In the Pan-Thames study, 59% of patients had already discontinued peritoneal dialysis before the diagnosis of EPS was made; in the series of Kawanishi *et al.*, 68.8% had discontinued PD [1]. These studies suggest that there is a group of patients on PD for 4 years or more who are at moderate (perhaps around one in 12) risk of developing EPS after stopping peritoneal dialysis. Currently, we have no way of identifying which long-term PD patient will go on to develop EPS. Identifying those at particular risk of EPS earlier in their time on dialysis might allow earlier renal replacement modality change, which is desirable since the mortality of EPS increases with the duration of peritoneal dialysis [3]. It would also facilitate appropriate counselling prior to transplantation since EPS has occurred after transplantation [4]. Patients are often reluctant to consider an elective modality change to haemodialysis, and a reliable screening test would allow those at low risk to continue on PD, while those at high risk could be advised to change to a different form of renal replacement therapy. CT scanning has not previously been fully evaluated as a screening test, although it is useful at the point of diagnosis of EPS [5].

There are no validated biochemical markers as yet to predict a high risk of EPS, although several have been suggested. Examples are matrix metalloproteinase-2 [6] and CCL18 [7]. Sampimon *et al.* investigated the utility of the combined measurement of IL-6 and CA125 in a group of 11 EPS and 31 control PD patients. The specificity of the combined measurement was 89%, but the sensitivity was only 70%, and these markers require validation in a larger cohort [8]. Some clinical features appear to be associated with a higher risk of developing EPS: a high and increasing membrane transport status, ultrafiltration failure, and severe episodes of peritonitis. However, membrane transport characteristics may simply reflect increased duration of peritoneal dialysis, and EPS has occurred in patients with no history of peritonitis [9].

Radiological screening is therefore of interest. One small study demonstrated a possible role for fluorodeoxyglucose positron emission tomography in the early detection of peritoneal inflammation [10], but this involved only a small number of patients, and the test is not universally available. CT scanning has been shown by our group [5] and by Vlijm *et al.* [11] to be both sensitive and specific at the point of diagnosis of EPS, when the patient has developed obstructive symptoms. However, we noted that CT scans were near normal in a small group of 13 patients who had had a CT scan 4 months or more before the diagnosis of EPS was made. As we have now seen further cases of EPS, we have extended these observations to a larger group of patients. Our aim was to determine whether CT scans can be used to screen peritoneal dialysis patients to identify those with a high risk of EPS.

Materials and methods

We defined EPS as a clinical syndrome of vomiting, ascites, anorexia, weight loss, abdominal pain and distension occurring in a patient with a history of long-term PD. The majority of our patients were managed conservatively, and most did not undergo laparotomy. We concur with the recent ISPD position paper; although laparotomy/laparoscopy is the only way to make a definitive diagnosis of EPS, it is associated with significant morbidity and mortality and is usually not performed [12].

Within our database of all patients with EPS, there were 20 PD patients who had had a 'pre-diagnostic' CT scan performed 3 months or more prior to developing the clinical features of EPS. All 20 of these patients had also had a CT scan at the time of diagnosis of EPS. All patients developed EPS after transfer from PD to haemodialysis or post-transplantation. The median time from cessation of PD to the development of clinical features of EPS was 4.7 months (range 0.2–36 months). Further demographic and clinical information is given in Table 1.

We reviewed the notes, electronic patient records, discharge summary database and CT request forms to establish the indication for the 'pre-diagnostic' CT scans in the group of patients that subsequently developed EPS. Twelve patients were formally documented to be asymptomatic at the time of the scan, which was performed for screening in long-term PD, as part of the work-up for transplantation or to investigate an unrelated condition such as a retroperitoneal haematoma. These patients developed symptoms of EPS at a median of 7.9 months after the 'pre-diagnostic' scan (range 3.4–32 months). Eight patients had had a transient episode of abdominal symptoms—constipation, pain, nausea or vomiting—which had resulted in a brief hospital admission, and which prompted the CT scan. All eight patients recovered from these symptoms and did not develop the severe, persistent features of EPS for a median period of another 11.6 months (range 5–37 months).

We used a historical 'control' group of 20 patients who had had a CT scan for various reasons such as screening in a long-term PD patient, investigation of possible malignancy, and fever of unknown origin (this group was described in our previous study). The median duration of PD in the control group at the time of the CT scan was 3.2 years; the patients in this group were followed up for a median duration of 2.5 years following the CT scans. Most had changed dialysis modality (10 to haemodialysis and 4 received transplants); 6 patients died while on peritoneal dialysis. None had developed EPS in this more prolonged follow-up period.

Table 1. Clinical characteristics of the EPS patients

	Diagnosis	Duration of PD (years)	RRT after PD	Symptoms of EPS	Surgery	TPN?	F/U since EPS (months)	Current status
1	GN	12.2	Cad Tx	Ascites, pain, vomiting	No	No	43.9	1–2 admissions/year with SBO
2	Unknown	11.4	HD	Weight loss, ascites, vomiting	No	No	2.6	Died (EPS)
3	DM	6.9	Cad Tx	Weight loss, ascites, vomiting	No	Yes	9.7	Died (EPS)
4	Membranous	7.2	Cad Tx	Pain, vomiting	Yes	No	61.1	1–2 admissions/year with SBO
5	DM	5.3	HD	Weight loss, vomiting	No	No	22.0	Died (non-EPS)
6	IgA	8.7	HD	Weight loss, pain ascites	No	No	47.4	Well
7	GN	6.7	Cad Tx	Ascites, vomiting	No	Yes	31.7	Well
8	Hypertension	5.3	HD	Ascites, vomiting	No	Yes	27.3	Well
9	DM	5.0	HD	Ascites, vomiting, pain	No	Yes	3.8	Died (EPS)
10	DM	7.0	HD	Ascites, vomiting, pain	No	No	22.1	Well
11	GN	8.0	HD	Ascites, vomiting, pain	No	Yes	24.0	TPN dependent
12	GN	10.4	HD	Weight loss, pain, vomiting	Yes	Yes	9.4	Died (EPS)
13	Unknown	4.2	Cad Tx	Ascites, vomiting, pain	No	Yes	56.1	Well, but limited diet
14	Reflux	7.3	LRTx	Ascites, vomiting, pain	No	Yes	29.6	TPN dependent
15	Alport's	6.2	LRTx	Ascites, vomiting, pain	No	Yes	27.6	TPN dependent
16	Goodpasture's	8.0	HD	Ascites, vomiting	No	Yes	28.9	Died (EPS)
17	GN	6.6	HD	Ascites, vomiting	Yes	No	11.3	Died (EPS)
18	PPH	4.6	HD	Ascites, vomiting, pain	No	Yes	55.6	TPN dependent
19	Unknown	5.0	Cad Tx	Ascites, vomiting, pain	Yes	Yes	7.2	Died (EPS)
20	Unknown	6.2	HD	Ascites, vomiting, pain	Yes	No	80.7	5–9 admissions/year with SBO

GN, glomerulonephritis; DM, diabetes mellitus; PPH, post-partum haemorrhage; Cad Tx, cadaveric transplant; LRTx, live-related transplant; HD, haemodialysis; TPN, total parenteral nutrition; SBO, small bowel obstruction; RRT, renal replacement therapy.

CT scans were performed at the Hammersmith Hospital using a GE Lightspeed 8 slice scanner (GE Milwaukee, WI, USA) and at Charing Cross Hospital using a Siemens Somatron Sensation 16 slice scanner (Siemens, Erlangen, Germany). Intravenous contrast was routinely given (100 mL of Ultravist 300); one pre-diagnostic scan had intraperitoneal contrast. Contrast was injected peripherally at 3 mL/s, and the abdominal and pelvic images were acquired at 60 s post-contrast in the venous portal phase.

The CT scans were scored independently by the same three radiologists who participated in our original validation study, who have extensive experience of abdominal imaging in renal patients and who are familiar with the scoring system (see Table 2). Our previous work showed good interobserver variability using this scoring system, with kappa values of 0.4–0.75 (moderate to substantial agreement) for peritoneal thickening, peritoneal calcification, bowel tethering and bowel dilatation; kappa values of 0.2–0.4 (fair agreement) were found for a number of loculations and peritoneal thickening. We used this system to score the 'pre-diagnostic' CT scans of the 20 patients who had had CT scans prior to the development of symptomatic EPS. We compared these 'pre-diagnostic' scores with those of the 20 'control' patients who did not develop EPS.

The data were analysed using SPSS version 17 and GraphPad Prism 4. Non-parametric statistics were used throughout the analysis. The Mann–Whitney *U*-test was used to compare CT scores between groups of patients; ROC analysis was used to examine the specificity and sensitivity of CT scanning as a screening tool for future EPS.

Results

There was a significant difference in the CT scores of control PD patients and patients with a clinical diagnosis of EPS, as we found in our previous study ($P < 0.001$). The median CT scores were 1 in the control group, 2 in the pre-diagnostic group and 9 at the time of diagnosis of EPS (Figure 1).

Although statistically the pre-diagnostic scans scored more highly than the control group ($P = 0.003$), in clinical practice, the high degree of overlap between these two groups makes it unlikely that a screening CT scan would be useful. A score of 1 or 2 was common in both the control and pre-diagnostic groups.

As EPS is associated with abdominal symptoms, we divided the pre-diagnostic group into two sections, determined by whether there was any history of abdominal symptoms at the time of the CT scan. Twelve patients were entirely asymptomatic, and the scan was performed either as screening, pre-transplant work-up or as part of the man-

Table 2. CT scoring system

Score	Peritoneal thickening	Peritoneal calcification	Loculations	Bowel wall thickening	Bowel dilatation	Bowel tethering
0	Not identified	Not identified	Not present	Not identified	Not identified	Not present
1	Localized area <20%	Localized area <20%	<3 locules	Localized area	Localized area	Mild tethering
2	>20%	>20%	3–6 locules	>20% of bowel	>20% of bowel	Moderate tethering
3	>50%	>50%	>6 locules	>50% of bowel	>50% of bowel	Marked tethering
4	>80% of peritoneum	>80% of peritoneum		>80% of bowel	>80% of bowel	

The maximum score is 22, and scores above 2.5 are abnormal.

agement of an unrelated condition. Eight patients had experienced a brief bout of abdominal symptoms which had prompted the CT scan. The duration of PD in both the symptomatic and asymptomatic groups was a median of 7.1 years. The time from the date of the scan to the diagnosis of EPS was not significantly different between the symptomatic and asymptomatic groups (11.6 months vs 7.7 months, P = 0.238) and, interestingly, was actually longer in the group who had had mild abdominal symptoms. This confirms the transient nature of these symptoms—the symptomatic group was not experiencing persistent, sustained abdominal problems, which should have elicited an earlier diagnosis of EPS.

When the CT scores were shown by symptom status (Figure 2), there was a significant difference between the asymptomatic patients and the patients who had had an episode of symptoms (median scores 1.75 and 4.5, respectively, P = 0.03).

Scores from patients with minor abdominal symptoms were significantly different from those of control PD patients (median scores 4.5 vs 1; P = 0.0016), but in the asymptomatic group, CT scan scores did not distinguish the pre-diagnostic patients from the control PD patients (median scores 1.75 vs 1).

Are abdominal symptoms predictors of EPS?

It is possible that the presence of symptoms alone is a good predictor of future EPS, without any useful information being gained from a CT scan. We therefore identified a group of eight patients who had abdominal symptoms at the time of their CT scan but who subsequently did not develop EPS. Four were in our original control group, and we also identified a further four PD patients who had a CT scan for abdominal symptoms without subsequently developing EPS. The indications for CT scanning and CT scores for these patients are shown in Table 3. When compared with the abdominal symptom group who did go on to develop EPS, the scores in the group that did not develop EPS were lower (median score pre-EPS = 4.5 and median score symptomatic but no EPS = 1; P = 0.02). This suggests that in symptomatic patients, a CT scan may aid the assessment of future EPS risk.

When all the pre-diagnostic scans were considered together, both the sensitivity and specificity to predict future EPS were 0.7 with a cut-off score of 1.5. Increasing the cut-off score to ≥2.5 increased the specificity to 0.9, but the sensitivity was only 0.45 (just 9 out of 20 of our pre-diagnostic patients would have met this criterion). Figure 3 shows the ROC curve for screening with CT scanning in all pre-diagnostic patients, which is clearly far from ideal.

Discussion

An increased awareness of the risk of EPS as a complication of long-term PD has engendered considerable debate regarding the optimal management of patients who remain on PD for extended periods of time. The recent ISPD guidelines emphasize the need to arrange for early transplantation [12]. Where transplantation is not an option, the clinician and patient should discuss the relative risks of remaining on PD or transfer to haemodialysis, balanced with quality of life issues. Thus, for an elderly, frail patient, a decision might be made to remain on PD, especially since EPS seems

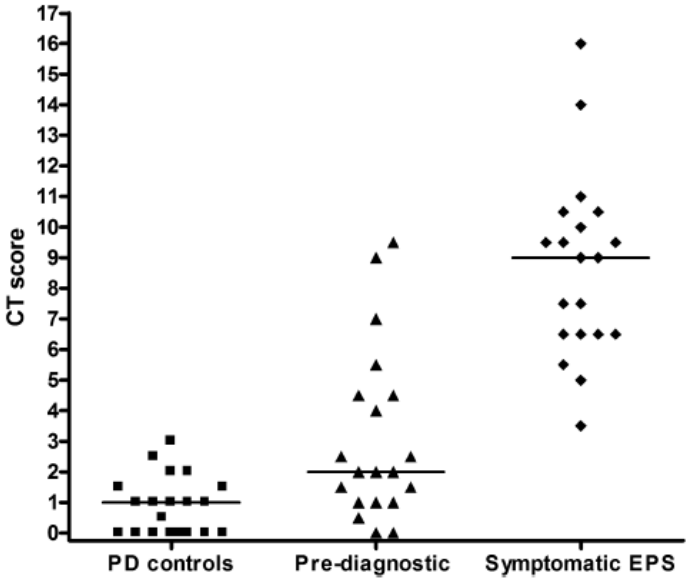


Fig. 1. CT scores in PD controls, pre-diagnostic scans (>3 months before the diagnosis of EPS) and scans performed at the time of presentation with the symptoms of EPS. Bars indicate median score.

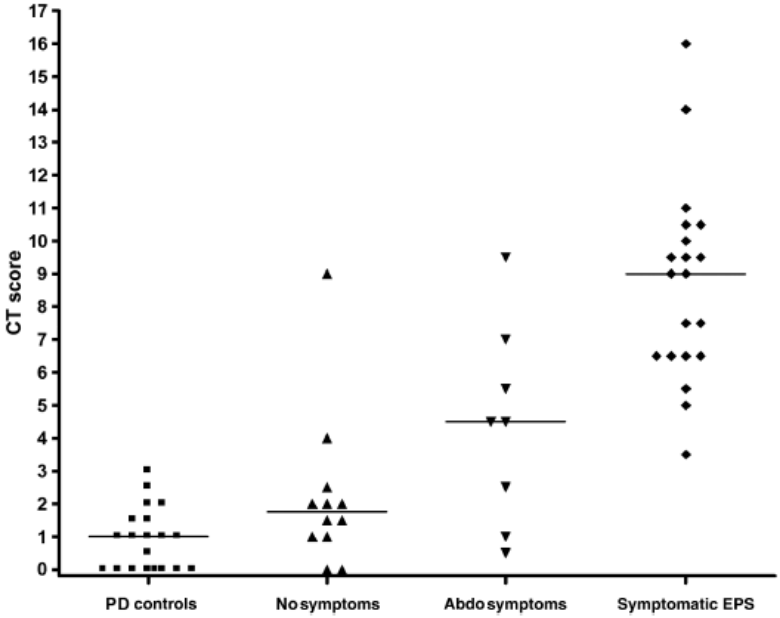


Fig. 2. CT scan scores for patients with (median score 4.5) and without (median score 1.75) minor abdominal symptoms. CT scores from control PD patients and patients with EPS are shown for comparison.

less likely to be symptomatic while the patient continues PD. Real-life clinical decision making is frequently more difficult, however. Younger patients on long-term PD cannot expect PD to provide adequate renal replacement therapy indefinitely—the incidence of membrane failure increases with duration of PD [13]—and hence will be exposed to a risk of EPS in the future when they require an alternative renal replacement modality. If early transplantation is not possible, the timing of transfer to haemodialysis becomes an important decision for both patient and clinician. There is a lack of well-validated biomarkers to predict EPS risk in the individual patient, so CT scanning has been used to attempt to detect early changes in the peritoneum that might indicate a higher risk of EPS and guide whether PD can be safely continued.

In this study, we have shown that CT screening for early changes of EPS in asymptomatic PD patients is not useful; 10 of 12 patients had CT scores in the same range as the control group but went on to develop EPS within a period of months (median 7.7 months). In only 2 of 12 patients who were completely asymptomatic on PD were the screening CT scans clearly abnormal.

In contrast, in the group of patients with documented minor episodes of abdominal symptoms, the CT scan was more likely to be abnormal. Using a cut-off score of 2.5, six of eight patients would have had an abnormal scan. However, the number of patients in this group is small. We emphasize that these symptoms were not severe and were those commonly seen in PD patients, such as constipation. The episode of symptoms was transient in nature (as evidenced by the time from the scan date to diagnosis of EPS being longer than in the asymptomatic group).

Abdominal symptoms alone, however, cannot be used as a predictor for EPS. CT scan scores in a group of patients with abdominal symptoms who did not develop EPS were lower than in the symptomatic pre-EPS group.

There are some drawbacks to our data—the study is retrospective in nature and thus is subject to confounding. Our control patients had not been on peritoneal dialysis for the same length of time as the EPS group (3.2 vs 7.1 years, $P < 0.001$), and we cannot exclude the possibility that the changes seen in the pre-diagnostic scans are simply related to duration of dialysis and do not represent pre-EPS abnormalities. However, the fact that the abnormalities in these scans are similar (although less severe) than the abnormalities seen in EPS patients suggests that there is a progression from near-normal scans to very abnormal scans at the time of symptomatic

Table 3. Indications for CT scans and scores in symptomatic patients who did not develop EPS

Nature of symptoms	CT scan score
Abdominal pain	0
Change in bowel habit	1.5
Weight loss	0
Abdominal pain	1.5
Abdominal pain, diarrhoea and vomiting	4
Abdominal pain	1
Abdominal pain and distension	1
Abdominal pain and satiety	0

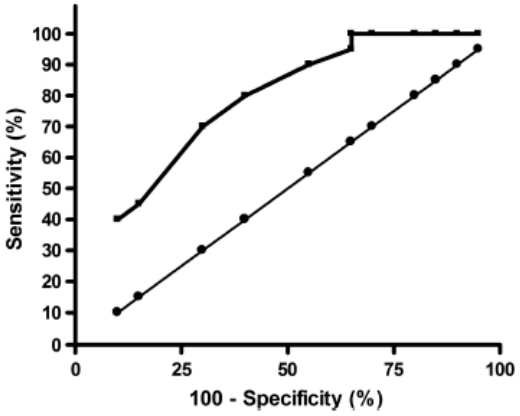


Fig. 3. ROC for CT scanning as a screening tool to predict future EPS; CT scans lack sensitivity and specificity.

EPS. The patients from the control group have largely transferred from PD to either haemodialysis or transplantation, and the duration of follow-up suggests that it is now unlikely that any of the control patients will develop EPS.

In summary, CT scans performed as a screening tool for EPS are not sufficiently sensitive in asymptomatic PD patients. Patients with even minor and transient abdominal symptoms who have an abnormal CT scan may be at an increased risk of EPS post-cessation of peritoneal dialysis. The challenge clinically is how to counsel such patients about transplantation or changing to haemodialysis.

Conflict of interest statement. E.A.B. has received speaker and consultancy fees from Baxter Healthcare. The results presented here have been published only in abstract form (Renal Association 2009).

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Appendix D

Encapsulating Peritoneal Sclerosis: What Have We Learned?

Catriona Goodlad, MBBS, and Edwina A. Brown, DM, FRCP

Summary: Encapsulating peritoneal sclerosis (EPS) is a rare complication of peritoneal dialysis (PD), but carries significant morbidity and mortality. We review the clinical features and radiologic and histologic changes found at diagnosis of EPS. Although EPS is strongly associated with the duration of PD, the pathogenesis remains only partly understood. We discuss the mechanisms thought to underlie the abnormally thickened, sclerotic peritoneal membrane seen in long-term PD patients including epithelial to mesenchymal transition and the molecular mediators of fibrosis and angiogenesis. We review how exposure to high-glucose, nonphysiological dialysis fluids, peritonitis, and uremia may be responsible for these changes. Much remains to be learned about optimal management of EPS, both medical and surgical, because the literature lacks controlled studies. Future research challenges include defining the role of surgery, immunosuppression, and antifibrotic agents in the management of EPS. We also need to understand why some patients progress from asymptomatic peritoneal sclerosis to the extreme levels of fibrin deposition and bowel encapsulation seen in EPS. Screening PD patients for potential future EPS remains difficult, and we need strategies for monitoring patients on longer-term PD that enable us to better quantify the risk of EPS for the individual patient.

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Keywords: Encapsulating peritoneal sclerosis, peritoneal dialysis

Encapsulating peritoneal sclerosis (EPS) is a rare complication of long-term peritoneal dialysis (PD). Because of the high morbidity and mortality associated with this condition, nephrologists and patients have become increasingly aware about the risk of EPS after four or five years on PD, and new questions have arisen in the management of PD patients. Here we review the available data on EPS and discuss what questions remain to be answered.

DEFINITION

EPS is a clinical syndrome of repeated episodes of bowel obstruction, which presents with nausea, vomiting, abdominal distension, anorexia, and weight loss. Ascites (often blood-stained), fever, and increased inflammatory markers may be present. The macroscopic appearance of EPS is characteristic, with a thick fibrotic capsule around the bowel. Radiologic changes include peritoneal thickening and calcification, with bowel tethering and dilatation.

EPIDEMIOLOGY

Historically, EPS has been seen with the use of practolol[1] and chlorhexidine.[2] EPS has been recognized as a complication of PD for many years, and is known to occur in cirrhotic liver disease with ascites.[3]

Incidence

The incidence varies in three prospective reports of EPS in long-term PD patients. Johnson et al[4] examined the incidence of EPS in 7,618 patients in Australia and New Zealand and found an overall incidence of 1.8 per 1,000 patient-years. The cumulative incidence of EPS

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after 3, 5, and 8 years of PD was 0.3%, 0.8%, and 3.9%. Most (79%) of the patients were on PD at the time of diagnosis of EPS in this series. Apart from duration of PD, the only risk factor that remained significant in multivariant analysis was age, with EPS being more common in younger patients (<50 y). The rates of PD peritonitis were low in the EPS population (which may have contributed to a more prolonged technique survival and higher risk of EPS overall). In the Japanese EPS registry, the overall incidence was 2.5%.[5] A clear relationship with the duration of PD was again seen: the incidence of EPS after 3, 5, 8, 15, and longer than 15 years on PD was 0%, 0.7%, 5.9%, 8.6%, and 17.2%. The mean duration of PD before EPS was 114.3 months (range, 36–201 mo) and the majority (68%) of cases were diagnosed after PD cessation. In the United Kingdom, the Scottish registry data found a similar pattern of increasing incidence with PD duration; although the rate at 4 to 5 years was high at 8.1%.[6] Most patients (72%) were diagnosed after cessation of PD with transfer to hemodialysis (HD) or transplantation. EPS is well recognized after renal transplantation.[7]

Outcomes

In the Australian study 18 of 33 EPS patients died; 7 were considered to have died of EPS. The mortality at 4 years was similar to that of matched dialysis control patients. However, the median duration of PD at the time of diagnosis was lower than reported elsewhere. It may be that early diagnosis and less severe disease contributed to better survival rates in this study.[4] The Japanese data show that mortality from EPS increases significantly with the duration of the preceding PD.[5] No patients who had performed PD for 5 years or less died of EPS, but after 8 years of PD the mortality rate was 8.3%, increasing to 28.6% after 10 years, 61.5% at 15 years, and 100% in the group who had been on PD for more than 15 years. A similarly poor prognosis was reported by the Scottish renal registry group; in their series the mortality rate of EPS was 42% at 1 year after diagnosis. The Pan-Thames group[8] looked retrospectively at 111 patients with EPS. Again, mortality increased with the length of time on PD; overall, 53% of patients died.

DIAGNOSIS

The diagnosis of EPS can be challenging; in renal replacement therapy patient weight loss may be masked by fluid gain and other diagnostic possibilities must be excluded (Table 1).

Clinical Features

The key clinical features are symptoms of obstructive ileus: vomiting, pain, and distension, often developing insidiously. A systemic inflammatory response also may be present, with fever, increased C-reactive protein level, and hypoalbuminemia. In patients still on PD, there may be blood in the effluent dialysate. Peritoneal thickening, encapsulation, and calcification may be confirmed radiologically.[9] Because EPS frequently presents after withdrawal from PD (the Kawanishi series finding EPS developed after cessation of PD in 68.8-94.0% of patients[10,11]) and the time from cessation of PD until the development of EPS has been reported as up to 5 years,[11] a prolonged period of clinical vigilance is required in long-term PD patients who change dialysis modality or receive a renal transplant. In some patients there appears to be a prodromal period associated with nonspecific symptoms and evidence of inflammation with a high C-reactive protein level, but this is by no means uniform, and in the majority of patients the onset of symptoms can be very rapid (eg, within a few weeks of stopping PD).

It is important to try to rule out alternative treatable causes of abdominal symptoms. Conditions that may present in a similar manner to EPS include malignancy (especially with peritoneal deposits) and mycobacterial disease. Repeated-interval imaging and microbiological/cytologic analysis of ascitic fluid might change the working diagnosis. Laparoscopy, with both histology and microbiology samples, may be required if there is significant diagnostic dilemma, but is not without risk (bowel may be adherent to the anterior abdominal wall, mandating careful review of imaging before undertaking laparoscopy).

Table 1. Diagnosis of EPS

Duration of PD	Incidence increases after 5 years (risk of 5%-8%); highly unlikely if <3 y
Peritonitis	EPS can be precipitated by severe peritonitis but can occur in patients with no peritonitis history
Residual renal function	Uncommon if significant urine output
Previous PET tests	Patients with poor UF are more at risk; not all patients with EPS will be high transporters
Length of time since stopping PD	Less likely the longer the time period since stopping PD (mean interval, 4.5 mo, maximum, 4-5 y)
Abdominal symptoms	Consistent with bowel obstruction: pain, vomiting, distension; weight loss is likely
Other potential causes of symptoms	Rule out other possible causes of obstruction and/or bowel dilatation such as postoperative ileus, infections such as *Clostridium difficile* or tuberculosis and malignancy
Ascites	May be a feature of EPS with associated CT scan changes; pleural effusions are not a feature of EPS (but may complicate loss of flesh weight in a HD patient)
CT scan	Seek expert review Bowel dilatation alone is not sufficient to diagnose EPS; other features such as bowel wall and peritoneal thickening and bowel tethering also will be present; peritoneal calcification is a nonspecific sign
Surgical findings	A fibrous cocoon wrapped around bowel is diagnostic; a thickened peritoneal membrane and intra-abdominal adhesions are common in long-term PD and after peritonitis and are therefore not diagnostic
Histology	Findings are nonspecific and overlap with membrane changes occurring in long-term PD with UF failure and infectious peritonitis

Peritoneal Membrane Function and Ultrafiltration

As well as considering the duration of PD, it may be helpful to review the peritoneal equilibration test (PET) results to assess the prior likelihood of EPS because patients with clinical evidence of peritoneal membrane damage seem particularly at risk of developing EPS. In the Stoke PD cohort, nine patients who had a diagnosis of EPS were shown to have had lower ultrafiltration (UF) capacity and greater glucose exposure (with less residual renal function) than 683 non-EPS patients during their final years on PD. However, dialysate to plasma creatinine ratios were higher than the non-EPS group only at the time of PD cessation.[12] The decrease in UF capacity in EPS patients was therefore disproportionate to the changes in peritoneal solute transport rate. Lambie et al[12] hypothesize that this loss of UF capacity may be owing to the effect of fibrosis on the membrane hydraulic conductance, and might be used as a marker of increased EPS risk. Another study from The Netherlands showed a high risk of EPS (50%) in patients who continued on PD for more than 3 years after the development of UF failure. In this group with UF failure, further more detailed analysis of peritoneal membrane transport characteristics did not help to predict which patient would develop EPS. The only potential marker was a low effective lymphatic absorption rate in the pre-EPS group, but this test requires the use of an intraperitoneal volume marker such as dextran 70 and is not performed routinely at most centers.[13] Regular PET tests during PD may thus at least help to identify a population with markedly impaired UF who are at higher risk of EPS. These patients could be considered for elective withdrawal from PD, especially because they are likely to develop clinical symptoms related to UF failure, and should be kept under close clinical review

both during and after PD. Case reports suggest that severe peritonitis (especially fungal) may precipitate EPS[14]; patients who cease PD in the context of severe peritonitis also might be considered high risk.

Radiologic Findings

Prolonged contrast transit may (but not always) be seen on small-bowel studies[15]; bowel loops may appear fixed in position. Magnetic resonance imaging with oral contrast can show bowel wall thickening.[16] Fluorodeoxyglucose positron emission tomography scanning showed peritoneal enhancement in the early stage of EPS in three patients; later in the course of the disease there was less peritoneal enhancement,[17] but neither of these imaging modalities are widely available. The imaging modality of choice in suspected EPS is computed tomography (CT) scanning. A series of 10 patients with EPS after continuous ambulatory peritoneal dialysis was described in 1998[18]; peritoneal thickening and calcification, bowel tethering, and loculated fluid frequently were seen. Tarzi et al[19] performed a study validating the use of CT scans to diagnose EPS. More than a hundred scans from control patients on PD and HD, and patients with EPS, were scored in a blinded manner by three radiologists. Interobserver consistency was high for bowel wall thickening, bowel tethering, bowel dilatation, and peritoneal calcification (Fig. 1). CT scores at the time of diagnosis of EPS were significantly higher than the scores of PD or HD patients without EPS, indicating that CT can be a useful adjunct to the diagnosis of EPS. Vlijm et al[20] in a case-control study of CT findings in EPS found similar characteristic CT abnormalities.



Figure 1. CT scan showing ascites, bowel dilatation, bowel wall thickening, peritoneal thickening, and bowel tethering.

They noted that the sensitivity and specificity of CT were higher when a radiologist with PD experience reviewed the images, but CT remained highly specific even without this advantage. The control patients in this study had all been on PD for a minimum of 4 years, showing that the CT changes seen at the time of development of EPS were not simply related to PD duration.

Macroscopic Findings

The laparoscopic findings of a thick membrane encapsulating the bowel are typical of EPS and appearances at surgery are the gold standard in the diagnosis of EPS. However, many patients do not undergo surgery; in these patients the diagnosis is made on the basis of clinical and imaging features.

Histology

Peritoneal biopsy specimens can be obtained at the time of catheter removal or insertion, or transplantation. EPS, however, is a disease primarily of the visceral peritoneum whereas biopsy specimens usually are taken from the parietal peritoneum. Biopsy studies in stable PD patients have shown mesothelial denudation, submesothelial interstitial fibrosis, and vasculopathy.[21-23] The thickness of the submesothelial zone increases progressively with the duration of PD.[22] Venules show hyalinization and the lumen may be obliterated; the severity of the vasculopathy also increases with PD duration.[22] Inflammation is not a prominent feature. These changes are often termed *peritoneal sclerosis* and are not associated with abdominal symptoms, although there may be changes in membrane transport status and UF capacity. Patients with EPS develop further visceral changes with extensive fibrin deposition and macroscopic bowel encapsulation, which results in the obstructive clinical features. Biopsy findings in asymptomatic PD patients and patients with EPS may be qualitatively similar; Honda et al[24] reviewed the findings in 69 peritoneal biopsy specimens including 12 from patients with EPS and compared these with non-EPS patients. The peritoneum of EPS patients showed formation of a fibrinous capsule. There was perivascular bleeding and capillary angiogenesis. An infiltrate of inflammatory mononuclear cells was frequently observed. Fibroblasts were swollen and proliferating. All of these changes could be seen in patients without EPS, although the frequency with which the abnormalities were observed, and also the severity of the lesions, was higher in patients with EPS. Immunohistochemistry was used to investigate the expression of markers including α-smooth muscle actin (α-SMA), fibroblast growth factor, and macrophage migration inhibitory factor. Although the expression of these markers was more prominent in the biopsy specimens from patients with EPS, these markers all could be found in the biopsies from non-EPS patients. Because biopsy findings from a limited region of the parietal peritoneum may not always be representative, and the changes in EPS overlap with findings in non-EPS patients, it may be difficult to distinguish EPS from uncomplicated peritoneal sclerosis on biopsy features alone. The overall clinical picture and macroscopic appearance of the bowel at laparoscopy may be more helpful in the confirmation of the diagnosis of EPS.[21]

PATHOGENESIS OF EPS

Epithelial to Mesenchymal Transition: The First Step In Peritoneal Membrane Damage

Within the peritoneal membrane, epithelial to mesenchymal transition (EMT) is one of the earliest histologic changes in the peritoneum and is a critical step in the development of an abnormal membrane. EMT is the process, first shown in cultures of mesothelial cells derived from PD effluent,[25] by which the peritoneal mesothelial cells lose their tight junctions, adherent junctions, desmosomes, and cell polarity. The cytoskeleton is reorganized and cells begin to express mesenchymal markers. Upregulation of matrix metalloprotein production permits degradation of the basement membrane and migration of mesothelial cells into the interstitium.[26] The morphology of effluent cell cultures is variable, with more nonepithelioid features found in cultures of cells taken from patients with a longer PD duration, but both mesothelial and fibroblast-type cells ex-

press high levels of intercellular adhesion molecule-1, indicating a mesothelial origin (intercellular adhesion molecule-1 is not normally expressed by fibroblasts). EMT can occur within the first 2 years on PD, before the development of vasculopathy, which is consistent with the hypothesis that EMT is one of the first steps in peritoneal sclerosis. The presence of EMT has been associated with a high peritoneal transport rate,[27] indicating a link to the clinical manifestations of membrane damage. EMT may underlie the appearance of myofibroblasts (a source of inflammatory cytokines and extracellular matrix constituents) in the peritoneum; this population of cells expressing α-SMA are not seen in the normal peritoneum but can be seen in biopsy specimens from PD patients.[28] EMT also is linked to the vascular changes seen in chronic peritoneal sclerosis; transdifferentiated mesothelial cells produce vascular endothelial growth factor (VEGF),[29] which is involved in peritoneal angiogenesis.[30]

There may be more than one stimulus for EMT; candidates include transforming growth factor-β (TGF-β), angiotensin II, fibroblast growth factor, and connective tissue growth factor (CTGF).[31] Overexpression of TGFβ-1 in the rat peritoneum using an adenovirus vector was shown to induce EMT; by day 7 cells could be seen that expressed both cytokeratin and α-SMA.[32] In uremic rats the degree of EMT was reduced when the rats were treated with antibodies to the receptor for advanced glycosylation end products (AGE),[33] linking EMT to dialysate containing glucose degradation products that lead to the formation of AGE. The precise molecular pathways that mediate EMT are the subject of investigation and are likely to be multiple; this rapidly developing area was reviewed recently.[31]

What Triggers Severe Fibrin Deposition and Symptomatic EPS?

The early histopathologic changes of EMT and membrane thickening, frequently seen in patients on long-term PD, clearly do not always progress to EPS. It remains unclear why some patients develop the gross fibrin deposition of EPS and become symptomatic. One possibility would be genetic variation in the molecular mediators of the fibrosis pathway. Investigation of this hypothesis would be aided by an international EPS registry and DNA bank[34] because large numbers of cases would be required. Another potential explanation is the "two-hit" model proposed by Honda and Oda,[21] in which a denuded, thickened peritoneum as a result of long-term PD undergoes a second, inflammatory insult. The severity of the second insult might be minor in patients with pre-existing severe peritoneal damage, whereas patients with only mild chronic changes would require a much stronger inflammatory stimulus to develop EPS. Fibrin deposition would occur more easily after the cessation of PD because there would be no washout of fibrin. Abnormal blood vessels might be more prone to leak fibrin in response to an inflammatory stimulus. It is unclear to what extent the processes of fibrosis and angiogenesis are linked. In a rat model of PD, Margetts et al were able to reduce fibrosis selectively using the TGF-β inhibitor decorin, and angiogenesis using angiostatin,[35] suggesting that to at least some extent these processes can proceed independently.

HOW DOES PD ITSELF PROMOTE PERITONEAL MEMBRANE CHANGE AND EPS?

Uremia

The peritoneal biopsy study of Williams et al[22] showed that uremia itself, in the absence of PD, can cause thickening of the mesothelial zone. Overt vasculopathy was noted in 20% of uremic non-PD patients (and none of the nonuremic samples, taken from kidney donors). Chronic uremia induces permeability changes in the peritoneum perhaps mediated by changes in nitric oxide synthase (NOS) expression[36] or increased carbonyl stress.[37]

Glucose

It is highly likely that the use of glucose as an osmotic agent is an important factor in the development of long-term changes in the peritoneal membrane. Data from the European APD Outcomes study showed more rapid changes in membrane transport rate and decline in UF ca-

pacity in patients who had greater glucose exposure.[38] Hyperglycemia can induce TGF-β and monocyte chemotactic protein-1 (MCP-1) production from mesothelial cells; this may occur by the induction of aldose reductase and the intracellular accumulation of sorbitol via the polyol pathway (known to be implicated in the generation of diabetic complications).[39] The metabolism of glucose results in a high ratio of reduced to oxidized nicotinamide adenine dinucleotide, which is a characteristic feature of cellular ischemia (pseudohypoxia) and promotes VEGF production and angiogenesis.[40]

High levels of glucose promote the nonenzymatic glycosylation of proteins to form stable structures called AGEs. Advanced glycosylation products have long been known to be formed in dialysate[41] and are deposited in the peritoneal membrane of patients undergoing PD.[42] AGEs are implicated in neoangiogenesis and VEGF release from mesothelial cells.[43]

In addition, the heat sterilization of glucose-containing PD fluids produces glucose degradation products (GDPs) such as methylglyoxal, 3 deoxyglucosone, and acetaldehyde, which may have toxic effects. Reactive carbonyl compounds are cytotoxic and GDPs promote the formation of AGE. Methylglyoxal was investigated in a rat model and shown to increase production of matrix metalloproteinase, collagen IV, and VEGF.[44] The degree of fibrosis was marked with higher concentrations of methylglyoxal and these rats even showed formation of an abdominal cocoon. Although the concentrations used experimentally were higher (20 mmol) than those found in PD fluids (range, 2-33 μmol), the duration of exposure was much shorter. Increases in TGFβ, VEGF, and collagen I production owing to conventional dialysate were reduced in rats given intraperitoneal N-acetyl cysteine (an anti-oxidant) to antagonize the formation of reactive oxygen species by GDPs.[45] In contrast, Mandl-Weber et al[46] found a more marked effect on the intraperitoneal fibrinolysis system when human peritoneal mesothelial cells were exposed to glycated albumin rather than to methylglyoxal. The relative contributions of glucose degradation products, acidic pH, and nonphysiological buffers to peritoneal damage is not known, but the evidence that these factors are harmful has led to the development of newer dialysate fluids with much lower levels of GDPs, neutral pH, and bicarbonate/pyruvate buffering systems. The use of icodextrin instead of a high-concentration glucose for the long dwell also might be expected to be protective; in the European APD Outcomes study population UF was better maintained in patients using icodextrin.[47]

Peritonitis

Patients with EPS have not always been found to have a higher frequency of peritonitis during their time on PD[4] than non-EPS patients and a history of peritonitis is not always present in patients with EPS.[4,6,8] These observations may reflect the fact that duration of PD is critical in the development of the membrane abnormalities that precede EPS. Patients who were experiencing severe or frequent bouts of peritonitis might be withdrawn from PD at an earlier stage.

Although on an epidemiologic basis peritonitis rates may not seem significant, for an individual patient peritonitis episodes may accelerate the process of membrane damage. During peritonitis the release of inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α increases the basal level of TGF-β production[48] by mesothelial cells above that owing to chronic exposure to dialysate. In addition, recurrent peritonitis is associated with higher levels of inflammatory cytokines with each subsequent infection, indicating a sustained alteration in peritoneal cytokine regulation.[49] In agreement with the two-hit hypothesis, an episode of severe peritonitis may precede the cessation of PD and development of EPS[14,50] when occurring on a background of long-term peritoneal dialysis and chronic membrane change.

WHAT ARE THE MOLECULAR MEDIATORS OF FIBROSIS AND ANGIOGENESIS?

A large number of chemokines and other mediators promoting inflammation, fibrosis, and angiogenesis have been shown to have a potential role in peritoneal membrane damage. TGF-β, IL-6, and VEGF are perhaps the best established

mediators, but many others are biologically plausible and it is likely that there are many pathways to a common end point.

As described earlier, TGF-β has been implicated in EMT, the early histologic change within the peritoneum. TGF-β has been shown to cause submesothelial fibrosis, increased matrix production, and increased VEGF production using an adenovirus gene transfer model.[51] It has been difficult to show a consistent association between the levels of TGF-β in dialysate and clinical outcomes, perhaps because TGF-β is found in both an active and inactive form. Human peritoneal mesothelial cells exposed to high glucose concentrations can be shown to produce both TGF-β[48] and an increased amount of fibronectin; this increase in matrix production is blocked by anti–TGF-β antibodies.[52]

IL-6 is well recognized as a mediator of inflammation. Mesothelial cells produce IL-6 but do not express a cognate IL-6 receptor. During peritonitis, infiltrating neutrophils produce the soluble IL-6 receptor; IL-6 bound to the soluble IL-6 receptor then interacts with cells via glycoprotein 130 to modulate chemokine expression[53] and promote T-cell recruitment.[54] Even early in the course of PD, in patients who have never had peritonitis, dialysate IL-6 levels correlate with the levels of chemokines involved in chronic inflammation and angiogenesis such as MCP-1 and VEGF, as well as with the peritoneal solute transport rate.[55]

VEGF promotes angiogenesis and alters vascular permeability. VEGF production is linked to chronic hyperglycemia because mesothelial receptor of AGE activation increases VEGF levels.[43] In a rat model, vascular changes and altered membrane characteristics resulting from hyperglycemia could be prevented with an anti-VEGF antibody.[30] VEGF production is increased in mesothelial cells that have undergone EMT[29] and is attenuated by angiotensin II blockade.[56] Patients possessing VEGF polymorphisms associated with higher PD effluent levels of VEGF have higher membrane transport rates.[57]

Other mediators are likely. MCP-1 is induced by glucose from human peritoneal mesothelial cell cultures in a dose-dependent manner[58] and is implicated in the development of vascular changes in diabetes.[59] Levels of MCP-1 increase after peritonitis episodes.[60] NO has been shown to be involved in changes in membrane permeability, vascular proliferation, and inflammation; mice deficient in endothelial NOS showed reduced vascular proliferation and lower membrane transport rates in a bacterial peritonitis model.[61] Alternative NOS isoforms may have differing roles within the peritoneum, however, and inhibition of NOS also can lead to fibrosis.[62] The matrix metalloproteinases, such as matrix metalloproteinase 2, are likely to be important in EMT.[32] Other profibrotic factors include cytokines such as CTGF, which may be a downstream mediator in the TGF-β pathway and is increased in the dialysate of patients with high transporter status,[63] chemokine ligand 18,[64] and angiotensin.[65] Inhibitors of fibrinolysis, such as plasminogen activation inhibitor,[66] are up-regulated by TGF-β in human peritoneal cell culture. A summary of the pathogenesis of EPS is given in Figure 2.

MANAGEMENT OF EPS

The management of EPS is summarized in Table 2, but there is much that remains unsupported by randomized trials. In particular, the role of medical therapies and the timing of surgery are poorly defined. The evaluation of different treatment regimens is difficult because the clinical course of EPS is very variable. Some patients can have a relatively benign course with resolution of abdominal symptoms after a few weeks and others progress (often rapidly) to complete bowel encapsulation and obstruction.

Supportive Management

Bowel obstruction may require long-term nasogastric tube placement and careful attention to fluid balance. The UK EPS guidelines highlight the need for early nutritional support,[67] with the use of total parenteral nutrition (TPN) when enteral feeding fails. Significant (>10%) weight loss and hypoalbuminemia are common at the time of diagnosis of EPS.[68] Patients with prolonged poor nutritional intake are at risk of refeeding syndrome and expert dietician input is required. In severely malnourished patients there is a role for TPN for 7 to 10 days[69] before planned surgery.

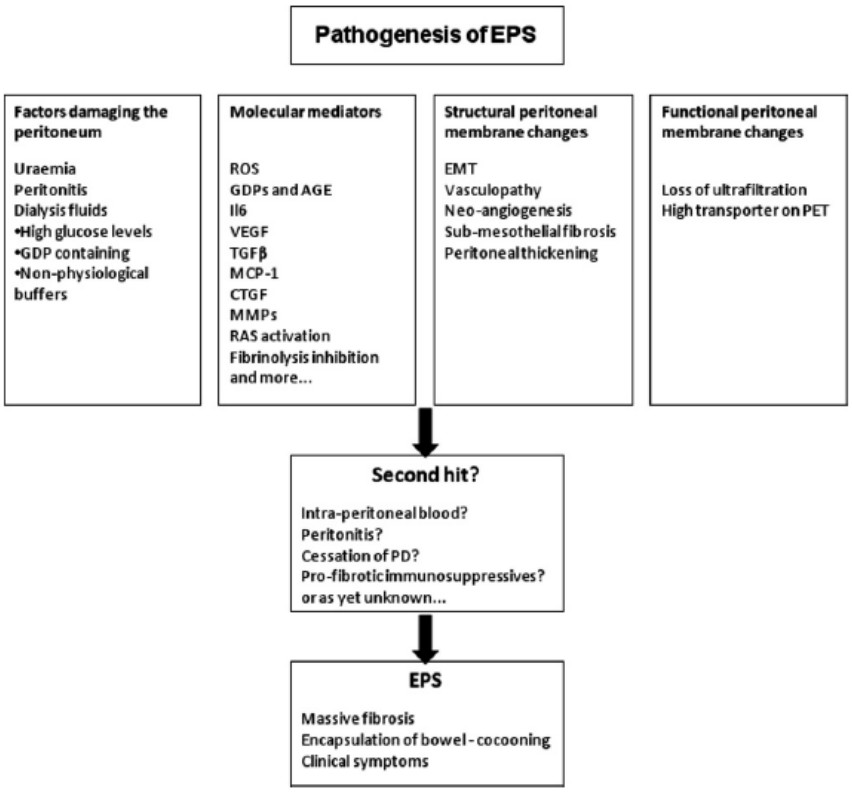


Figure 2. Pathogenesis of EPS.

Steroid and Immunosuppressive Treatment

There have been no controlled trials of corticosteroid therapy in EPS patients. Maruyama and Nakayama[70] summarized 60 articles and abstracts describing the use of corticosteroids in Japanese patients. In this group of patients, the mean initial dose used was 27.5 mg and steroids were continued for 8 months (range, 1-36 mo). Patients were stratified by phase of EPS: early ascites through to established encapsulation. The majority of patients receiving steroids early in the course of EPS achieved a good functional outcome and the mortality was low; in patients with established bowel encapsulation 50% had bowel function and mortality was 25%, which compares favorably with the historical mortality data of around 30% across all patients with EPS. Both favorable and unfavorable reports can be seen in the literature. Kuriyama and Tomonari[71] compared a group of 5 patients treated with 30 to 40 mg of prednisolone who all survived with functioning bowel, with a historical control group of 6 non-steroid-treated patients who all died of EPS. Less encouragingly, Yamamoto et al[72] described the use of steroids and outcome of EPS in 15 patients with established ileus and encapsulation. Five patients recovered function with TPN alone. Ten were treated with steroids, of whom four died. The remaining six did not improve sufficiently to avoid surgery. In the Pan-Thames study, steroids had been used in 29 of the 111 patients with EPS. No beneficial effect on survival was observed, although the investigators noted that it would be difficult to assess any steroid effect given heterogenicity of other treatments.[8] The timing of steroid use may at least partly explain variations in observed efficacy because corticosteroids would not be expected to treat established fibrosis.

Table 2. Management of EPS

Clinical diagnosis confirmed by CT or at surgery
Current PD patients
EPS can worsen on stopping PD, so consider age and comorbidities and discuss options with the patient
Change modality to HD if possible
If life expectancy is otherwise limited it may be appropriate to remain on PD
All patients
Assess nutritional status: involve renal dietician
Start oral supplements, enteral or parenteral nutrition as appropriate
Consider treatment with tamoxifen 20-40 mg/d (no randomized controlled trial evidence of benefit)
Steroid therapy is unlikely to be effective in the treatment of established fibrosis
The role of lavage remains unclear and carries the risk of infection in immunosuppressed patients
Monitor closely for sepsis
If dependent on parenteral nutrition and no improvement in symptoms after 3-6 mo consider surgery at a specialist center

The use of immunosuppression in the treatment of EPS has been questioned in the context of patients who develop EPS after transplantation. These patients clearly already are receiving immunosuppression to prevent graft rejection, yet still develop EPS.[7] Complications of surgery at the time of transplantation, such as intraperitoneal bleeding, might act as a second-hit inflammatory stimulus in at-risk patients. In addition, some immunosuppressants commonly used after transplantation might be expected to have a profibrotic effect, such as the calcineurin inhibitors. In a rat model of PD, cyclosporin exposure has been shown to increase angiogenesis, fibrosis, CTGF, VEGF, and TGF-β.[73]

Tamoxifen

Tamoxifen has been used as an antifibrotic in retroperitoneal fibrosis.[74] In a peritoneal mesothelial cell culture model, Selgas et al[75] showed that tamoxifen reduced the production of extracellular matrix components, CTGF and EMT, when cells were stimulated with TGF-β. Oncology research also has shown an anti-angiogenic effect of tamoxifen.[76] The efficacy of tamoxifen has been described only in case series. The largest study is from The Netherlands. This study investigated the outcomes of patients diagnosed with EPS between 1996 and 2007. Tamoxifen had been given to 24 patients, and not used in 39 patients. The two groups were otherwise well matched. The duration of tamoxifen use was for 4 weeks or more. Mortality in the tamoxifen group was 45.8% as compared with 74.4% in the untreated group ($P = .03$), and multivariate Cox regression analysis confirmed a trend to better survival in the treated group.[77] In a much smaller series,[78] tamoxifen was used in four patients (in whom infection was thought to preclude the use of steroids) at a dose of 40 mg/d. All four patients had clinically severe EPS on TPN and two had undergone an attempt at surgical correction without resolution of symptoms. All four patients experienced a marked improvement in symptoms, with three resuming a normal diet and one a low-residue diet. In the Pan-Thames data, a total of 31 patients received tamoxifen, either alone or in combination with other immunosuppressive drugs. Patient outcomes were similar between treatment groups, although the severity of disease in each group was not known.[8] del Peso et al[79] also described the use of tamoxifen in patients with peritoneal sclerosis. Nine received tamoxifen 20 mg twice day and 14 did not. Death as a result of EPS was seen only in the control group (4 of 14 patients). There are further case reports of successful use of tamoxifen[14] but some caution is necessary. The use of tamoxifen entails a risk of thromboembolism,[80] which might be increased in patients with inflammation and fluid balance issues.

Surgical Management

Mortality after surgery for EPS has varied hugely in the published literature. Partly this reflects an increasing surgical expertise. Early experience suggested a high mortality when enterectomy and bowel anastomosis was required,[81] but more recently centers have reported improved

outcomes using enterolysis and painstaking removal of the adherent fibrous sheath. Kawanishi et al[82] described a perioperative mortality rate of 7% (9 of 130 patients) treated with enterolysis; a further 3 patients died of recurrent EPS. Of note, re-operation was required in 33 patients and 11 patients required 3 or more laparotomies. Identifying the plane of separation could be difficult, especially where extensive calcification was present. Improvement was noted in all patients but long-term nutritional and symptom control outcomes were not discussed extensively. The Manchester group reported a series of 72 patients who underwent peritonectomy.[83] Mortality was 32% and 10% of patients required re-operation. Surviving patients did not require ongoing TPN or enteral feeding. Mortality was much higher in the patients requiring surgery as an emergency procedure (ischemic bowel, peritoneal sepsis, or bleeding) and early referral for peritonectomy was advocated. The timing of surgery is thus an important and as yet unresolved factor. Surgery should follow a trial of medical therapy,[67,82] yet is more difficult with established severe peritoneal calcification, and outcomes are worse with prolonged preoperative malnutrition.

PREVENTION OF EPS

Duration of PD

Because the duration of PD is the best established risk factor for EPS, elective withdrawal from PD after a standard time period is one strategy for EPS prevention. A recent position statement of the International Society for Peritoneal Dialysis points out that the incidence of EPS is low and currently available screening methods do not adequately predict the risk of EPS in an individual patient. Elective withdrawal from PD might best be targeted at those with increasing dialysate-to-plasma (D/P) creatinine and failing UF for whom there is a high risk of technique failure. Patient age and comorbidities also should be taken into account and a single rule mandating withdrawal from PD for all was not supported,[84] particularly because the clinical features of EPS often only occur or are exacerbated after stopping PD.

Biocompatible Solutions

There is considerable interest in the role of newer dialysate fluids, with much reduced levels of glucose degradation products and bicarbonate/pyruvate buffering, to reduce chronic damage to the peritoneum. These fluids yield similar creatinine clearance and UF as conventional fluids,[85] but there is a reduction in dialysate IL-6 and an increase in cancer antigen 125,[86] which may indicate reduced inflammation and increased mesothelial cell numbers. In theory the neutral pH should improve leukocyte function and perhaps reduce peritonitis rates; a decrease in peritonitis rates was observed in one single-center study,[87] but this was not confirmed in the Euro-Balance study, a large multicenter cross-over trial.[88] As yet there are no outcome data regarding the incidence of EPS in patients using the newer fluids, and so no assumption should be made about their use in preventing this complication.

Inhibition Of the Renin-Angiotensin System

The renin-angiotensin system is thought to be involved in the progression of renal fibrosis[89] and evidence suggests it also may be involved in the development of peritoneal fibrosis through up-regulation of TGF-β and fibronectin.[90] Angiotensin converting enzyme inhibitors can improve peritoneal morphology in the rat model[65,91] and long-term function in PD patients.[92] Aliskiren also had a beneficial effect in a rat model of EPS induced by chlorhexidine.[93] Angiotensin converting enzyme inhibitors and angiotensin receptor blockers attenuate VEGF production[56] and might reduce vasculopathy. The use of angiotensin converting enzyme inhibitors/angiotensin receptor blockers to prevent EPS in human beings has not been studied.

Peritoneal Lavage

The rationale of peritoneal lavage (periodic washout of the peritoneum via a retained PD catheter after the cessation of PD) is that removal of inflammatory molecules and fibrogenic factors may help to reduce the degree of fibrosis in the bowel wall. Moriishi et al[94] et al

followed up D/P creatinine ratios and cancer antigen 125 levels in eight patients using twice weekly peritoneal lavage for more than a year. The mean duration of PD was 10 years and three of the eight patients went on to develop EPS. D/P creatinine ratios decreased in non-EPS patients and some increase in cancer antigen 125 levels was seen, suggesting increased mesothelial cell viability. However, lavage was ineffective in a high proportion of patients. In a nonrandomized study, Yamamoto et al[95] compared 73 patients not given lavage with 174 patients treated with peritoneal lavage. Duration of PD, D/P creatinine, and mesothelial cell area were similar in the two groups. EPS occurred in 15.1% of the nonlavage group and 6.9% of the lavage group. Of note, the rate of EPS in patients with a PD duration of more than 96 months was significantly reduced in the lavage group at the 2 year follow-up evaluation; however, follow-up evaluation of high-risk patients after lavage was not complete and the lavage protocol was not standardized. The investigators hypothesized that the lavage process aided mesothelial recovery, as evidenced by changes in the mesothelial cell population in dialysate effluent. Beneficial effects of lavage on membrane function were seen from 6 months after cessation of PD, when repair had occurred. Peritonitis rates during lavage were not discussed and patients receiving immunosuppression were excluded.

WHAT DO WE STILL NEED TO KNOW ABOUT EPS?

Etiology

Future research will investigate the possibility of inhibiting the molecular mechanisms of peritoneal sclerosis using specific inhibitors of fibrosis or angiogenesis. The identification of EMT as a key and potentially reversible step in the development of membrane structural alterations has permitted investigation of inhibitors of this process such as bone morphogenetic protein 7 and hepatocyte growth factor.[96] Similarly, the use of inhibitors of the renin-angiotensin pathway could be investigated further in PD patients because efficacy has been shown in animal models[91] and preservation of peritoneal membrane function has been noted in patients taking such medications.[92] Other areas of research include inhibition of the TGF-β pathways using pentoxyphylline, diltiazem, dipyridamole, or tranilast (reviewed by Kaneko et al[97]), inhibition of angiogenesis, and the role of antioxidants.

Screening

Although the long-term membrane changes that predispose to EPS in PD patients are better understood, we do not know why some patients go on to develop florid fibrin deposition with bowel encapsulation and become symptomatic and others do not. This makes it more difficult to counsel patients adequately regarding duration of PD or the likelihood of post-transplantation EPS. For the PD patient, the absence of a screening test that could quantify the risk of future EPS remains a significant problem. Clinicians can give advice based on epidemiologic data (duration of PD) and PET results (especially reduced UF). Radiologic screening may not be helpful given the observation in the Tarzi et al[19] study that several patients developed EPS within a year of a normal CT, although the development of even mild transitory abdominal symptoms if accompanied by some CT changes may predict development of EPS on cessation of PD.[98] As yet there are no validated biomarkers from the dialysate effluent that can predict the risk of future EPS. Several groups have suggested potential markers[99,100] and this would seem a useful area for future research. The combination of low levels of cancer antigen 125 and high levels of IL-6 in dialysate effluent had a sensitivity of 70% and a specificity of 89% for the prediction of EPS,[100] but these markers require validation in a larger cohort. Furthermore, the low sensitivity is a concern.

For patients with diagnosed EPS, further information on the outcomes of surgery versus conservative medical therapy with long-term TPN will be of great help. Surgical expertise is likely to remain highly center-specific and it may not be possible to conduct randomized controlled trials in this area. Likewise, the role of immunosuppression and antifibrotic therapy still is unclear, but a better understanding of the pathogenesis of EPS may allow therapies to be

targeted to an appropriate stage in the evolution of EPS-related changes.

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