# Local and systemic endothelial injury in renal failure treated with peritoneal dialysis

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**Abstract**

Excess fluid and waste products of metabolism, as well as protein, are removed from the peritoneal cavity in Peritoneal Dialysis (PD). Increased peritoneal protein clearance (Pcl) is associated with a greater risk of mortality. It is not clear whether this association reflects systemic endothelial injury or local peritoneal capillary damage and inflammation, or both.

To investigate this problem a series of analyses were undertaken in different incident, prevalent and longitudinal patient cohorts. Transcapillary escape rate of albumin (TERalb) was measured to determine systemic capillary permeability. Luminex assays combined with principle component analysis were applied to measure endothelial biomarker patterns.

It was demonstrated that: (1) Pcl is a function of both local peritoneal inflammation, membrane area (PSTR) and comorbidity (especially cardiovascular) but only its association with the latter predicted survival. (2) There is a progressive uncoupling of the Pcl, (indicative of large pore pathway) and PSTR (effectively the small pore area) with time on PD. (3) Isolated small pore ultrafiltration (due to icodextrin) decreases with prolonged time on PD and is also uncoupled from the increase in peritoneal membrane area. (4) The systemic endothelial barrier function is decreased in PD patients, especially diabetics, but not associated with hypoalbuminaemia which is linked to systemic inflammation. (5) Hydration status is related to plasma albumin concentration but not endothelial dysfunction as measured by soluble biomarkers.

Pcl is a function of both local peritoneal factors, e.g. inflammation and progressive fibrosis, and systemic patient characteristics, e.g. age and comorbidity. The influence of comorbidity is complex depending on type, associated patterns of endothelial injury and causes of associated hypoalbuminaemia. The importance of plasma colloidal pressure in determining fluid status was emphasized. Strategies to improve fluid distribution should focus on reducing peritoneal protein loss and increasing albumin synthesis rather than blocking systemic vascular leak.

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Dr Boon Kay Tan, whom I spent most time with in the research office, was the named research fellow for the Bioimpedance study, who set up the study and was the main person coordinating data and sample collection in the UK centres. The analysis from the Bioimpedance study was based on his work.

Dr Mark Lambie, who worked on the data coordination of the GLOBAL Fluid Study, supplied me the data for the analysis based in Chapter 3. I am also grateful to him for debating the three pore model and discussions about statistical issues.

The transcapillary albumin leak, including absolute TBW (headspace D2O measurement), was determined from measurements done in another project before I joined the team. It was contributed by Biju John, Kay Tan, Stephen Dainty, Professors Patrik Spanel and David

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Last but not least, to my parents, whom I have been away from for all these time. They enable and encourage me doing things I enjoy. Without their support I would not be who I am.

**List of abbreviations**

ACE angiotensin-converting enzyme

ALB albumin

ANOVA analysis of variance

APD automated peritoneal dialysis

AR appearance rate

BMI Body Mass Index

BNP Brain Natriuretic Peptide

BIA Bioelectrical Impedance Analysis

BUN Blood Urea Nitrogen

CAPD Continuous Ambulatory Peritoneal Dialysis

CKD Chronic Kidney Disease

cAMP cyclic monophosphate

Cr creatitinine

Ccr creatitinine clearance

CRP C-Reactive Protein

CVD cardiovascular disease

D Deuterium

D/P dialysate/plasma

D2O Deuterium Oxide

DM Diabetes Mellitus

ECF Extracellular Fluid

ECW Extracellular Water

ELISA enzyme-linked immuno sorbent assay

ESRD end stage renal disease

F female

FA-MS Flowing Afterglow Mass Spectrometer

FMD Flow-mediated vasodilation

GDPs glucose degradation products

Hb Haemoglobin

HCA Hierarchical clustering analysis

HD Haemodialysis

HSA human serum albumin

Hz Hertz

ICAM inter-cellular adhesion molecule

ICF Intracellular Fluid

ICO icodextrin

ICW Intracellular Water

IHD ischemia heart disease

IL interleukin

INF interferon

Kt/V Dialysis Adequacy

Lp hydraulic conductance

LVD Left Ventricular dysfunction

LVH Left Ventricular Hypertrophy

M male

MMP matrix metalloproteinase

MCP monocyte chemotactic protein

PC principal component

PCA principal component

Pcl protein clearance

PD Peritoneal Dialysis

PET peritoneal equilibration test

PSTR peritoneal solute transport rate

PV plasma volume

PVD peripheral vascular disease

RRF residual renal function

R Resistance

S surface area

SD standard deviation

SE standard error

TBW Total Body Water

TERalb transcapillary escape rate of albumin

TNF tumor necrosis factor

UF ultrafiltration

V Volume

VCAM vascular cell adhesion protein

VEGFV ascular endothelial growth factor

Xc Reactance

(σ) Reflection coefficient

## Publications and Abstracts related to this thesis

### Peered review publications

1. Zanzhe Yu, Boon Kay Tan, Stephen Dainty, Derek L. Mattey, Simon J. Davies， Hypoalbuminaemia, systemic albumin leak and endothelial dysfunction in peritoneal dialysis patients. Nephrol Dial Transplant 2012; 27:4437-4445.

### Oral Presentations at national and international academic meetings

1. ISBP (international society of blood purification) Sep 2009
    a. “Peritoneal protein leak, systemic and local inflammation: sub-analysis from two centres of GLOBAL cohort”
2. International Centre of Biocybernetics-Peritoneal Transport Meeting March 2011
    a. "Systemic peritoneal protein leak in PD patients and markers of endothelial dysfunction"
    b. "Determinants of UF using icodextrin"
3. BRS/RA conference 2011(British Renal Society and renal association) June 2011
    a. “Understand the variability in Ultrafiltration obtained by Icodextrin”
4. 10th Euro PD 2011 Oct 2011
    a. “Systemic Endothelial Albumin Leak is Associated with Markers of Platelet Activation Independent of Systemic Inflammation in Prevalent Peritoneal Dialysis Patients”
5. BRS/RA conference 2012(British Renal Society and renal association) June 2012
    a. Aquaporin 1 genotype has clinically important impact on ultrafiltration (Nominated for the Renal Young Scientist Award)
6. 14$^{th}$ International Society for Peritoneal Dialysis (ISPD) Sep 2012

a. Late break clinical trial-Developing Bioimpedance (BIA) as a tool for fluid management in Peritoneal Dialysis Patients: A validation Study (joint presentation with Dr Kay Tan)

**Posters**

1. “Peritoneal protein leak, systemic and local inflammation: sub-analysis from GLOBAL fluid study” poster presentation in 2010 BRS/RA conference
2. “Systemic endothelial albumin leak is associated with markers of platelet activation independent of systemic inflammation in prevalent peritoneal dialysis patients” poster presentation in 2010 American Society of Nephrology (ASN) meeting and 2011 BRS/RA conference
3. “Understand the variability in Ultrafiltration obtained by Icodextrin” poster presentation in 2011 American Society of Nephrology (ASN)
4. “Progressive uncoupling of peritoneal small solute transport and protein clearance with time on peritoneal dialysis” in 2012 RA conference and ISPD 2012

# chapter 1.**Introduction and Hypotheses**

## 1.1 Background

### 1.1.1 What is the main cause of death for peritoneal dialysis (PD) patients?

Renal replacement therapy is the most successful organ replacement therapy at present. Thousands of end stage renal disease (ESRD) patients are saved from death by the therapy. Unfortunately, the expected life span of the ESRD patients is still much shorter than the general population. Cardiovascular disease (CVD) is the leading cause of death in patients with ESRD. Cardiovascular mortality is markedly greater in patients treated by renal replacement therapy than in general population. [1, 2] Although the high risk of CVD had been recognized early after introduction of maintenance dialysis, [3] the mechanisms behind the high prevalence of CVD observed in these patients are still far from understood. The prevalence of Framingham's traditional risk factors for CVD (age, hypertension, diabetes, dyslipidemia, and physical inactivity) is high in chronic kidney disease (CKD) patients. Some CKD-specific risk factors, such as fluid overload, anemia, hyperparathyroidism, oxidative stress, hypoalbuminemia, accumulation of pro-inflammatory cytokines, elevated asymmetric dimethylarginine (ADMA), hyperhomocysteine, and modification of protein by non-enzymatic advanced glycation are also thought to play roles in the high prevalence of CVD in dialysis patients [4, 5]. Moreover, several factors associated with PD therapy, such as advanced altered lipid

profile, hyperinsulinemia, and formation of advanced glycation end-products may increase the risk in PD patients.[5]

### 1.1.2 What plays the key role in the development of cardiovascular disease (CVD) in PD patients?

The vascular endothelium is a single cell barrier, located between the plasma and the underlying tissue. It actively regulates vascular tone and permeability, the balance between coagulation and fibrinolysis, the extravasation of leukocytes, and the proliferation of vascular smooth muscle. Different risk factors, such as hyperlipaemia, smoking, hemodynamic stimuli, may destroy the normal balance followed by endothelial dysfunction. Endothelial dysfunction is the main pathway in most forms of CVD not only in the general population but also in chronic renal failure. Impaired vasodilation has been found in hypertension, type 1 and type 2 diabetes, coronary artery disease, congestive heart failure and also chronic renal failure. [6] Moreover, according to a study in the offspring of hypertensive patients, endothelial dysfunction may not be only associated with its existence but also precede its development. [7] Endothelial dysfunction is an important early event in the pathogenesis of atherosclerosis and it also contributes to vascular remodeling. [4, 6, 8] Although non-atherosclerotic CVD may also contribute to the high cardiovascular mortality rate in ESRD, much evidence suggests that ESRD patients are subjected to the process of accelerated atherogenesis. [9] Different measurements of

endothelial function, including biomarkers and functional measurements of endothelium, show endothelial dysfunction in ESRD patients and predicts patients' outcome. [10-13]

With the understanding of the importance of endothelial dysfunction in patients' outcome in PD, we have reviewed the measurement methods of endothelial dysfunction in patients and investigate the possibility that peritoneal protein leak is a marker of endothelial function in PD patients. For this reason, we have also discussed the present understanding of the pathophysiology and longitudinal changes of the peritoneal membrane and map the gap in our current knowledge.

## 1.2 How is endothelial function measured?

The importance of the endothelium in CVD has been recognized for many years. Despite this the optimal methodology for investigating the multi-aspects of endothelial dysfunction is still under debate. Generally speaking, endothelial function can be measured by two ways. One is to measure the concentration of the biomarkers that mediate endothelial cell function. The other is to measure the functional consequences of endothelial cell activity. From a clinical perspective, it can also be divided into invasive and non-invasive methods. We will mainly focus on non-invasive methods here.

### 1.2.1 Normal function and dysfunction of endothelium

Normal endothelial function includes the regulation of hemostasis and fibrinolysis, vasomotor activity, angiogenesis, permeability to macromolecules, leukocyte adhesion and vascular smooth muscle cell proliferation. Endothelial dysfunction can be defined as any change in endothelial functions that are inappropriate to the organ function. There are many types of endothelial dysfunction depending on which function is affected.

It should also be noted that endothelial function in different organs is very variable. For example, in the glomeruli, capillaries normally allow small amount of protein to cross. In contrast, albumin permeability in hepatic sinusoids is much higher than that in glomerulus. A normal endothelial function is defined as a suitable condition for a definite organ circumstance.

### 1.2.2 Biomarkers and their limitations

Biomarkers are most widely used for estimating endothelial dysfunction in clinical research for practical reasons. A wide range of biomarkers have been established so far. It is predictable that more biomarkers will be recognized with our increasing knowledge of endothelial dysfunction. However, generally speaking, they are either endothelium-derived regulatory proteins, or may not derive from endothelium but are documented to take part in the pathogenesis of endothelial dysfunction or reflect the endothelial dysfunction. Detailed reviews of this have been done by others. [14-17]

Some limitations should be realized when using biomarkers. Firstly, each of these biomarkers reflects an aspect of endothelial function, but none of them can be regarded as the gold standard and can replace each other. Secondly, it is also not clear whether the predictive value of endothelium derived regulatory proteins is due to their specific function (for example vascular cell adhesion protein 1 (VCAM 1) for leukocyte adhesion) or reflects general endothelial dysfunctions. For these two reasons, measuring the pattern of the biomarkers might be a better strategy than separate biomarkers. Thirdly, high plasma levels of biomarkers may reflect increased synthesis and/or decreased clearance. Data on the latter process is limited but it may be an important issue in renal disease. Fourthly, they cannot give information about where endothelial dysfunction occurs. The endothelium derived products may mainly reflect dysfunction at the level of

small resistance arteries, capillaries or venules, because of the large area of them, while clinical events such as myocardial infarction mainly occur in larger conduit arteries.

### 1.2.3 Measurement of functional consequences of endothelial cell activity

#### 1.2.3.1 Vasoactivity

##### 1.2.3.1.1 Flow-mediated vasodilation (FMD) – the most widely used method

The technique of assessing flow-mediated vasodilation (FMD) has been developed for years. It measures the change in diameter of a conduit artery in response to increased flow, typically induced by a period of ischemia in the distal circulatory bed, which is predominately nitric oxide (NO)-mediated. There is evidence that it is depressed in subjects with atherosclerosis and cardiovascular risk factors and is thought to be correlated with coronary vascular endothelial vasodilator function. [18-20] The equipment and protocol of FMD are well established by the working group on endothelin and endothelial factors of the European Society of Hypertension. [15] Briefly, for endothelium-dependent FMD, the baseline image is recorded after a blood pressure cuff is inflated to supra-systolic pressure for 5 min followed by deflation of the cuff, and recording of the post cuff deflation image. The effect of an endothelium-independent stimulation is accessed by sublingual administration of nitroglycerin. After the baseline image is recorded, nitroglycerin is administered sublingually. The image is recorded for a further 3

to 5 min after that. The endothelium-independent dilation, which is also known as nitroglycerin-mediated dilation, is expressed as the percentage of maximum change from baseline in diameter. FMD of the brachial artery is the most widely used and best validated, however similar principles can also be applied to other conduit artery, such as radial, femoral and posterior tibial arteries.

#### 1.2.3.1.2 Other methods

Non-invasive tests for assessment of coronary endothelial function have been described including new ultrasound technology and contrast echocardiography, positron emission tomography and magnetic resonance imaging. [21] The 'gold-standard' test for the evaluation of coronary endothelial function requires invasive coronary angiography. No non-invasive assessment method of endothelial dysfunction in the microvasculature has been widely accepted in humans until now. The Landis-Michel micro-occlusion technique, which involves capillary cannulation and occlusion[22], does allow in vivo measurements in animals. But it is not feasible in human. The laser Doppler imaging technique provides a possible non-invasive approach to the evaluation of microcirculation. [23] The effect of beraprost sodium, a prostacyclin analogue, on skin blood flow measured by laser doppler flowmetry decreases with an increase in the severity of retinopathy and nephropathy in a group of non insulin dependent diabetes patients. [24] The results of laser Doppler flowmetry are also consistent with the other risk assessments (Framingham, CRP, etc.) in ESRD patients. [13] The method of nail fold capillaroscopy is used in autoimmune rheumatic

diseases, especially in scleroderma. [25] But it has not been widely validated in CVD. A study using computerized videocapillaroscopic technique has shown the morphological difference of gingival microcirculation between smokers and non-smokers. [26] Further data about the predictive power of cardiovascular risk for these methods is needed, and standardization of protocols would be helpful to improve the reproductive power.

Whether endothelial dysfunction occurs in all vascular beds is difficult to test in human but obviously it is an important question. One study found that a disturbed endothelial vasomotor response was associated with microalbuminuria in not only the brachial artery but also the renal interlobar arteries. [27] However we are not aware of any studies that have been done, for example, on whether protein leak in peritoneum is related to endothelial dysfunction in coronary artery.

#### 1.2.3.2 Barrier function

##### 1.2.3.2.1 Transcapillary escape rate of albumin

The transcapillary escape rate of albumin (TERalb) is the most widely used method to identify the barrier function of macromolecules in endothelium. The method was described as early as 1973. [28] In brief, the TERalb, which is defined as the fraction of intravascular mass of albumin that passes to the extravascular space per unit time, is determined from the disappearance of intravenously injected radiolabelled albumin and usually expressed as the one hour fall of intravenous radiolabelled albumin expressed as percentage. Transcapillary permeability to albumin is found to be increased in patients

with diabetes, hypertension and atherosclerosis [29-32] and also in acute inflammation status, such as sepsis. angiotensin-converting enzyme (ACE) D/D homozygosity, which is related to an increase risk of atherosclerotic vascular disease in essential hypertension, is also associated with higher TERalb despite identical 24 hour blood pressure readings. [33] High-dose simvastatin reduces low-density lipoprotein cholesterol by 39%, and normalizes TERalb. [34] However, we have to realize that the TERalb is a complex parameter which combines the effect of hemodynamic forces, endothelial surface area and endothelial permeability.

#### 1.2.3.2.2 Microalbuminuria

##### ***1.2.3.2.2.1*** *Microalbuminuria is closely related to CVD*

Microalbuminuria is thought to reflect the systemic endothelial function and considered to be a marker of endothelial dysfunction. Microalbuminuria is usually defined as the urinary albumin excretion rate of 30-300mg/24h urine, or of 30-300(mg/g creatinine) in spot urine sample. Microalbuminuria, even below the conventional cut-off values is demonstrated to be a predictor for cardiovascular events in both diabetic and general population. [35-38] Microalbuminuria in diabetes has been shown to relate to endothelial dysfunction as measured by biomarkers, eg von Willebrand factor, tissue-type plasminogen activator, soluble vascular cell adhesion molecule 1 (VCAM-1), and soluble E-selectin, and functional measurement, FMD. [39] This association between microalbuminuria and endothelial dysfunction has also been found in non-diabetic

individuals. [40-42] The observation that changes in albuminuria during treatment translate into changes of cardiovascular events further suggests that albuminruia reflects a systemic endothelial dysfunction. [43]

#### *1.2.3.2.2.2 Is there a common pathway of microalbuminuria and CVD?*

The Steno Hypothesis, published in 1989,[44] introduced the concept that microalbuminuria might reflect a systemic transvascular leakage of albumin.

Data from Jensen, et al have shown that in patients with microalbuminuria the fractional disappearance rate of albumin from the plasma compartment is higher than in the normal group. It is positively correlated with urinary albumin excretion rate and is independent of age, sex, smoking status, blood pressure, body size, plasma volume, plasma albumin concentration and blood glucose, serum insulin and serum lipids. [45] Falqui has shown that in a group of never-treated non-diabetic hypertensive patients, microalbuminuria is associated with higher capillary permeability to albumin. [46] Furthermore, Viazzi has shown that in primary hypertension patients, 1% increment in TERalb or 10mmHg increase in systolic BP results in an almost three times higher risk of having microalbuminuria. [47]

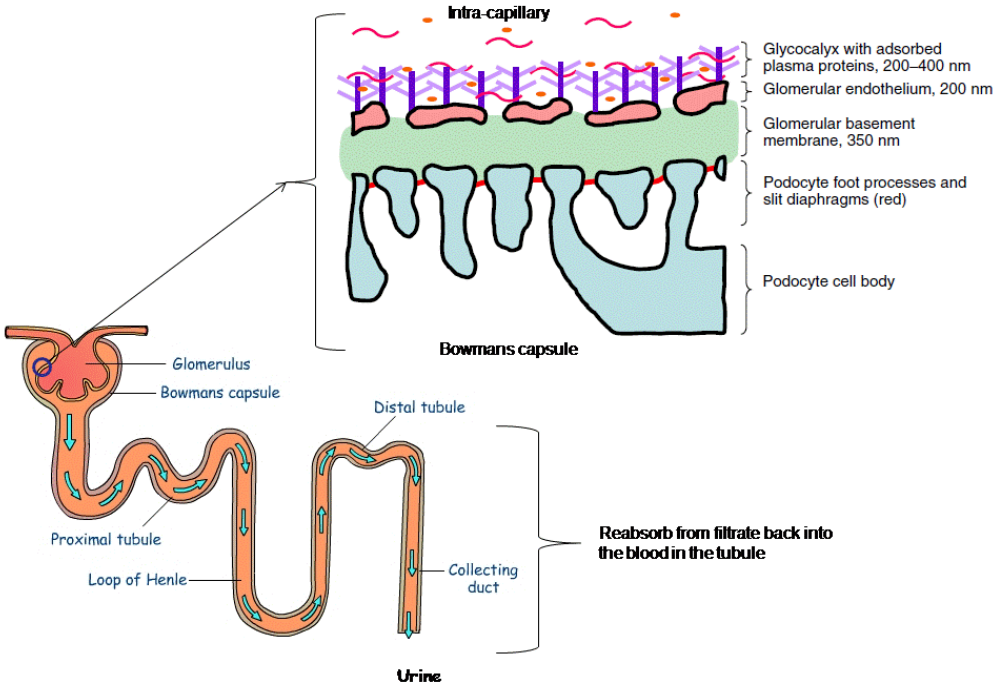
However, the association between increased TERalb and microalbuminuria is not present in all circumstances. Pedrinelli and his colleagues have shown that in essential hypertension and in atherosclerotic and hypertensive men transcapillary albumin leakage

is altered, but the abnormality is dissociated from the amount of albuminuria. [31, 48] They found that microalbuminuria was associated with the hemodynamic response to acetylcholine in the forearm, another assessment of endothelial function. [49]

In the case of glomerular protein leak, apart from the endothelium, it also depends on glomerular basement membrane composition, slit diaphragm structure, podocyte and interstitial-tubular lesion, which may partly explain the paradoxical results. (Figure 1-1) On the other hand, as mentioned before, hemodynamic factors could also affect the protein leak, and the intra-glomerular-capillary pressure is not only regulated by systemic blood pressure but also by the local factors. Actually, in the data from Pedrinelli, both TERalb and albuminuria were related to blood pressure [48], but they are not related to each other. [31, 48]

Figure 1-1 The main filtration barrier of glomerulus

It includes glomerular endothelial, glomerular basement membrane, podocyte and slit diaphragms. After filtration, the tubule of kidney selectively reabsorbs substances back into the blood.




Adapted from http://www.health.bcu.ac.uk/physiology/renalsystem.htm and [50]

## 1.3 Could peritoneal protein clearance (Pcl) be regarded as a marker of endothelial function in PD patients?

### 1.3.1 Is peritoneal Pcl related to CVD in PD patients?

#### 1.3.1.1 The small solute clearance is the important but not the only important factor for patient survival in PD

Removal of extra fluid and toxin is the major function of dialysis. Kt/V and the creatinine clearance rate (Ccr) represent the small molecular solutes clearance in dialysis patients and are the most widely used measurement of dialysis adequacy. Kt/V is the product of dialyzer (which in the case of PD is the peritoneal membrane) urea clearance and treatment time divided by the volume of distribution of urea, and Ccr is the volume of blood plasma that is cleared of creatinine per unit time.

Kt/V

K - dialyzer clearance of urea

t - dialysis time

V - volume of distribution of urea, approximately equal to patient's total body water

$$\text{Kt/V} = \frac{24hour\ dialysate\ urea\ excretion \times 7}{total\ body\ water}$$

$$\text{Ccr}\left(\frac{L}{week}\right) = \frac{24hour\ dialysate\ creatinine\ excretion \times 7 \times 1.73m^2 BSA}{Patient's\ BSA}$$

The importance of small molecular solutes clearance has been well established in recent years. Almost all the guidelines for clinical practices on PD recommend a dialysis adequacy target in terms of Kt/V and Ccr. Studies have shown that the greater the small molecular solute clearance the better the patients survival is, although the variance in clearance that explains this survival effect is entirely due to residual renal function. The data from the Stoke Study showed that weekly Kt/V was an independent predictor of survival in CAPD patients. [51] Increasing the delivered dialysis dose may have a beneficial effect on nutrition in malnourished patients without comorbidity [52] but not in patients with comorbidities. Studies have also shown that increasing peritoneal Kt/V and Ccr above the minimum recommended threshold cannot improve the survival rate in PD [53, 54], and numerous studies have tried and are trying to find the underlying contributors for patient survival other than small solute clearance.

#### 1.3.1.2 The changing role of high peritoneal membrane transport status (PSTR) as a strong predictor of worse patient outcome

In addition to total small solute clearance, the rate at which small solutes diffuse across the peritoneal membrane, usually referred to as peritoneal solute transport rate (PSTR), usually measured by the dialysate to plasma creatinine ratio (D/Pcrea) at four hours, is also regarded to associate with patient

failure, independent of comorbidity and small solute clearance, [55] at least historically. Data from our own centre showed a relationship between increased PSTR and reduced survival in CAPD patient before 1995. [51] The underlying reasons for this relationship may include a reduced peritoneal membrane ultrafiltration capacity resulting from a more rapid dissipation of the glucose osmotic gradient and fluid reabsorption, [56] which consequently causes extracellular water (ECW) expansion and hypertension. [57] More recently high PSTR is no longer a risk factor for technique failure or death in contemporary PD patients treated with dialysis methods such as automated peritoneal dialysis (APD) and polyglucose solutions, such as icodextrin, which counter balance the negative effects on ultrafiltration. [58-61].

With the improved understanding of the peritoneal membrane, it has been hypothesized that the high PSTR patients may not be a homogeneous group. They could be subdivided into at least three groups. Type I is an early inherent type which is potentially associated with increased mortality and is usually associated with inflammation and comorbidity. This group of patients would also have a poor outcome even if they were treated by haemodialysis, although this has not been shown. Type II is also an early inherent type which is mainly related to a large peritoneal surface area. Most of these patients are not inflamed. Type III is a late acquired type, which develops over time of PD. Inflammation and comorbidities are also not necessary in this group of patients although local (as opposed to systemic) inflammation may be the driving mechanism. (Table 1-1) The use of icodextin and APD improves the fluid management especially in high PSTR patients [62-64]

which help to convert the poor outcome tendency in type II and type III high PSTR but is not helpful to type I patients.

Table 1-1 characteristics of the three types of high PSTR [58]

| | Early inherent phenotype | | Late acquired phenotype |
|---|---|---|---|
| | Type I | Type II | Type III |
| Onset | Beginning of PD | Beginning of PD | Over time on PD |
| Peritoneum | Vasculopathy, endothelial dysfunction, high protein loss | Large peritoneal surface area | Structural changes |
| Causes | Genetics<br>Inflammation<br>Comorbidity | Genetics | Genetics?<br>Local effects on the peritoneal membrane due to<br>Peritonitis<br>Bioincompatibility of PD solutions?<br>Loss of substance like hyaluronan, phospholipids? |
| Prognosis | Poor | Better | Better |

### 1.3.1.3 Peritoneal protein clearance (Pcl) is an independent predictor of mortality in incident PD patients

More clearance of mid-molecular weight toxin is regarded as an advantage of PD compared with haemodialysis. It should be noted that the clearance of large molecular weight substances are also increased in PD patients. PD patients may lose 5-15 gram of protein per day through the peritoneum. [65] This extrarenal protein loss contributes to the low albumin level in PD patients to some degree on the population base [66] and it also likely explains why for a given degree of hypoalbuminaemia survival in PD patients is superior to those on haemodialysis. [67]

Recent studies have shown that increased peritoneum Pcl may predict a poor outcome in incident PD patients even with the use of icodextin and APD. Johansson used the method

of personal dialysis capacity test [68] which was based on the three-pore theory of capillary transport to identify large-pore flow in a group of 249 patients. They found that large-pore flow is greater in patients with severe comorbidity than in patients with fewer comorbid conditions. [69] This is in keeping with the observation by Szeto, C.C., that a single measure of dialysate albumin >300mg/l is associated with increased cardiovascular events.[70] Van Biesen has shown that a large-pore flux higher than expected from the membrane area parameter is related to an increased mortality and is weakly correlated with systemic inflammation. [71] Heaf also has found that mortality is increased significantly with increased large pore flow and is related to hypoalbuminaemia after PD initiation. [72] More recently, Pcl has been confirmed to relate to peripheral arterial disease in a cohort of 133 PD patients. [73] Data from our centre show that peritoneal Pcl is an independent predictor of mortality and is related to increased pulse pressure and the presence of peripheral vascular disease while PSTR is no longer a predictor. [61] Most of the clinical observations support that peritoneal Pcl is correlated with poor outcome and vascular disease. However, data from a Netherland group, showed that baseline peritoneal albumin and protein clearances from a 4 hour dwell with 3.86% glucose dialysate were associated with signs of comorbidity but no measurable effect on patient survival could be found. [74] The reasonable number of patients and duration of follow up in that study make it unlikely that the negative result is due to a lack of power. The reason for the different observations calls for further investigation.

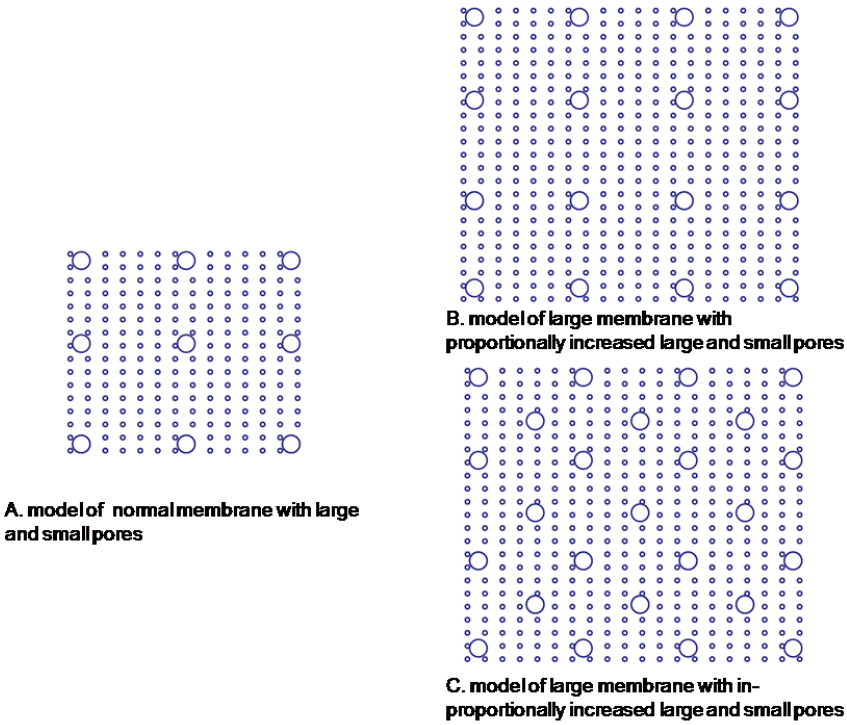
#### 1.3.1.4 The predictive power of peritoneal Pcl is independent from PSTR

A larger membrane tends to have a higher PSTR, measured as the diffusive transport rate for small solute, for example, D/Pcrea, and also tends to have a higher peritoneal Pcl. The membrane transport status would change proportionally with the peritoneal Pcl in an anatomic large membrane (probably type II high PSTR) while if the high PSTR is a result of inflammation or vascular injury (probably type I high PSTR) they may change in-proportionally. (Figure 1-2)

One way to distinguish these two possibilities is to dissociate the PSTR from peritoneal Pcl by using the three-pore model. According to the three-pore model, PSTR depends on effective membrane area while peritoneal Pcl mainly reflects large pore function. Several studies have dissociated the effective membrane area from large pore flow and found that large pore flow was an independent predictor of survival while membrane area parameters tend to lose its predictive value in multivariate models. [61, 71, 72] It is likely that the membrane transport status has lost its power to predict worse survival, because of the use of icodextin and APD which relieve the disadvantages of high PSTR in fluid control, and reveals an underlying relationship between peritoneal Pcl and survival.

Figure 1-2 A) model of normal membrane with large and small pores B) model of anatomic large membrane with proportionally increased large and small pores C) model of inflamed membrane with more small pores and even much more large pores. In this situation, small pores and large pores increased in-proportionally.

(This diagrammatic representation shows a much lower ratio of small to large pores than seen in reality.)




## 1.3.2 What are the main determinations of peritoneal Pcl?

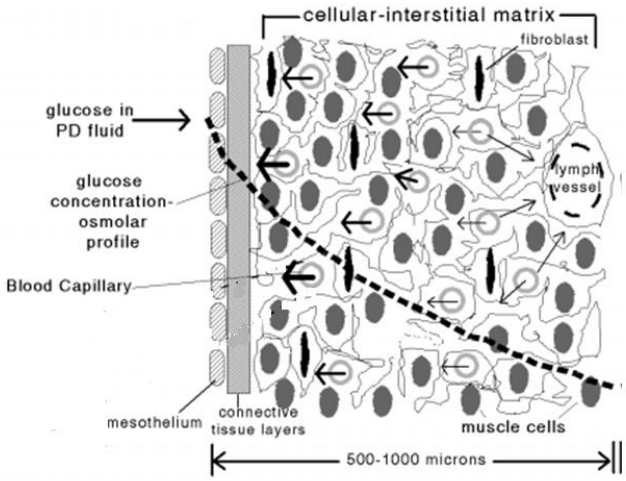
### 1.3.2.1 Main barrier in peritoneal membrane

As shown in Figure 1-3, the anatomic barrier separating the blood from the peritoneal fluid in PD contains a number of structures, including the peritoneal capillary walls, the cellular-interstitial matrix and the single layer of mesothelial cells. The mesothelium is not a major transport barrier to solute and water transfer. Complete removal of the

mesothelium in rodents does not affect the solute or water transfer significantly. [75] The capillary endothelium is the most important resistance in PD.

Figure 1-3 The anatomic barrier separating the blood from the peritoneal fluid in PD

It contains the peritoneal capillary walls, the cellular-interstitial matrix and the mesothelium.




Adapted from ref [76]

## 1.3.2.1Physiology and pathophysiology of trans-capillary protein leak

Over the last century several physiologists including Starling, who formulated Starling equation in 1896, [77] Pappenheimer, Michel, Curry, Rippe, Bates, Renkin, Crone, Levick, and Landis [78-83] have investigated the mechanisms of transport across the vascular barrier. Gases, water, and other small molecules cross the endothelial cell barrier freely whereas larger molecules such as plasma protein are tightly restricted.

Diffusion is the most important mechanism for the exchange of small molecules and is driven by the molecular concentration gradient across vascular endothelium. This is not the case for large molecular weight substances, such as protein. The diffusion of albumin across the vessel is estimated to be 1000-fold less than that of water. [81] Convection is the most important determinate for the flux of large molecules such as plasma proteins, which is determined by the forces of the Starling equation.

The Starling equation reads as follows:

$$Jv = LpA[(Pv - Pi) - \sigma(\pi v - \pi i)]$$

Jv is the net fluid movement between compartments.

Lp is hydraulic conductance.

A is surface area available for molecular exchange.

(Pv-Pi) is the hydrostatic pressure gradient between capillary and interstitial.

(πv-πi) is the oncotic pressure gradient between capillary and interstitial.

(σ) is reflection coefficient.

[(Pv-Pi)- σ(πv-πi)] is the net driving force.

As established by Rippe B, according to the three-pore model, the size-selectivity for macromolecules larger than albumin is compatible with the presence of large pores by

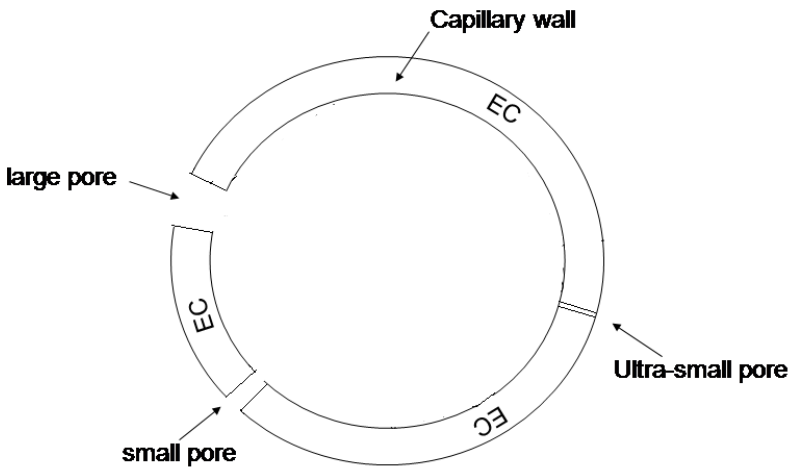
pressure-driven flow, without being affected by the oncotic or colloid osmotic pressures acting across small and transcellular pores. In other words, large pore transport is affected only by changes in the transcapillary hydrostatic pressure gradient and/or the number of large pores available for transport. [84]

#### 1.3.2.1.1 Three-pore model

In the three pore theory developed by Rippe and his colleagues, the capillary endothelium is modeled as a barrier with pores. There are three types of pores in capillary endothelium: ultra-small pores, small pores and large pores. (Figure 1-4) The presence of ultra-small pores is supported by the discovery of aquaporin-1, a molecular structure that penetrates the cell wall and provides a water only pathway. [85] The small pores, which are 40-55 Å in radius, are responsible for small solute transport and are thought to be the intercellular clefts between the endothelial cells. Most of the protein is thought to go through large pores which are approximately 250 Å in radius. The nature of the large pores is not clear.

Figure 1-4 three-pore model

According to the three-pore model, there are three kinds of pores in capillary endothelium: small pores, large pores and ultra-small pores. The ultra-small pores are probably the transendothelial aquaporin-1 and provide a water only pathway. The numerous small pores, which are 40-55 Å in radius, are the principal pathway for water-solute substances and water. The small pores are thought to be the intercellular clefts between the endothelial cells. Most of the protein is thought to go through large pores which are approximately 250 Å in radius and fewer in numbers. The nature of the large pores is not well known. [82]



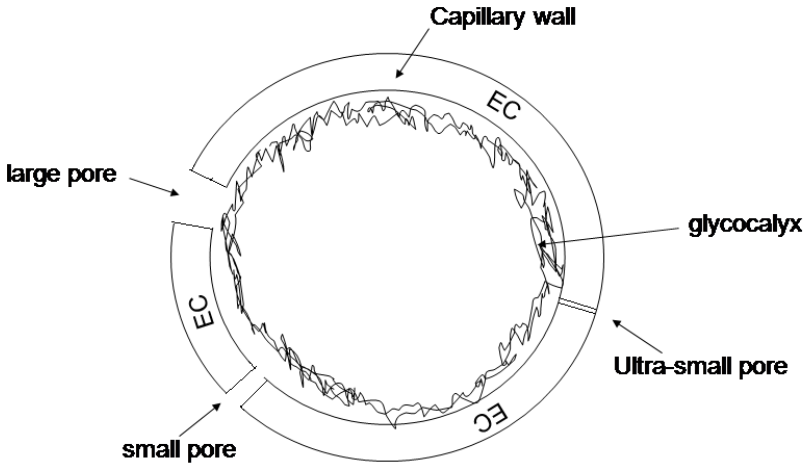

EC, endothelial cell

### 1.3.2.1.2 The glycocalyx

The glycocalyx is a thin layer of proteoglycans, glycoprotein and glycosaminoglycans located on the luminal surface of endothelial cells. (Figure 1-5) It conveys a negative charge to the endothelium, which may be important for the glomerular filtration barrier. [86] It is also possible that it affects the peritoneal permeability for macromolecules through the endothelial barrier. The glycocalyx appears to be damaged in several clinical

situations such as inflammation, ischaemia/reperfusion and hyperglycaemia, all of which are likely to be associated with an increased capillary permeability. [87, 88] Hyaluronan, a major component in the interstitium, has been found to make up a major part of the glycocalyx endothelial barrier. Treatment with hyaluronidase results in a marked increase in permeability of 70- and 145-kDa dextrans. [89] Pro-inflammation cytokines, such as TNF-α, cause modification of the glycocalyx and result in increased permeability to macromolecules, even when TNF-α-enhanced white cell adhesion is inhibited. [90] Glycocalyx modification also occurs in ischemia-reperfusion injury, which may be reversed by pretreatment with adenosine. [91, 92] Short-term hyperglycemia results in a rapid decrease of the ability of glycocalyx to prevent 70-kda dextran from transporting across the endothelial barrier. [93] Although the effect of short-term hyperglucemia on decreased endothelial barrier function is complex. The cytoskeleton of the cells in the glomerular barrier may also be involved in the alterations. [94]

Figure 1-5 glycocalyx - a thin layer of proteoglycans, glycoprotein and glycosaminoglycans located on the luminal surface of endothelial cells, which conveys a negative charge to the endothelium




## 1.3.2.1.3 The longitudinal change of peritoneal membrane

### *1.3.2.1.3.1 Functional changes*

Different from haemodialysis, the dialysis membrane we use in PD, the peritoneum, changes with time. Membrane failure is the main cause for technical failure in long term PD. It has been shown that ultrafiltration decreases and PSTR increase with time on PD. [95, 96] The increase in PSTR with time is preceded by increased peritoneal exposure to hypertonic glucose. [96, 97] Apart from dialysate, severe and repeated peritonitis may also contribute to the change of membrane function. [98]

Membrane failure, as a result of membrane deterioration, can be subdivided into two group, type I and type II. Type I membrane failure is the most common cause of ultrafiltration failure and is characterized as an increase in PSTR. The increased PSTR causes the rapid loss of the osmotic gradient and insufficient fluid ultrafiltration combined with increased reabsorption in the long dwells. Type II membrane failure is relatively rare and usually seen in long-term patients and is characterized by a reduction in osmotic conductance of the membrane. Clinically, it is evidenced by the observation that in long term PD patients, UF capacity decrease disproportionally to the increase of PSTR. [95]

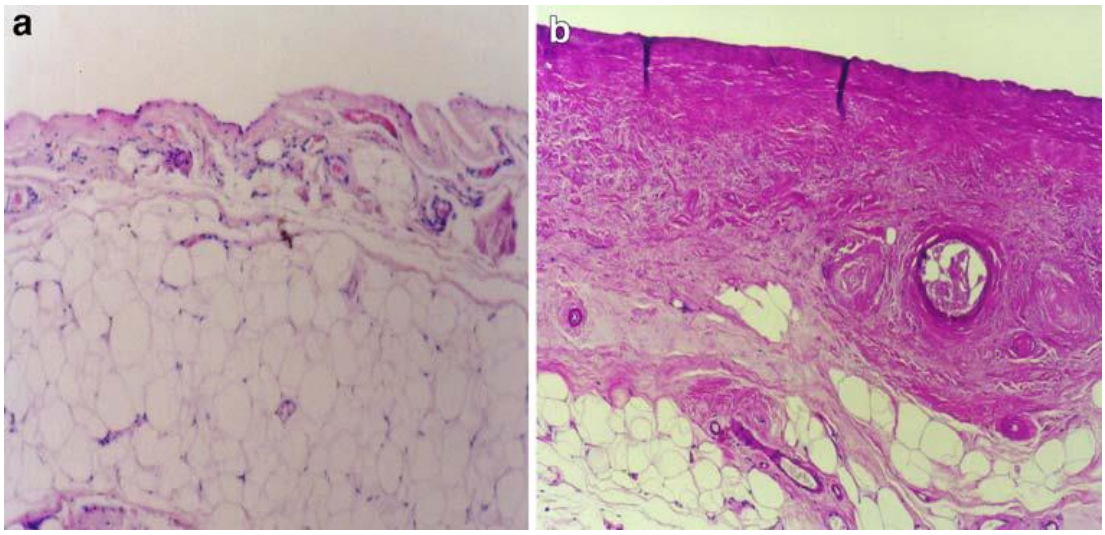
Data on longitudinal change of macromolecular transport is limited. Large observational cohort study is needed to understand the longitudinal change of the peritoneal protein permeability.

#### *1.3.2.1.3.2 Morphological change*

It is known that peritoneal membrane changes in morphology with prolonged time on PD. The morphological changes include mesothelial cell denudation, interstitial fibrosis, vascular changes and neovascularization. (Figure 1-6) Vascular changes include progressive subendothelial hyalinization, with luminal narrowing or obliteration. The vascular changes are similar to diabetic vascular changes but are unique in terms of the fact that it affects both venules and arterioles. Williams and colleagues examined biopsy samples from four groups of patients, including those who had normal renal function,

patients who had been treated only with haemodialysis, uremic patients who had never been dialyzed and PD patients. The results have shown that membrane morphology changes are present even in uremic patients who are not yet on dialysis and more severe in patients undergoing PD. In the PD group, the dialysis regimen is likely to associate to the morphology change of peritoneum. The prevalence of vasculopathy increases significantly with time on therapy. The degree of fibrosis and thickness of the overlying structure under the peritoneum are also directly related to the duration of PD. [99] The increase of the submesothelial fibrous tissue was related to cumulative glucose load on PD. [100] Patients on automatic PD (APD) tend to have increased submesothelial stroma and vascularization, in spite of the fact that they are treated for a shorter period of time than the continuous ambulatory PD (CAPD) group. Peritonitis is also a contributor for morphological change. A marked loss of mesothelial cells has been observed in cases with two or more episodes of peritonitis. [100]

Figure 1-6 typical morphology change of long term PD




a) at the beginning of PD b) after 6 years of PD

Sub-mesothelial fibrosis, increased numbers of vessels and vasculopathy are obvious in long term PD. [101]

#### *1.3.2.1.3.3 Link between functional change and morphological change*

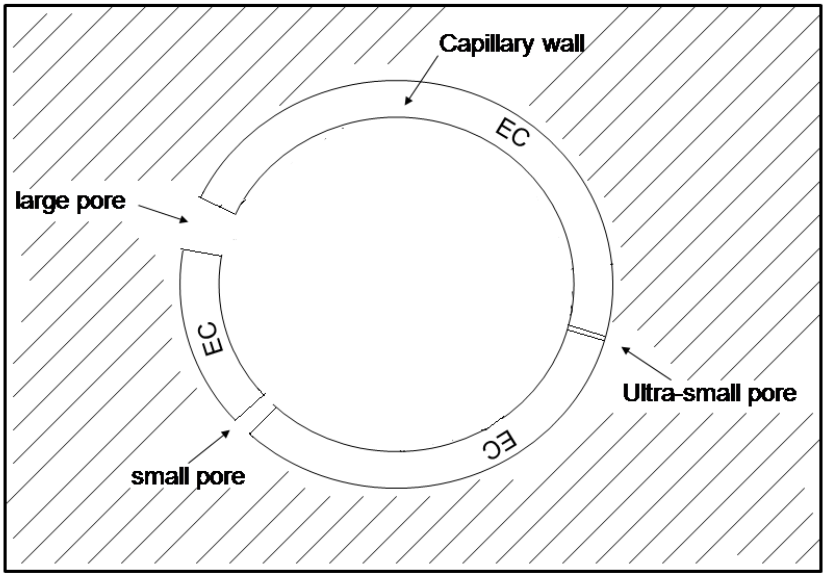
Plum and colleagues further reported the relation between functional changes and histological changes in the peritoneum. Patients with a high PSTR have significantly thicker submesothelial fibrous layers. [100] Numata and his colleagues gave more data on the link between morphology change and transport rate of β2-microglobulin and albumin. They have found that both PSTR of creatinine and β2-microglobulin is associated with relative microvessel area (RVA) which is calculated as total area of microvessels/total area of peritoneal field but not with relative microvessel number (RVN), while no statistic relationship is found between the dialysate and plasma ratio for albumin and RVA or RVN.

[102] The synchronous data for the relationship between peritoneal Pcl and fibrosis and vasculopathy is not seen.

#### *1.3.2.1.3.4 The three-pore membrane/fiber matrix model*

Recently, the three-pore model has been extended into the three-pore membrane/fiber matrix model (Figure 1-7), the three-pore model to the capillaries and the fiber-matrix model to the intestitium. [103] It fits the observation in severe ultrafiltration (UF) failure (type II membrane failure) by uncoupling PSTR (peritoneal mass transfer area coefficient for glucose (PS(g))) from peritoneal glucose osmotic conductance ($L_pS\sigma_g$). When increases in vascular surface area are combined with further increase in fiber density, peritoneal UF coefficient (L(p)S) remains largely unchanged. However, it is not known whether this fiber matrix restrict the large molecular flow.

Figure 1-7 three-pore membrane/fiber matrix serial barrier model




## 1.3.3 What causes protein leak in capillary through an "endothelium-dependent" pathway?

### 1.3.3.1 Is there a link between systemic factors and peritoneal Pcl?

In PD, Van Biesen and colleagues have shown that patients with systemic inflammation have a higher peritoneal Pcl. Furthermore, there was no difference in membrane area parameters between patients with and without systemic inflammation in that study raising the possibility that this relationship may not couple with PSTR. [71] It is not clear whether the relationship between systemic inflammation and peritoneal Pcl is because the patients with systemic inflammation also have inflamed membranes.

One study in a group of pediatric PD patients has shown that peritoneal protein losses in patients with steroid-resistant nephrotic syndrome (SRNS) are twice as great as in those without nephrotic syndrome which is thought to be the result of the systemic effect of a "circulating factor" in SRNS. [104] It gives us another example of how a systemic factor might affect local protein leak.

#### 1.3.3.2 Is there a link between local peritoneal membrane injury and peritoneal Pcl?

Peritoneal membrane inflammation does occur in PD and is thought to be the main cause of membrane deterioration in PD. The non-physiologic nature of dialysate includes different factors, eg. high osmolarity, buffer composition, high dextrose concentrations, and glucose degradation products (GDPs) formed during heat sterilization have been shown to damage different type of peritoneal cells, [105-109] all of which may trigger an inflammatory response. GDPs and subsequent formation of advanced glycation end products (AGE) induce inflammatory activation of cells. [110-112] The use of a more biocompatible, neutral pH PD solution with a low concentration of GDPs has been shown to result in a significant decrease in dialysate effluent IL-6 levels compared to a conventional acidic PD solution, suggesting a reduction of intra-peritoneal inflammation.[113, 114]

Several studies have shown that local chronic inflammation is associated with PSTR in incident patients [115, 116] and may be related to PSTR change with time. [117] The

synchronous data on large pore flux is limited. As the absolute large pore flux is a function of membrane area, it is also important to understand whether the change of large pore flux is proportional to the change of membrane area. The peritoneal Pcl is sharply increased in acute peritonitis [68] and clearly it is not proportional to the increase of PSTR in this condition. Data in stable CAPD patients have shown that the dialysate appearance of IL-6 correlates with albumin and IgG clearances, which is also related to small solute transport at the same time. [118] It is not clear whether the inflammation associated peritoneum Pcl increase is coupled with the change in PSTR through that study.

#### 1.3.3.3 The association between local peritoneal membrane factors and systemic factors

Another point need to be noted is the intraperitoneal inflammation is related to systemic inflammation [117] but cannot be completely explained by systemic inflammation. Actually, dialysate IL-6 levels are even higher than plasma levels despite dilution by dialysate. Other pro-inflammation factors, such as IL-1, TNF-α, IFN, are usually low but can be elevated above plasma. (unpublished data from the GLOBAL study) A short-term randomized controlled study has shown that a new biocompatible bicarbonate/lactate-based solution having low concentration of GDPs, resulted in decreased peritoneal appearance rate of IL-6 compared with conventional lactate-based PD solution, whereas systemic levels of inflammatory markers did not differ between the two solutions. [119] The clinical benefit of the biocompatible solution is still under debate. [120] Actually, the largest

randomized study comparing biocompatible and conventional solutions did not detect any clinically significant advantages in terms of technique survival or peritonitis. [121]

#### 1.3.3.4 The potential cause of protein leak in capillary

##### 1.3.3.4.1 Inflammation and others

###### *1.3.3.4.1.1 Inflammation*

Inflammation is the predominant cause of capillary hyperpermeability. Capillary permeability is increased both in acute and chronic inflammation. TNF-α, as an example of classical pro-inflammation cytokine, has been well investigated in this area and it regulates vascular permeability through different pathways. Koga et al has shown that TNF increases endothelial cell permeability by decreasing adenosine 3', 5'-cyclic monophosphate (cAMP). [122] The TNF-α induced hyperpermeability can be suppressed by adiponectin via a cAMP/protein kinase A (PKA) signaling. [123] TNF-α also up-regulates the phosphodiesterase2 expression in a p38 mitogen-activated protein kinase (MAPK)-dependent manner and results in destabilization of endothelial barrier function. [124] Others have found that TNF-α induces endothelial actin microfilament rearrangement, microtubule destabilization and intercellar gap formation that parallels the development of transendothelial permeability. [125-127] TNF-α is also thought to disrupt the glycocalyx and lead to an increase in macromolecular permeation. [90]

#### *1.3.3.4.1.2 Platelet activation*

There is a link between inflammation as a trigger for platelet activation in vascular lesions. These pathways may however act either independently or sequentially; activated platelets can induce secretion of chemokines for monocyte recruitment [128] whereas single microvessel perfusion with TNF-$\alpha$ alone, without platelet activation, does not alter the endothelial permeability [129]. In an aseptic animal injury model, systemic depletion of neutrophils with antibody failed to prevent the increase in vascular permeability, whereas antiplatelet pre-treatment reduced this by 25% [130]. These findings suggest that platelet activation may have a critical role in endothelial hyper permeability that may not necessarily be through inflammation, especially in the low grade inflammatory status seen in uremia.

#### *1.3.3.4.1.3 Others*

In addition to inflammation and platelet activation, hyperglycemia [131], reactive oxygen species [132] and AGEs [133] are also thought to increase endothelial permeability.

### 1.3.3.4.2 Angiogenesis

The importance of angiogenesis to protein leak is not only the increase in the endothelial surface area but also the increasing permeability. New vessels tend to be leaky vessels. Vascular endothelial growth factor (VEGF), which was initially discovered as a prime regulator of angiogenesis, has also been found to effect vascular permeability. [134, 135]

Brown and colleagues have shown a possible link between angiogenesis and hyperpermeability. The study in rat has shown that after electrical stimulation which resulted in the growth of new microvasculature. The glycocalyx is continuous in only 10% of the capillaries and totally absent on 44%-58% of the angiogenic capillaries. The fact that new born vessels have less glycocalyx may lead to hyper permeability. [136]

ESRD patients have a significantly increased density of small vessels and capillaries in the peritoneal membrane compared with normal controls and this morphological change tends to be more severe in PD patients. [99, 100] Angiogenesis is an important cause of increase in membrane surface area and is thought to contribute to type III high PSTR.

In fact, it is found that the dialysate appearance of VEGF correlated with albumin and IgG clearances, as well as with small solute transport, while it is not clear whether it is a result of the increased effective surface area. [118] In theory, as mentioned before, new vessels also tend to be leaky. However based on the limited longitudinal data about peritoneal leak, the peritoneal Pcl tends to be stable through the time on PD [69, 72] while the small solutes transport rate, a measure of membrane area, increases with time. Actually, it is in keeping with the findings by Numata, M. that no statistical relationship was found between the dialysate and plasma ratio for albumin and relative microvessel area or relative microvessel number, which represents the degree of angiogenesis. [102]

#### 1.3.3.4.3 Hemodynamic factors

According to the Starling Equation and three-pore model, intracapillary hydrostatic pressure is one of the determinate factors of capillary Pcl. Local intracapillary hydrostatic pressure is determined by both systemic and local factors and it is different between organs. We will focus on peritoneal cavity.

Intracapillary hydrostatic pressure is highest at the arteriolar end of the capillary and lowest at the venular end. The average capillary hydrostatic pressure is determined by arterial and venous pressures and also by the ratio of post-to-precapillary resistances. The signaling molecules released by endothelial cells act on the smooth muscle cells in the walls of nearby vessels and take part in the control of vascular resistance. It is also affected by the autonomic nervous system and the viscosity of blood. Pre-capillary (mainly arteriolar) vasodilation increases intracapillary hydrostatic pressure and post-capillary vasodilation decreases it. An increase in either arterial or venous pressure will increase intracapillary pressure. Change in venous pressure has more effect on intracapillary pressure changing because venous resistance is relatively low and pressure in veins is easily transmitted to the capillary in general. [77] However, in the case of PD, at least for visceral peritoneum, because the venous pressure is coupled with intraperitoneal hydrostatic pressure, which is also coupled with interstitial hydrostatic pressure, the importance of venous pressure in net Starling force is decreased. As a whole,

both systemic artery pressure and local control of vascular resistance may contribute to Starling force of protein in PD.

Few data are available about the relationship between systemic artery pressure and peritoneal Pcl. Data from our centre have shown that pulse pressure is positively related to peritoneal Pcl. [61] (Figure 1-8) However, it is difficult to distinguish whether the relationship between Pcl and pulse pressure is from the hemodynamic effect or as it reflects general vascular damage.

Figure 1-8 The association between peritoneal protein clearance (Pcl) and pulse pressure. [61]

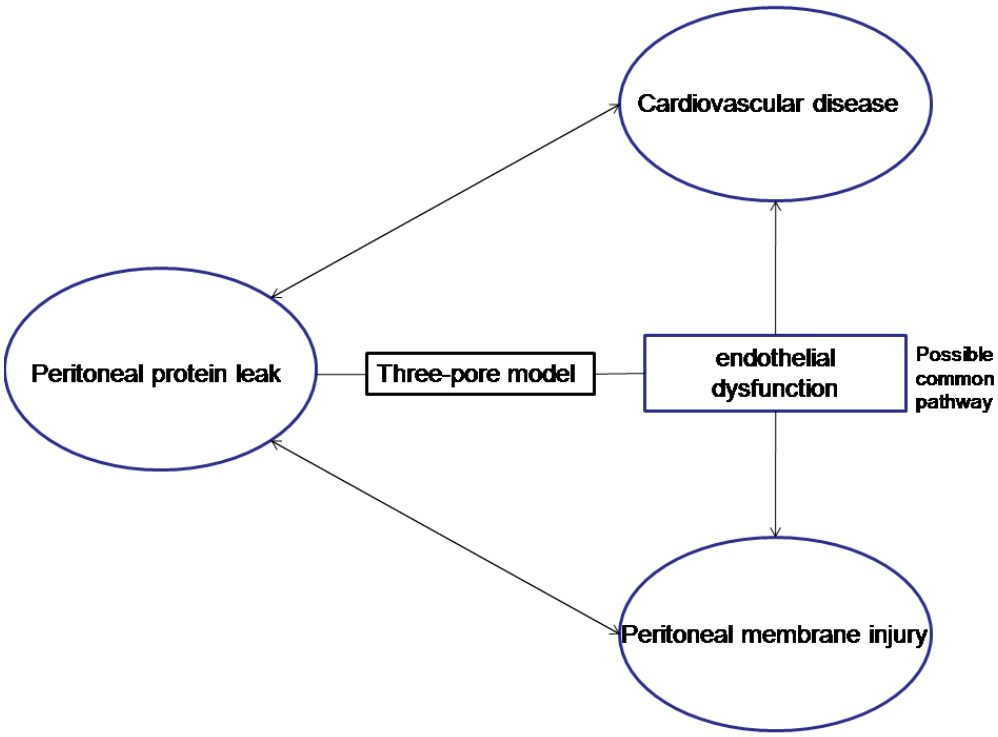
Peritoneal vasodilatation is thought to occur in the early phrase of the dwell in PD in most of commercially available clinical PD solutions. [137-139] Data from rats have shown that

infusion with glucose 4.25% Dianeal (Baxter Healthcare), the most widely used standard, low biocompatibility dextrose based commercially available dialysate, results in a doubling of the arteriolar flow and a 20% increase of the perfused capillary length per area. The glucose 1.5% Dianeal (Baxter Healthcare) fluid induces similar but less pronounced changes. [140] No significant difference in Pcl between different dialysates was found in acute exposure human studies [141] in spite of the fact that their vasodilatation effect is different. Peritoneal blood flow seems to have minor effect on Pcl, [137, 142, 143] while small solute transport is blood flow limited. [143] It is not clear whether long term exposure to elevated blood flow and perfusion pressure will cause structural and functional changes as seen in hypertension and diabetic nephropathy.

## 1.4 Hypothesis and research questions

It is hypothesized that peritoneal Pcl reflects a systemic abnormality of the capillary circulation, specifically at the level of the endothelium, rather than evidence of the local effects of the process of dialysis treatment which is known to cause local peritoneal membrane injury especially with time on therapy. This systemic injury has important implications for patient management through its effects on plasma albumin. (Figure 1-9)

Figure 1-9 hypothesis of the thesis




To test this hypothesis, several focused research questions were developed:

1. Is peritoneal Pcl related to comorbidity, local peritoneal membrane inflammation or both at the commencement of PD? Which of these are predictors of patient survival?
2. Does peritoneal Pcl change with time on PD? If so, is this change over time coupled with the known increase in peritoneal membrane area?

3. Does the UF obtained with icodextrin – which only occurs via the small pore pathway, change with time on PD? If so, is this change over time dissociated from the increase in peritoneal membrane small pore area?
4. What is the relationship between soluble biomarkers of inflammation and endothelial damage, systemic transcapillary albumin leak and hypoalbuminaemia in PD patients?
5. What is the relationship between soluble endothelial biomarker pattern, hydration status and hypoalbuminaemia in PD patients?

# chapter 2.**Methodology**

## 2.1 Study setting and cohorts

Three different patient cohorts were used in this project-the Stoke PD cohort, the GLOBAL fluid study (Longitudinal evaluation of peritoneal membrane function, inflammation and structural integrity in peritoneal dialysis MREC 02/9/14) cohort and the BIA study (Developing Bioimpedance (BIA) as a tool for fluid management in Peritoneal Dialysis Patients: A validation Study ClinicalTrials.gov NO: NCT00801112) cohort. The study specific eligible and exclusive criteria were described in each study.

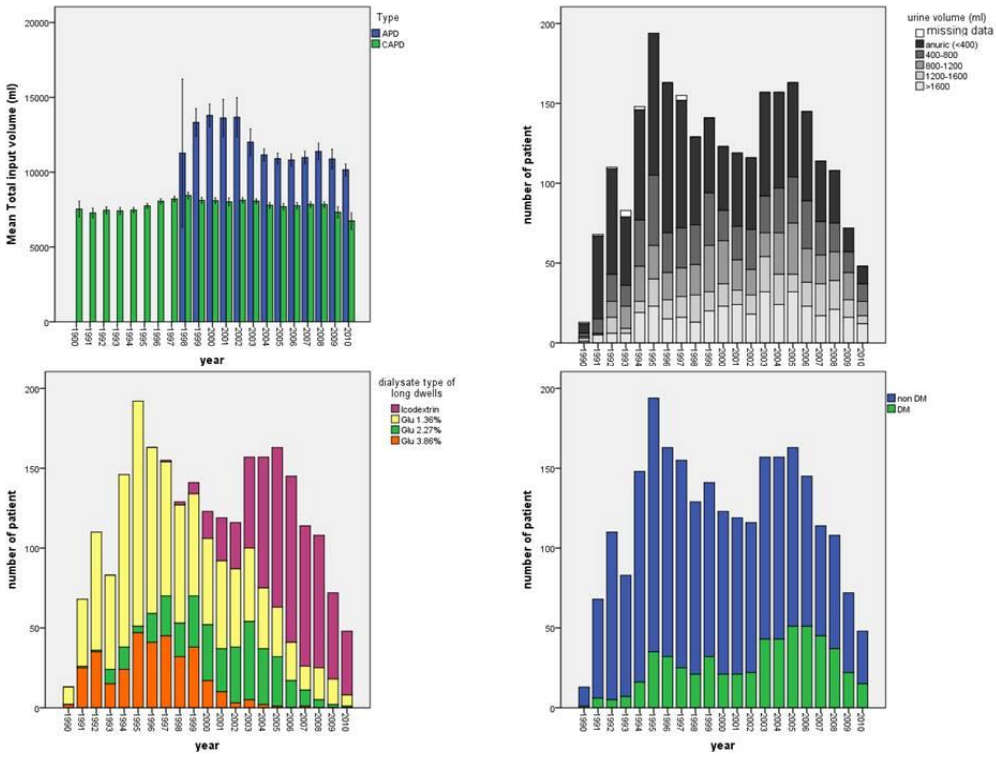
### 2.1.1 The Stoke PD cohort

This was a single centre, longitudinal, observational cohort. The data were prospectively collected since 1990. Peritoneal membrane function using peritoneal equilibration test (PET) and dialysis adequacy were routinely measured within the first one or two months of treatment, and then usually at 6 monthly interval unless prevented by acute illness or subject availability. The information about PD prescription, ultrafiltration (UF) of each exchange and urine volume on the day of dialysis adequacy test was recorded. Baseline data including demography, primary renal disease and comorbidity, were recorded when commencing PD.

There was a major change in clinical policy in June 1998. Before June 1998, vast majority of the patients had been treated with CAPD and glucose based solutions. The clinical policy has changed since then. In brief, an increasing proportion of patients were treated

with APD, particularly when anuric and with difficulties in achieving clearance target of 60L/week/1.73m$^2$, as described in the European Automated Peritoneal Dialysis Outcome Study. Patients' choice, mainly related to their life style, was also taken into concern in the modality choice (APD or CAPD). Icodextrin was more likely to be used in faster transporters, especially when combined with clinical difficulties of fluid management. No more than one bag of icodextrin was used per day. [95] Apart from this major practice change in 1998, it is also important to note the drift of the prevalent PD cohort, which generally accompanied with the global change of PD therapy. For example, there were more diabetic patients on PD therapy compared with 20 years ago (Figure 2-1). (Data censored at Oct 2010)

Figure 2-1 the prevalent Stoke PD cohort and the change of practice pattern (Jan 1990-Oct 2010)



### 2.1.2 The GLOBAL fluid study cohort

The GLOBAL Fluid Study was a multi-centre, multi-national prospective, cohort study. Samples of dialysate and plasma and complete clinical phenotype were prospectively collected when patients were undergoing routine pre-determined clinical assessments of membrane function. Baseline data and samples were collected within three months of treatment start. The clinical characteristics, including membrane function, biochemistry profiles and comorbidity were estimated locally. Dialysate and plasma samples were sent to central lab for inflammation cytokine measurements.

### 2.1.3 The BIA study cohort

The BIA study was a multicentre, prospective, randomized controlled trial. The detailed study design is shown in appendix. (chapter 9.3) It was a prevalent cohort. The baseline data in the three UK centres was used in this project. The three UK centres were University Hospital of North Staffordshire, St. James's University Hospital and Sheffield Kidney Institute Northern General Hospital. All of them were university hospitals with well established PD programmes. The patients in the study were clinically stable and with optimal fluid status from clinical point of view.

The clinical characteristics, including membrane function, biochemistry profiles and comorbidity were estimated locally. The blood samples were sent to University Hospital of North Staffordshire shortly after being collected for biomarker analysis.

## 2.2 Measurement of biomarkers

### 2.2.1 Luminex

Luminex suspension array system (Bio-Rad) was used in this project to measure a panel of soluble endothelial biomarkers. The system is fundamentally a bead-based ELISA. The microsphere beads contain a 2-dye fluorescent variable intensity, which creates a 100-distinct bead set, each of which is coated with a specific capture antibody. The capture antibodies bind to analyte, which are then tagged by fluorescent reporter labels. The beads pass through 2 laser column. One laser column excites the internal dyes marking the microsphere set. The other laser column detects the fluorescent dye on the reporter molecules. (see Figure 2-2)

Figure 2-2 principle of Lumenix

(Adapted from http://www1.imperial.ac.uk/resources/6DB4DBCF-7A48-4655-85ED-21C3E2C63932/)

The assay procedure were followed the manufacturer's recommendation. It was read on a Bio-Plex 200TM system (Bio-Rad). Duplicate measurement was carried out. For those with more than 10% difference between the duplicated wells, the measurements were repeated. The quality was also controlled by the in-house Quality Control. Two Quality Controls with known concentration were available in each kit. Interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), CD40 ligand (CD40-L) and

vascular endothelial growth factor (VEGF) were measured by Human cytokine/chemokine MAP multiplex kit (Millipore corporation, USA). The brief description of the protocol was as following.

#### 2.2.1.1 Preparation of samples

The plasma samples were removed from storage at -20°C and were thawed at 4°C. Samples were centrifuged at 13,200 rpm for 10 minutes at 4°C to clear the precipitate.

#### 2.2.1.2 Preparation of antibody-immobilized beads

1. Sonicate each antibody-bead vial for 30 seconds and vortex for 1 minute.
2. Add 60 µl from each antibody bead vial to the mixing bottle (60×5=300µl) and bring final volume to 3.0ml with bead diluent (2.7ml).
3. Vortex the mixed beads for 1 minute.

#### 2.2.1.3 Preparation of Quality Controls

Reconstitute quality control 1 and quality control 2 with 250 µl deionized water. Invert the vial several times and vortex for 30 seconds.

#### 2.2.1.4 Preparation of Wash Buffer

Vortex the 10X Wash Buffer for 1 minute to bring all salts into solution. Dilute 30 ml of 10X Wash Buffer with 270ml deionized water.

#### 2.2.1.5 Preparation of Serum Matrix

Add 1.0 ml deionized water to the bottle containing lyophilized Serum Matrix. Invert the vial several times.

#### 2.2.1.6 Preparation of Standard

Reconstitute the Human Cytokine Standard with 250 µl deionized water. Invert the vial several times and vortex for 10 seconds. After sitting the vial for 10 minutes, transfer the standard to the polypropylene microfuge tube labeled as S1, which serve as the highest standard (10000pg/ml).

Add 200 µl of Assay Buffer to each of the five tubes labeled as S2-S6. A 1 in 5 series dilution was carried out by transferring 50 µl of solutions from the tube ahead to the next one. The Standard Concentration was as following, S1-10000pg/ml, S2-2000pg/ml, S3-400pg/ml, S4-80pg/ml, S5-16pg/ml, S6-3.2pg/ml. Assay Buffer was used for background (S7-0pg/ml).

#### 2.2.1.7 Assay procedure

1. Bring all diluted standards, samples and controls, and buffer solutions to room temperature 20 minutes prior to use. Sit the plate on a plate holder so that the bottom of the plate does not touch any surface during the whole assay process.

2. Pre-wet the 96-well filter plate with 200 µl of Assay Buffer. Seal and shake the plate on a plate shaker for 10 minutes. Remove Assay Buffer by vacuum. Blot excess fluid from the bottom of the plate with paper towels.
3. Add 25 µl of each Standard or Control into the appropriate wells.
4. Add 25 µl of Assay Buffer to the sample wells
5. Add 25 µl of Samples to the sample wells
6. Add 25 µl of Serum Matrix to background, standards and control wells
7. Vortex Mixing Bottle for 1 minute. Add 25 µl of the Mixed Beads to each well.
8. Seal the plate with a plate sealer and wrap it on the plate shaker together with the plate holder. Shake on the plate shaker for 1 hour at room temperature.
9. Remove fluid by vacuum.
10. Wash the plate with 200 µl of Wash Buffer per well and remove Wash Buffer by vacuum. Repeat the wash/vacuum step for 2 times. Blot excess fluid from the bottom of the plate with paper towels.
11. Add 25 µl of Detection Antibodies into each well.
12. Seal the plate with a plate sealer and wrap it on the plate shaker together with the plate holder. Shake on the plate shaker for 30 minutes at room temperature.
13. Add 25 µl Streptavidin-Phycoerythrin to each well.
14. Seal the plate with a plate sealer and wrap it on the plate shaker together with the plate holder. Shake on the plate shaker for 30 minutes at room temperature.
15. Remove fluid by vacuum.

16. Wash the plate as in step 10 for 2 times.

17. Add 150 µl of Sheath Fluid to each well.

18. Resuspend the beads on a plate shaker for 5 minutes.

19. Run the plate on Bio-Plex 200TM system (Bio-Rad).

## 2.2.2 ELISA

ELISA was applied in this project to measure MMP 8, MMP 9 (in the BIA study) and platelet factor (PF) 4. Duoset Human ELISA kits (R&D Systems, Minneapolis, USA) based on a sandwich ELISA method were used. It was read on a TiterTek Multiskan Plus MKII microplate reader (Flow Laboratories Ltd., Richmansworth, UK). The assay was run following the manufacturer's standard protocol. Duplicate measurement was carried out. For those with more than 10% difference between the duplicated wells, the measurements were repeated. Plasma samples were used in all three biomarkers. A pre-assay was run to decide the best sample dilution rate. The dilution rate for MMP8, MMP9 and PF4 was 1:2, 1:400 and 1:4900.

A brief MMP8 ELISA procedure is descripted as following.

### 2.2.2.1 Plate Preparation

1. Capture antibody (Mouse anti-human MMP-8) was reconstituted with 1 ml of PBS. The reconstituted capture antibody (360 µl/ml) was stored at 4°C. It was further diluted to a working concentration of 2.0µg/mL in PBS. 100 µl of the diluted

capture antibody was added into each well of a 96-well microplate (Costar 3590, Corning Incorporated, NY, USA) to coat the plate. The plate was sealed with foil and incubated overnight at room temperature.

2. Aspirate each well and wash with was wash buffer. Washing was carried out 4 times. Each wash step was performed by filling each well with 300 µl of Wash Buffer (0.05% Tween® 20 in PBS) and removing the liquid by inverting the plate and blotting it against clean paper towels.
3. 300 µl of Reagent Diluent was added into each well and the plate was incubated at room temperature for a minimum of 1 hour to block the plate.

#### 2.2.2.2 Sample preparation

The plasma samples were removed from storage at -20°C and were thawed at 4°C. For MMP8, sample dilution was done on the plate by adding 50 µL of plasma and 50 µL of reagent diluent into each sample well. (For MMP9 and PF 4 sample dilutions were done in the sample preparation step.)

#### 2.2.2.3 Standard preparation

Reconstitute Recombinant Human MMP-8 with 0.5 mL of deionized water to a concentration of 120ng/mL. A seven point standard curve was prepared by using 2-fold serial dilutions with reagent diluent. The high standard was at 4000 pg/mL.

#### 2.2.2.4 Biotinylated Detection Antibody preparation

Reconstitute Biotinylated goat anti-human MMP-8 with 1.0 mL of reagent diluent to a concentration of 36µg/mL. A working concentration of 200 ng/mL was prepare 1-2 hours prior to use with reagent diluent with 2% heat inactivated normal goat serum (NGS).

#### 2.2.2.5 Streptavidin-HRP

Dilute to the working concentration of 1:200 using reagent diluent.

#### 2.2.2.6 Assay Procedure

1.Add 100 µL of standards in Reagent Diluent or diluted samples (50 µL plasma and 50 µL reagent diluent) per well. Cover with an adhesive strip and incubate 2 hours at room temperature.

2.Repeat the aspiration/wash as in step 2 of Plate Preparation.

3.Add 100 µL of the Detection Antibody, diluted in Reagent Diluent with NGS, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.

4.Repeat the aspiration/wash as in step 2.

5.Add 100 µL of the working dilution of Streptavidin-HRP to each well. Cover the plate with a lightproof adhesive strip to avoid direct light and incubate for 20 minutes at room temperature.

6.Repeat the aspiration/wash as in step 2.

7.Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature with a lightproof adhesive strip to avoid direct light.

8.Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

9.Determine the optical density of each well immediately, using the TiterTek Multiskan Plus MKII microplate reader set to 550 nm for wavelength correction and 450 nm for result-obtaining.

### 2.2.3 Mesoscale Discovery Platform (in GLOBAL cohort)

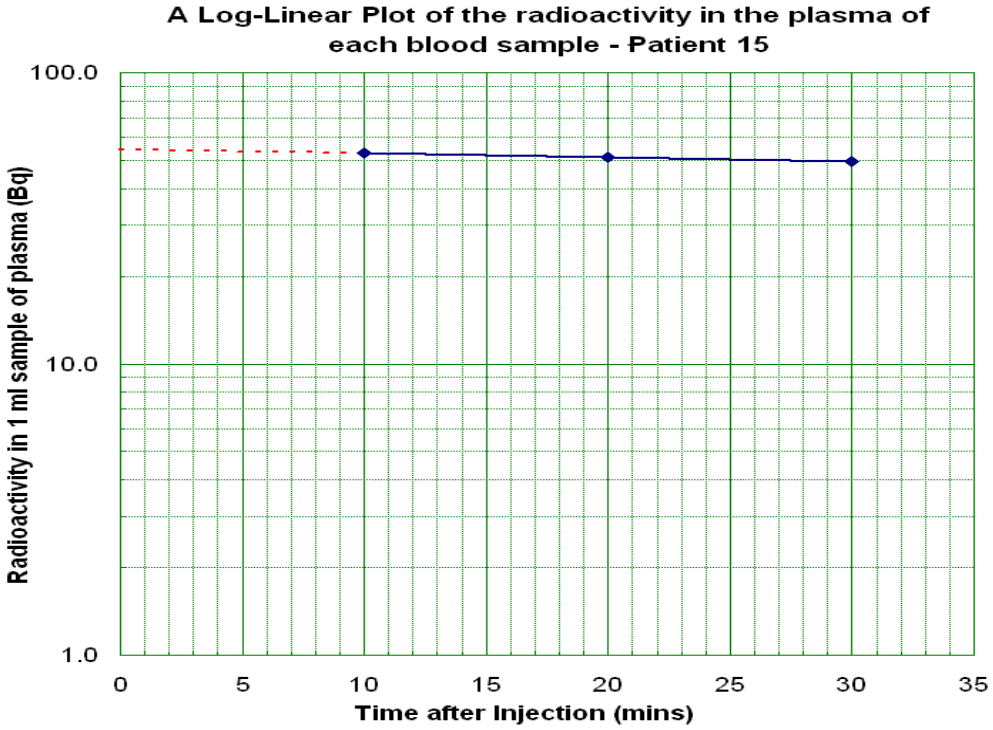
IL-1β, TNF-α, IFN-γ and IL-6 in GLOBAL cohort were measured by the GLOBAL study group. Electrochemiluminescence immune assay was used. It was carried out by the commercially available Pro-Inflammatory I 4-plex (Meso-Scale Discovery, Gaithersburg, Maryland, USA).

## 2.3 Measurement of systemic albumin leak – Transcapillary escape rate of 125-I albumin (TERalb)

Transcapillary escape rate of 125-I albumin ($TER_{alb}$) was measured by the Medical Physics department in University Hospital of North Staffordshire. The method was also applied for clinical purposes as a measurement of plasma volume to normalise the red cell mass mainly for diagnosis of primary polycythaemia.

In brief, after a bolus intravenous injection of 10ml 0.185MBq $^{125}$I-human serum albumin (HSA), blood samples were taken at 10, 20, and 30 min at a remote venous site. The isotopic concentration in each case was plotted against time on a semi-logarithmic scale and the best linear fit line was performed from these points. $TER_{alb}$, expressed as percentage loss per hour (%/h) was estimated from the disappearance rate of the $^{125}$I HSA from the gradient of this line. The plasma volume was calculated by extrapolating the line to time zero. (Figure 2-3)

Figure 2-3 calculate TERalb from the disappearance rate of the 125I HSA from a on a semi-logarithmic plot



## 2.4 Measurement of body composition

### 2.4.1 Deuterium Oxide (D2O) dilution technique

The D2O dilution technique was used for absolute measurement of total body water (TBW) in the project (chapter 6). It has been described in detail previously.[144, 145] In brief, following a baseline blood sample, an oral dose of 99.8% D2O (15-45 ml according to body weight, Cambridge Isotope Laboratories) was administered. Two and a half hours later, when D2O was equilibrated to TBW, a second blood sample was taken from the same cannula. Whole blood samples were stored for further measurement. Based on the facile exchange of deuterium/hydrogen with the hydronium ions, H3O+, their hydrates $H_3O^+.(H_2O)$ and water vapour in the gas phase, the blood headspace D abundance was measured by Flowing Afterglow Mass Spectrometer (FA-MS). The more detailed principle of FA-MS has been described previously. [146] The difference between baseline and equilibrated blood D2O concentration was used to determine TBW. A 4% reduction was taken to account for the H-D exchange that occurs between the HDO with carboxyl and amino groups in body proteins. [147, 148]

### 2.4.2 Bioimpedance analysis (BIA)

Estimated total body water (TBW) and extra-cellular water (ECW) were evaluated by the multi-frequency bioimpedance device (BIA, multi-frequency Xitron Hydra device, Model 4200, Xitron Technologies, San Diego, CA) in pilot biomarker study (Chapter 6). No dialysis

fluid was in the peritoneal cavity during measurement.TBW and ECW were derived based on the manufacturer's impedance algorithm.

The single frequency (50Hz) BIA device (Akern BIA 101 Anniversary) was used in the multicentre BIA study (chapter 7) for estimated TBW and ECW measurement. The patient might present with or without PD fluid in the peritoneal cavity when the measurement was carried out but this was consistent during the follow up. The resistance and reactance of the whole body were recorded. TBW and ECW were calculated by formula which had good agreement with the manufacturer's algorithm. (see appendix for detail - chapter 9.4)

The measurements were carried out according to the operating manuals, respectively. The measurement procedure was similar. In brief, patients lay in a supine position on the couch for at least 2 minutes. The electrodes were placed between the ipsilateral bony prominences of the wrist and of the ankle with at least 5 cm apart. (Figure 2-4) An alternating current passed through the body. The resistance and reactance of the whole body were measured for further calculation of TBW and ECW.

Figure 2-4 A schematic diagram showing the placement of adhesive pads in BIA measurement

Adapted from the manual of Akern BIA 101 Anniversary

## 2.5 Measurement of peritoneal membrane function, ultrafiltration and peritoneal Pcl in Stoke

### 2.5.1 Peritoneal equilibration test (PET)

We estimated PSTR through measuring dialysate:plasma ratio of creatinine (D/P creatinine) at the end of a standard 2L, 2.27%, 4 hour dwell in peritoneal equilibration test (PET). Net UF from this standard dwell was used as a measure of UF capacity.

The clinical laboratory upgraded the methodology of measuring plasma creatinine from the Oleary method to an enzymatic method on 29th Oct 2008 and the methodology of dialysate creatinine had not changed during the study period. This methodology change gave an about 0.06 decrease in D/P creatinine. The data had been carefully adjusted for longitudinal analysis. See appendix (chapter 9.2) for detail about this methodology change.

### 2.5.2 Peritoneal protein clearance (Pcl)

The peritoneal dialysate protein loss was measured from the collection of 24-hour peritoneal dialysate effluent. A validated correction factor was used for the calculation of peritoneal Pcl: 24 hour dialysate protein loss / (serum albumin/0.4783). [68] Pcl was expressed as mL/day.

Plasma albumin levels had been measured using the bromocresol green method before 22th Aug 2007 and was switched to bromocresol purple method afterward. Both

methods were run on the Siemens ADVIA 2400 platform. In 86 patients, who had plasma albumin measured once within 12 months before and once within 12 months after the switch data (22th Aug 2007), an average difference of 5.45g/L was found by paired t test ($P<0.0001$). It fits with the observation by Catherine M. Clase et al, where the average difference between the two methods in the same sample was 5.5g/L[149] So the convert equation, $Alb_{bromocresol\ green}=5.5+Alb_{bromocresol\ purple}$ was used in the study.

### 2.5.3 Measurement of ultrafiltration (UF)

For APD, the UF volume was measured by the APD device. Net UF volume from CAPD was accessed by weighing the drainage bag, converting the weight into volume by multiplying the specific gravity of 1g/ml and subtracting the expected input volume accounting for the average 'overfill', which had consistently been measured at 200mls in Stoke.

Two common issues in measurement of UF have been noticed and carefully addressed or acknowledged. Firstly, the 'overfill' has been accounted in all of the Stoke cohort based studies. It may explain part of the centre effect in the multicentre cohorts. Secondly, the specific gravity issue has been noted. The specific gravities of the input and output dialysate are different from pure water and it is related to the glucose concentration and type of dialysate. See details in appendix. (chapter 9.1)

## 2.6 Statistical analysis

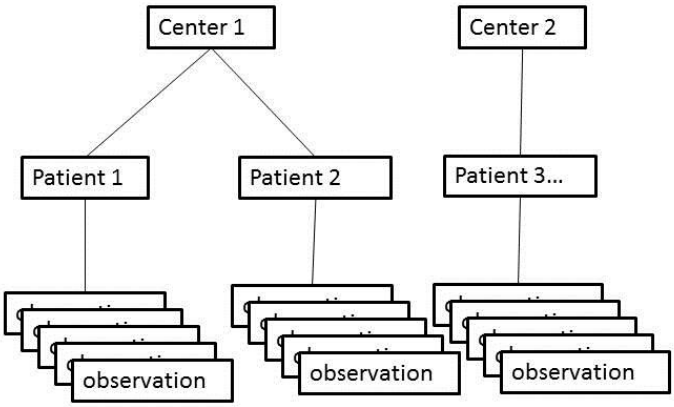
The study specific statistical methods were described in each study. Multilevel modelling and principal component analysis (PCA) combined with hierarchical clustering analysis (HCA) were the two main statistical strategies used in the project. The characteristics and general approach of the two strategies are summarized as follows.

### 2.6.1 Multi-level analysis

#### 2.6.1.1 Characteristics

Multi-level modeling is essentially an extension of ordinary multiple regression modeling but with special design for data sets with a clustered (hierarchical) structure. (Figure 2-5) The importance of recognizing the clustering nature of the data has been recognized for long time and has been viewed as a problem in the statistics world. Multi-level modeling is a strategy specially designed for this and more importantly, it allows additional features of the data to be exploited. (refer to http://www.bristol.ac.uk/cmm/software/mlwin/)

Figure 2-5 an example of mulit-level structure




### 2.6.1.1.1 The situations that multi-level structure has to be considered

One critical assumption of the single-level multiple regression model is that each observation should be independent. From a statistical point of view, the residuals should be uncorrelated with one another. It is obviously not the case in the longitudinal cohort study. The series observations from the same individual would be expected to highly correlate with each other. One potential option is to have a set of dummy variables for groups. This approach works well when there are only a few groups. However, in the longitudinal cohort studies (eg. chapter 4 and 5), each patient would be a group and numerous additional dummy variables would be needed in the model. Because of the small number of the observation from each patient the estimates of the coefficients of

the dummy variables for each patient may be unreliable. Hence, a multi-level structure has to be considered here.

#### 2.6.1.1.2 The missing data issue

The repeated measures ANOVA or paired t-test is the traditional way of analyzing longitudinal data. However, they both encounter the problem of missing data. To fit the assumption of these methods, each individual in the study should have measurement in each measurement occasion. The general recommendation is to delete the cases with missing data in one or more observations in a list-wise or case-wise manner or fill the gap with some assumptions. It works reasonably well especially in short term randomized controlled trial. However, it is clearly not suitable in our long term observational cohort study.

#### 2.6.1.1.3 Not just controlling for the clustering but also exploring it

If one just wants to control the clustering problem, eg, auto-correlation within individual in repeated measurement, some other approach can also be used. However, those methods generally treat clustering as a nuisance rather than something of potential interest on its own. In our case, the effect of patient level characteristics on the observational level dependant variable is clearly very much of our interest. Whether the change of the dependant variables vary across individuals is also part of our research question. The proportion of variance in each level in the total variance is also of interest,

eg, within patient variance vs between patient variance. The multi-level approach enables us to answer these questions.

#### 2.6.1.2 General approach

The multi-level modeling was performed using MLwin software (Version 2.22, Centre for multilevel modeling University of Bristol). Repeat observations (level 1) were nested within individuals (level 2).The target of constructing a model was searching for the best, most simple combination of independent variables to predict the dependent variable. Wald tests were used to test the statistical significance of the fixed effects. Nested models were compared by likelihood ratio test. (A nested model is an extreme case or restricted version of a more complex model. In other words, nested models can be obtained by restricting a parameter in a more complex model to be zero.) Intercepts were set as random in level 1 if without specific notice. The independent variables were tested and gradually included in the model if they were significantly related to either the dependent variables on the uni-level bivariate correlation or the plausible explanatory variables according to theoretic model or other studies.

## 2.6.2 Hierarchical clustering analysis (HCA) and principal component analysis (PCA)

### 2.6.2.1 HCA

#### 2.6.2.1.1 Characteristics

The concept of cluster analysis is to separate a set of objects into groups (clusters) in a way that the objects in the same cluster are more similar to each other than to those in other clusters. HCA is one of the most widely used methods of clustering analysis especially in a relatively small data set. Clustering is a main step of explorative data mining. No assumption is made about the underlying distribution of the data and even the number of groups. The objects clustered together depend on the similarity or distance of their characteristics. [150]

Cluster analysis has been more popular in large scale gene expression analysis and it enables the investigation of mechanisms of fundamental processed by identifying differential gene expression pattern. [150] This idea was used in biomarkers in the present project. The pattern of biomarkers was identified and enabled further investigation of underlying process.

#### 2.6.2.1.2 General approach

Genesis software (version 1.7.2, Alexander Sturn, Institute for Genomics and Bioinformatics, Graz University of Technology) was used to carry out HCA. The biomarker

concentrations were first converted to Log2 and expressed relative to the mean value for normalization. Heat maps were used to visualize the biomarker levels. A two-way hierarchical clustering method was run with an agglomerative procedure and followed the average linkage clustering agglomeration rule.

#### 2.6.2.2 PCA

##### 2.6.2.2.1 Characteristics

PCA is a useful statistic technique to reduce a number of observed variables into a smaller number of artificial variables and contain most of the variance in the data set. It makes no assumptions about the underlying causal structure.

1. A variable reduction procedure

   In brief, a principal component is a linear combination of optimally weighted original variables. The phrase 'linear combination' refers to the fact that scores on a component are the sum of the scores on original variables. The phrase 'optimally weighted' refers to the fact that the observed variables are weighted in such a way that the output components account for a maximal amount of variance in the data set. The weight is in proportion to the strength of the relationship between the generated PC and original variables.

   The first component extracted in a PCA accounts for a maximal amount of total variance in the data set. The second component extracted accounts for a maximal

amount of variance in the residual variance after extracting the first component. Eigen value is a measure of the amount of variance that is accounted for by a certain PC.

2. no assumptions about the underlying causal structure

   Factor analysis is similar to PCA in the sense that both are variable reduction methods. However, the assumption behind it is quite different. Factor analysis assumes that the covariation in the observed variables is due to the presence of latent variables (factors) that exert causal influence on the observed variables, while PCA makes no assumption about an underlying causal structure. The PCs are selected based on an eigen value cut-off of 1.0.

#### 2.6.2.2.2 General approach

PCA were carried out with IBM SPSS Statistics version 19. Principal components (PCs) were extracted using varimax rotation, with the factor selection based on an Eigen value cut-off of 1.0. A score was calculated for each subject on each given PC, so called PC score. The calculated PC scores were used in further analysis.

chapter 3.**Is peritoneal protein clearance related to comorbidity, local peritoneal membrane inflammation or both at the commencement of PD? Which of these are predictors of patient survival?**

## 3.1 Summary

### 3.1.1 Purpose

This study was to investigate whether peritoneal protein clearance (Pcl) was related to comorbidity, local peritoneal membrane inflammation or both at the commencement of PD and further clarify the link between Pcl and patient survival.

### 3.1.2 Method

Incident PD patients from three centres of the GLOBAL Fluid Study were included in this analysis. The patients were enrolled from 2002-2008 and followed up until death or censoring at the end of 2011. Demography, co-morbidities and biochemical data were collected prospectively as well as baseline peritoneal equilibration test (PET), measurement of dialysis adequacy and 24-h dialysate Pcl. Paired plasma and dialysis effluent samples were collected at the time of PET. Interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interferon-γ (INF-γ) were measured by the GLOBAL study group. Multivariate mixed linear analysis identified factors associated with Pcl. A Cox model was used to identify factors associated with survival.

### 3.1.3 Results

A total of 257 incident PD patients were studied. Peritoneal Pcl was positively related to age and higher in those with severe comorbidity. It was positively related to PSTR, plasma

and dialysate IL-6 in the univariate analysis. Dialysate IL-6 appearance rate, comorbidity grade and PSTR were independent explanatory variables for Pcl in the multivariate model. Both Pcl and daily peritoneal protein loss predicted survival in the Cox's proportional hazards regression. Pcl, plasma IL-6, age and comorbidity were independent predictors for survival. Daily peritoneal protein loss lost the significance as a predictor of survival, especially when adding plasma albumin into the model.

### 3.1.4 Conclusion

Peritoneal Pcl is a function of peritoneal membrane area, local membrane inflammation and comorbidity in patients commencing PD. The effect of comorbidity on Pcl is partly explained by the association between hypoalbuminaemia and comorbidity, but they are different by type of comorbidity. The high peritoneal Pcl predicts worse outcome. This prediction is not due to the local factors but to its relationship to hypoalbuminaemia and comorbidity. It is again complex and different by type of comorbidity.

## 3.2 Introduction

Peritoneal protein clearance (Pcl) has been proved to be related to comorbidity in several studies. Johansson used the method of personal dialysis capacity (PDC) test [69] which is based on the three-pore theory of capillary transport to identify large-pore flow, termed the large pore fluid flux ($J_{VL}$). They found that large-pore flow was greater in patients with severe comorbidity than in patients with fewer comorbid conditions. Heaf, using the same method observed that $J_{VL}$ was greater in older patients and those with cardiovascular comorbidity and also a predictor of poor survival. [72] Van Biesen showed that $J_{VL}$ was related to inflammation and predicted survival. [71] The observation by Szeto showed that a single measure of dialysate albumin >300mg/l was associated with increased cardiovascular events. [70] More recently, peritoneal Pcl was confirmed to relate to peripheral arterial disease by Sanchez-Villanueva [73] and Perl[61]. Regarding the relationship to survival, most of the above studies also showed that high Pcl is related to worse outcome. However, data from a Netherland group, showed that baseline peritoneal albumin and protein clearances from a 4 hour dwell with 3.86% glucose dialysate were associated with signs of comorbidity but no measurable effect on patient survival could be found. [74]

The potential link between peritoneal Pcl and comorbidity or survival was not clear. This study was to investigate whether Pcl was related to comorbidity, local peritoneal

membrane inflammation or both at the commencement of PD and further clarify the link between Pcl and patient survival.

## 3.3 Methods

### 3.3.1 Study design and patient population

This was an analysis based on GLOBAL Fluid study. In short, the GLOBAL Fluid study is a multi-centre, multi-national prospective, cohort study. Samples of dialysate and plasma and complete clinical phenotype were prospectively collected when patients undergoing routine pre-determined clinical assessments of membrane function. Baseline data and samples were collected within three months of treatment start. The clinical characteristics, including membrane function, peritoneal Pcl, biochemistry profiles and comorbidity were estimated locally. Dialysate and plasma samples were sent to central lab for inflammation cytokine measurements.

Three centres were in this analysis, two UK centres and one Korea centre, due to data availability. All the patients first commencing PD in these three centres in the GLOBAL Fluid study during 2002 to 2008 were included. The patients were followed and outcome data were censored at the end of 2011. There were two patients using amino acid based solution and were excluded from the analysis because of the interfering of measurement.

### 3.3.2 Measurement of membrane function and peritoneal protein clearance (Pcl)

Both of the two UK centres used the original 2.27% PET for the measurement of PSTR (D/P creatitine) and the Korea centre calculated the PSTR based on 4 hours result from a 3.86% exchange.

The peritoneal dialysate protein loss was measured from the collection of 24-hour peritoneal dialysate effluent. A validated correction factor was used for the calculation peritoneal Pcl: 24 hour dialysate protein loss / (serum albumin/0.4783). [68] Pcl was expressed as mL/day.

### 3.3.3 Comorbidity

Comorbidity was documented according to Stoke comorbidity score. [151] Briefly, 7 comorbid domains were considered, including noncutaneous malignancy, ischemic heart disease (IHD), peripheral vascular disease (PVD) (including cerebrovascular and renovascular disease), left ventricular dysfunction (LVD), diabetes mellitus (DM) , systemic collagen vascular disease, and any other condition known to reduce life expectancy. The comorbidity score for each patient was defined as the number of these domains affected. The comorbidity grade was then derived from the comorbidity score. Grade 0 (low risk) was a zero score, grade 1 (medium risk) was a score of 1-2, and grade 2 (high risk) a cumulative score of ≥ 3.

### 3.3.4 Measurement of inflammatory cytokines

IL-1β, TNF-α, IFN-γ and IL-6 in GLOBAL Fluid study cohort were measured by the GLOBAL Fluid study group. In brief, paired plasma and dialysis effluent samples were measured in the study. Four pro-inflammation cytokines were selected to represent the inflammatory status, which were IL-1β, TNF-α, IL-6 and INF-γ. Both plasma and dialysis effluent samples were measured by electrochemiluminescence immune assay. It was carried out by the commercially available Pro-Inflammatory I 4-plex (Meso-Scale Discovery, Gaithersburg, Maryland, USA). For correlation and comparisons, dialysate appearance rate (AR) of cytokines was calculated as dialysate concentration times the drained volume and divided by dwell time.

### 3.3.5 Statistical Analysis

Continuous data were expressed as mean values ± SD for normal distributed variables and medium (inter-quartile range) for non-normal distributed variables. One way ANOVA or Student's t-test were used to examine differences in normal distributed continuous data among baseline categorical variables, while Mann-Whitney or Kruskal-Wallis test for non-normal distributed variables. The relationship between peritoneal Pcl and continuous variables was examined by Pearson correlation coefficient. Variables not in normal distribution were logarithms transformed for further analysis.

Mixed linear model was used to identify the determinants of peritoneal Pcl. Multi-level strategy was applied to account for the centre effect. Centre was set as a second level with patient nested within centre. Cox regression was applied for survival analysis.

Significance was considered at P values ≤ 0.05. All statistical analyses were performed using SPSS 16 (SPSS Inc., Chicago Ill., USA) apart from the mixed linear model, which was performed by MLwin software (Version 2.22, Centre for multilevel modeling University of Bristol).

## 3.4 Results

### 3.4.1 The patient and membrane characteristics

A total of 257 patients in the three centres were included in the study. Table 3-1 displays the main baseline characteristics. There were significant differences between centres in a variety of variables, including BMI, comorbidity, PSTR, residual renal function and biochemical characteristics and all the plasma and dialysate cytokines levels. The dialysis regime was also different among centres.

Table 3-1 Patient demographic and biochemical characteristics, peritoneal membrane function and systemic and dialysate inflammatory cytokines

| | G05 (n=77) | G01 (n=57) | K03 (n=123) | P value[a] |
|---|---|---|---|---|
| Age (yr) | 56.3±15.3 | 57.1±14.3 | 53.3±14.6 | NS |
| Gender (M/F) | 47/30 | 37/20 | 72/51 | NS |
| BMI (kg/m$^2$) | 27.0±5.4 | 28.2±4.5 | 23.4±2.9 | *P*<0.01 |
| Comorbidity Grade n (%)<br>Low<br>medium<br>high | <br>23 (29.9%)<br>43 (55.8%)<br>11 (14.3%) | <br>26 (45.6%)<br>27 (47.4%)<br>4 (7.0%) | <br>49(40.0%)<br>73 (59.2%)<br>1 (0.8%) | *P*<0.01 |
| DM (yes/no) | 27/50 | 14/43 | 59/64 | *P*<0.01 |
| Day of PET(day) | 42±20 | 25±19 | 38±14 | *P*<0.01 |
| Alb (g/L) | 35.8±4.2 | 37.6±4.7 | 33.4±5.1 | *P*<0.01 |
| Hgb (g/L) | 11.9±1.5 | 11.4±1.4 | 8.7±2.4 | *P*<0.01 |
| Urine volume (ml) | 1203±785 | 1324±813 | 1027±620 | *P*<0.05 |
| PSTR | 0.77±0.14 | 0.60±0.12 | 0.73±0.10 | *P*<0.01 |
| CAPD/APD | 59/18 | 57/0 | 123/0 | *P*<0.01 |
| icodextrin (with/without) | 20/57 | 0/57 | 16/107 | *P*<0.01 |
| bicarbonate buffered solution (with/without) | 2/75 | 28/29 | 21/102 | *P*<0.01 |
| Peritoneal Pcl (ml/day) | 89.9±33.6 | 89.7±46.2 | 95.5±48.3 | NS |
| Dialysate IL-1β AR (pg/min) | 0(0-0) | 0(0-2.12) | 0(0-0.32) | *P*<0.01 |
| Dialysate TNF-α AR (pg/min) | 0(0-1.78) | 3.30(1.15-8.64) | 1.69(0.28-5.62) | *P*<0.01 |
| Dialysate IL-6 AR (pg/min) | 32.91(8.81-59.58) | 41.66(23.05-90.59) | 73.89(30.65-134.98) | *P*<0.01 |
| Dialysate INF-γ AR (pg/min) | 10.52(0-46.42) | 0(0-15.15) | 0(0-3.28) | *P*<0.01 |
| plasma IL-1β (pg/ml) | 0.12(0.06-0.26) | 0.01(0-0.07) | 0.05(0-0.21) | *P*<0.01 |
| plasma TNF-α (pg/ml) | 7.21(5.69-8.71) | 8.30(6.68-9.66) | 17.61(15.38-21.98) | *P*<0.01 |
| plasma IL-6 (pg/ml) | 1.53(0.70-2.76) | 0.80(0.24-2.25) | 1.97(1.20-3.70) | *P*<0.01 |
| plasma INF-γ (pg/ml) | 0.97(0.37-1.56) | 0.65(0-2.21) | 2.16(1.33-3.95) | *P*<0.01 |

a, One way ANOVA in normal distributed continuous data, Mann-Whitney test for non-normal distributed variables, chi-square test for the comparison of categorical variables

BMI, body mass index; PSTR, dialysate/plasma creatinine; Pcl, protein clearance.

Comorbidity Grade – Davies Comorbidity grade as defined previously

AR, appearance rate

### 3.4.2 Univariate correlation to peritoneal Pcl

The univariate correlations between peritoneal Pcl and patient and membrane characteristics, as well as dialysate and plasma inflammatory cytokines are presented in Table 3-2. Peritoneal Pcl was positively related to age and PSTR (Figure 3-1). A strong negative correlation was seen between peritoneal Pcl and serum albumin, in part a function of mathematical coupling. There was no statistically significant correlation between peritoneal Pcl and 24 hours urine volume. No significant difference of peritoneal Pcl was found between patients using icodextrin or not, using biocompatible solute or not and on CAPD or APD.

Table 3-2 univariate associations between peritoneal Pcl and patient and membrane characteristics, plasma and dialysate inflammatory cytokines

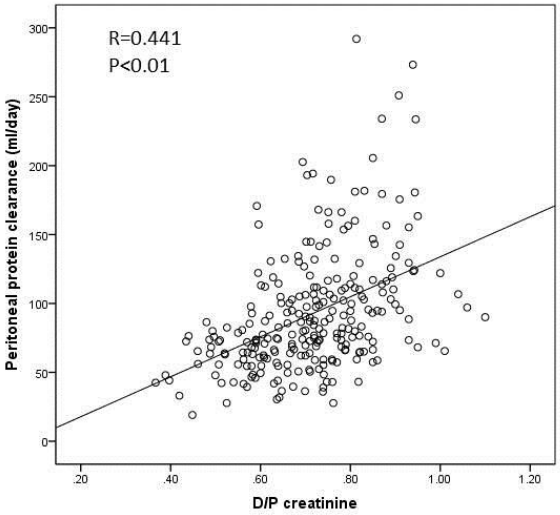
| | | β[a] | P value |
|---|---|---|---|
| Age (yr) | | 0.145 | 0.02 |
| BMI (kg/m2) | | 0.045 | 0.477 |
| Serum albumin (g/L) | | -0.474 | <0.01 |
| PSTR | | 0.441 | <0.01 |
| 24 hours urine volume (ml) | | -0.023 | 0.711 |
| Dialysate IL-1β AR (pg/min) | | 0.037 | 0.551 |
| Dialysate TNF-α AR (pg/min) | | 0.141 | 0.024 |
| Dialysate IL-6 AR (pg/min) | | 0.204 | 0.001 |
| Dialysate INF-γ AR (pg/min) | | 0.037 | 0.551 |
| plasma IL-1β(pg/ml) | | 0.02 | 0.747 |
| plasma TNF-α(pg/ml) | | 0.059 | 0.346 |
| plasma IL-6 (pg/ml) | | 0.127 | 0.043 |
| plasma INF-γ (pg/ml) | | -0.013 | 0.834 |
| | | mean±SD | P value[b] |
| Gender | Male | 96.3±45.5 | NS |
| | Female | 87.4±41.1 | |
| Comorbidity grade | Low | 79.4±37.0 | <0.01 |
| | Medium | 99.6±43.1 | |
| | High | 109.7±67.0 | |
| PD modality | CAPD | 92.4±44.6 | NS |
| | APD | 94.5±33.9 | |
| icodextrin | with | 92.9±45.1 | NS |
| | without | 90.2±35.5 | |
| bicarbonate buffered solution | With | 93.6±45.1 | NS |
| | without | 88.1±38.5 | |

a, Pearson correlation coefficient for normal distributed variables and Spearman correlation coefficient for non-normal distributed variables

b, independent samples t-test

BMI, body mass index; PP, pulse pressure; PSTR, dialysate/plasma creatinine; Ccr, creatinine clearance; IL, interleukin; TNF, tumor necrosis factor; INF, interferon; AR appearance rate.

Figure 3-1 Pearson correlation between peritoneal Pcl and PSTR (D/P creatinine)




Both dialysate and less strongly plasma IL-6 appearance rate (AR) was positively related to peritoneal Pcl. Dialysate but not plasma TNF-α were related to peritoneal Pcl. The relationship between peritoneal Pcl and other cytokine profile was not significant. (Table 3-2 and Figure 3-2)

Peritoneal Pcl was associated with the severity of comorbidity status. (Figure 3-3) Higher peritoneal Pcl was seen in the presence of IHD, DM, and LV dysfunction. No significant difference was found in the other comorbid domains. (Figure 3-4) Plasma albumin level according to different comorbidity status is also shown in Table 3-3.

Figure 3-2 Spearman correlation between peritoneal Pcl and dialysate IL-6 appearance rate

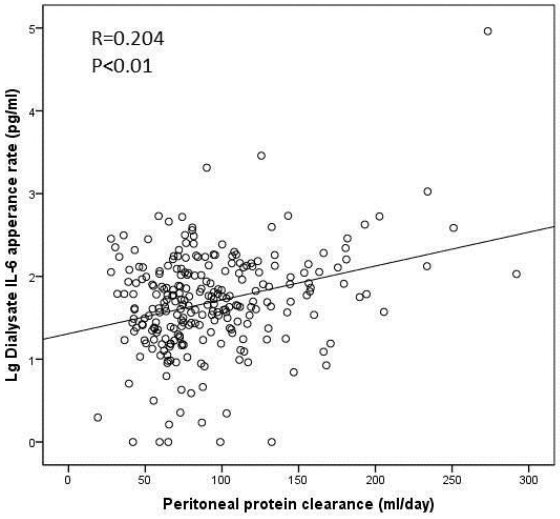



Figure 3-3 peritoneal Pcl and comorbidity grade (one way ANOVA)

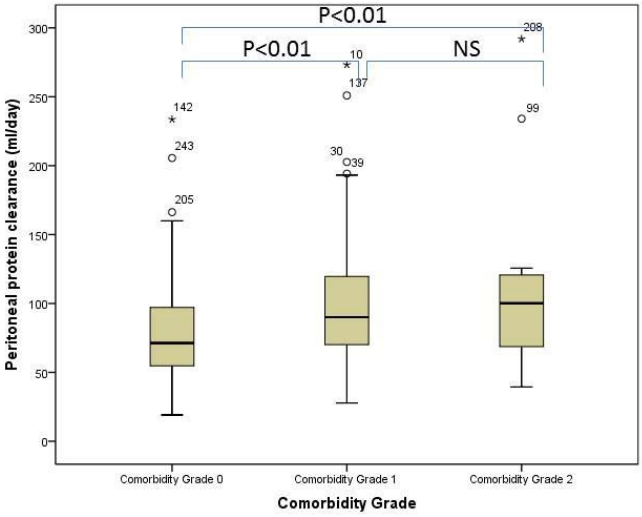
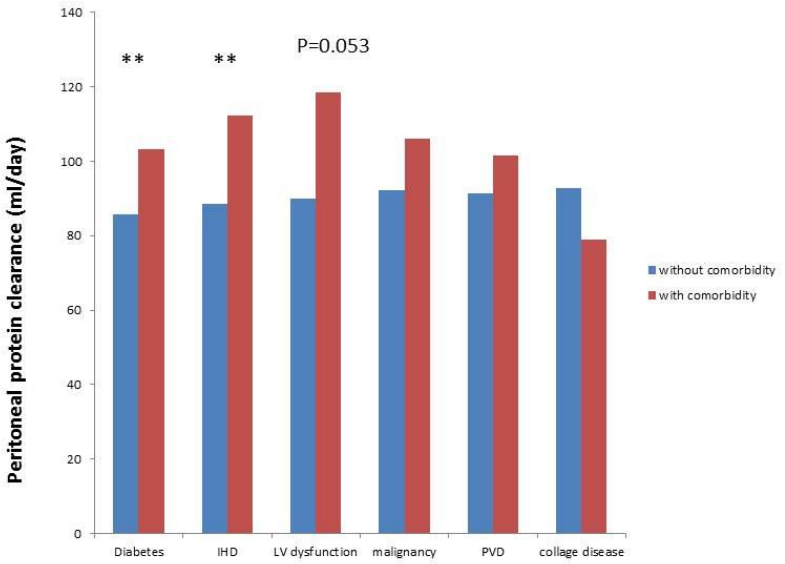


Figure 3-4 Pcl and individual comorbid domains (independent samples t-test)




**, P<0.01

IHD, ischemic heart disease; PVD, peripheral vascular disease; LVD, left ventricular dysfunction

Table 3-3 Plasma albumin according to comorbidity status

| | DM | IHD | LVD | Malignancy | PVD | collagen disease |
|---|---|---|---|---|---|---|
| without comorbidity | 36.77±4.37 | 35.36±5.1 | 35.21±4.98 | 35.04±5.11 | 35.28±5.03 | 35.02±5.06 |
| n | 157 | 213 | 234 | 250 | 228 | 254 |
| with comorbidity | 32.37±4.89 | 33.59±4.57 | 33.57±5.66 | 35.71±2.36 | 33.31±4.99 | 38±3.46 |
| n | 100 | 44 | 23 | 7 | 29 | 3 |
| P value[a] | <0.01 | <0.05 | NS | NS | <0.05 | NS |

a, unpaired t test

DM, diabetes; IHD, ischemic heart disease; PVD, peripheral vascular disease; LVD, left ventricular dysfunction

### 3.4.3 Multivariate model for peritoneal Pcl

A Multi-level strategy was taken in the multivariate analysis: Because of the observed centre effects, patients (level 1) were nested within centres, (level 2), with constant set to be random at both levels to balance the centre difference. Gender, age, comorbidity grade, $Lg_{10}$ dialysate IL-6 AR, $Lg_{10}$ plasma IL-6 and PSTR were included in the multivariate model.

In the model with peritoneal Pcl as the dependent variable, dialysate IL-6 appearance rate (AR), comorbidity grade and PSTR were independent explanatory variables. (Table 3-4) Further models substituting separate comorbid domains instead of the overall comorbidity grade showed that IHD and LV dysfunction were predictors for peritoneal Pcl, but not DM or other comorbidities.

Table 3-4 multivariate model for peritoneal Pcl without albumin as one of the dependent varible[a]

| | peritoneal Pcl | | |
|---|---|---|---|
| comorbidity grade | β | standard error | P value |
| constant | 84.778 | 7.383 | <0.01 |
| PSTR (for each 0.1 increase) | 15.05 | 2.03 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 11.042 | 4.179 | <0.01 |
| lg Plasma IL6 (for each unit increase) | -0.502 | 2.25 | NS |
| albumin (for each 1g/L increase) | | | |
| Age (year) | 0.14 | 0.167 | NS |
| gender (compare with female) | -1.849 | 4.806 | NS |
| Comorbidity Grade 1 (compared with Grade 0) | 14.823 | 4.992 | <0.01 |
| Comorbidity Grade 2 (compared with Grade 0) | 24.775 | 10.211 | <0.05 |
| diabetes | | | |
| constant | 91.125 | 6.54 | <0.01 |
| PSTR (for each 0.1 increase) | 146.954 | 20.875 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 11.488 | 4.231 | <0.01 |
| lg Plasma IL6 (for each unit increase) | -0.766 | 2.279 | NS |
| albumin (for each 1g/L increase) | | | |
| Age (year) | 0.272 | 0.166 | NS |
| gender (compare with female) | -0.978 | 4.873 | NS |
| diabetes (compared with if not) | 7.793 | 4.971 | NS |
| IHD | | | |
| constant | 91.361 | 6.58 | <0.01 |
| PSTR (for each 0.1 increase) | 155.361 | 20.253 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 10.182 | 4.221 | <0.05 |
| lg Plasma IL6 (for each unit increase) | -0.582 | 2.254 | NS |
| albumin (for each 1g/L increase) | | | |
| Age (year) | 0.155 | 0.169 | NS |
| gender (compare with female) | -1.738 | 4.813 | NS |
| IHD (compared with if not) | 17.897 | 6.378 | <0.01 |
| LVD | | | |
| constant | 92.771 | 6.334 | <0.01 |
| PSTR (for each 0.1 increase) | 154.214 | 20.22 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 10.779 | 4.201 | <0.05 |
| lg Plasma IL6 (for each unit increase) | -1.025 | 2.254 | NS |
| albumin (for each 1g/L increase) | | | |
| Age (year) | 0.224 | 0.165 | NS |
| gender (compare with female) | -2.294 | 4.826 | NS |
| LVD (compared with if not) | 22.306 | 8.128 | <0.01 |

a, mixed linear model

DM, diabetes; IHD, ischemic heart disease; LVD, left ventricular dysfunction; Pcl, protein clearance; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6

To exclude the possible impact of plasma albumin, negatively mathematically related to peritoneal Pcl, another two sets of models were constructed in which albumin was an independent variable in one set (Table 3-5) and daily peritoneal protein loss a dependent variable for the other set. (Table 3-6)

Plasma albumin was strongly related to Pcl in the multivariate model. It diluted the effect of the other variables that had been in the model. When presented as comorbidity grade, comorbidity was not a significant predictor for Pcl. When looked into each comorbidity domain, IHD and LV dysfunction was still significant, although with some decrease in beta value. (Table 3-5)

When replace Pcl by daily peritoneal protein loss as the dependent variable, albumin was not an independent predictor for daily peritoneal protein loss. Neither was comorbidity grade. However, when looked into separate comorbidity domains, IHD and LV dysfunction took over dialysate IL-6 and was the independent predictor. (Table 3-6)

Table 3-5 multivariate model for peritoneal Pcl with albumin as one of the dependent varible[a]

| | peritoneal Pcl | | |
|---|---|---|---|
| comorbidity grade | β | standard error | P value |
| constant | 90.111 | 7.444 | <0.01 |
| PSTR (for each 0.1 increase) | 11.6 | 2.05 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 9.442 | 3.984 | <0.05 |
| lg Plasma IL6 (for each unit increase) | -1.754 | 2.15 | NS |
| albumin (for each 1g/L increase) | -2.807 | 0.537 | <0.01 |
| Age (year) | 0.057 | 0.16 | NS |
| gender (compare with female) | 0.353 | 4.585 | NS |
| Comorbidity Grade 1 (compared with Grade 0) | 6.124 | 5.026 | NS |
| Comorbidity Grade 2 (compared with Grade 0) | 10.46 | 10.087 | NS |
| diabetes | | | |
| constant | 95.22 | 7.065 | <0.01 |
| PSTR (for each 0.1 increase) | 115.511 | 20.62 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 9.603 | 3.982 | <0.05 |
| lg Plasma IL6 (for each unit increase) | -2.12 | 2.144 | NS |
| albumin (for each 1g/L increase) | -3.184 | 0.535 | <0.01 |
| Age (year) | 0.084 | 0.159 | NS |
| gender (compare with female) | 0.602 | 4.571 | NS |
| diabetes (compared with if not) | -2.766 | 4.978 | NS |
| IHD | | | |
| constant | 91.723 | 6.836 | <0.01 |
| PSTR (for each 0.1 increase) | 116.404 | 20.354 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 8.385 | 3.973 | <0.05 |
| lg Plasma IL6 (for each unit increase) | -1.709 | 2.12 | NS |
| albumin (for each 1g/L increase) | -2.948 | 0.498 | <0.01 |
| Age (year) | 0.017 | 0.16 | NS |
| gender (compare with female) | 0.387 | 4.526 | NS |
| IHD (compared with if not) | 13.943 | 6.013 | <0.05 |
| LVD | | | |
| constant | 92.746 | 6.707 | <0.01 |
| PSTR (for each 0.1 increase) | 115.362 | 20.309 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 8.737 | 3.951 | <0.05 |
| lg Plasma IL6 (for each unit increase) | -2.069 | 2.114 | NS |
| albumin (for each 1g/L increase) | -2.98 | 0.496 | <0.01 |
| Age (year) | 0.067 | 0.156 | NS |
| gender (compare with female) | -0.087 | 4.53 | NS |
| LVD (compared with if not) | 18.636 | 7.622 | <0.05 |

a, mixed linear model

DM, diabetes; IHD, ischemic heart disease; LVD, left ventricular dysfunction; Pcl, protein clearance; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6

Table 3-6 multivariate model for daily peritoneal protein loss[a]

| | daily peritoneal protein loss | | |
|---|---|---|---|
| comorbidity grade | β | standard error | P value |
| constant | 6.334 | 0.508 | <0.01 |
| PRST (for each 0.1 increase) | 0.83 | 0.14 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 0.552 | 0.277 | <0.05 |
| lg Plasma IL6 (for each unit increase) | -0.123 | 0.15 | NS |
| albumin (for each 1g/L increase) | -0.006 | 0.037 | NS |
| Age (year) | 0.009 | 0.011 | NS |
| gender (compare with female) | 0.008 | 0.319 | NS |
| Comorbidity Grade 1 (compared with Grade 0) | 0.557 | 0.35 | NS |
| Comorbidity Grade 2 (compared with Grade 0) | 0.672 | 0.702 | NS |
| diabetes | | | |
| constant | 6.728 | 0.48 | <0.01 |
| PSTR (for each 0.1 increase) | 8.207 | 1.436 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 0.554 | 0.278 | <0.05 |
| lg Plasma IL6 (for each unit increase) | -0.156 | 0.15 | NS |
| albumin (for each 1g/L increase) | -0.033 | 0.037 | NS |
| Age (year) | 0.012 | 0.011 | NS |
| gender (compare with female) | 0.043 | 0.319 | NS |
| diabetes (compared with if not) | -0.145 | 0.347 | NS |
| IHD | | | |
| constant | 6.497 | 0.463 | <0.01 |
| PSTR (for each 0.1 increase) | 8.291 | 1.416 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 0.467 | 0.277 | NS |
| lg Plasma IL6 (for each unit increase) | -0.128 | 0.148 | NS |
| albumin (for each 1g/L increase) | -0.018 | 0.035 | NS |
| Age (year) | 0.007 | 0.011 | NS |
| gender (compare with female) | 0.026 | 0.315 | NS |
| IHD (compared with if not) | 1.008 | 0.419 | <0.05 |
| LVD | | | |
| constant | 6.583 | 0.455 | <0.01 |
| PSTR (for each 0.1 increase) | 8.206 | 1.417 | <0.01 |
| lg Dialysate IL6 AR (for each unit increase) | 0.5 | 0.276 | NS |
| lg Plasma IL6 (for each unit increase) | -0.154 | 0.148 | NS |
| albumin (for each 1g/L increase) | -0.022 | 0.035 | NS |
| Age (year) | 0.01 | 0.011 | NS |
| gender (compare with female) | -0.001 | 0.317 | NS |
| LVD (compared with if not) | 1.178 | 0.533 | <0.05 |

a, mixed linear model

DM, diabetes; IHD, ischemic heart disease; LVD, left ventricular dysfunction; Pcl, protein clearance; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6

### 3.4.4 Survival analysis

In the Kaplan-Meier plot, the survival rate was compared between patients with Pcl lower and higher than medium value of the whole group. Higher Pcl was showed to be related to worse overall outcome and this was also true for daily peritoneal protein loss. (Figure 3-5, Figure 3-6)

Figure 3-5 Kaplan-Meier curve for peritoneal protein clearance (Pcl) (log rank test)

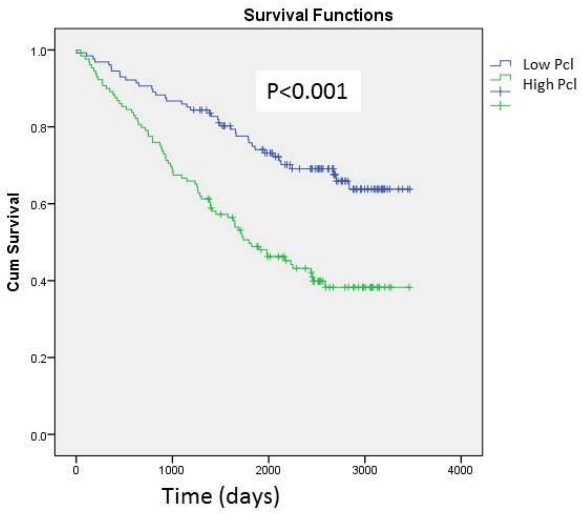
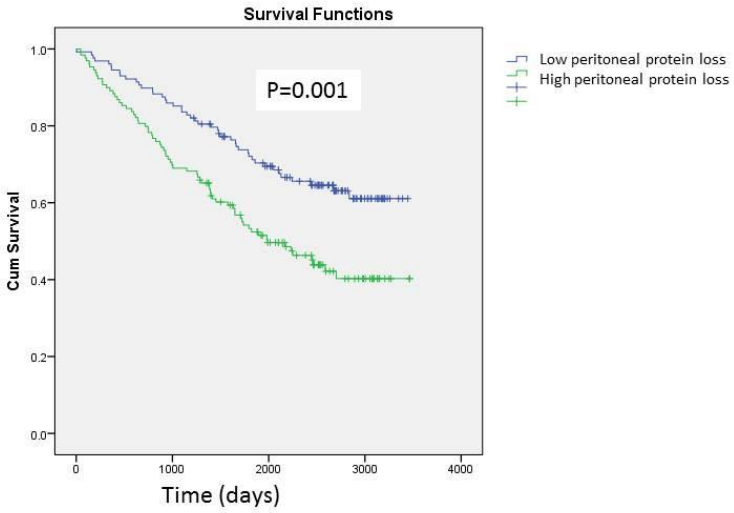


Figure 3-6 Kaplan-Meier curve for daily peritoneal protein loss (log rank test)




In Cox regression, in the basic model with age, gender, comorbidity, PSTR, local and systemic IL-6, Pcl was an independent predictor of survival in incident PD patients. As shown in Table 3-7, the predictive value of PSTR disappear when adding peritoneal Pcl into the model. By adding plasma and dialysate IL-6 into the model, both Pcl and plasma IL-6, but not dialysate IL-6 AR, were independent predictors for survival. (Table 3-8)

Table 3-7 Cox regression model of patient survival - with and without peritoneal Pcl as one of the confounder in the model

| | RR | 95%CI | P value | RR | 95%CI | P value |
|---|---|---|---|---|---|---|
| age | 1.069 | 1.05-1.09 | <0.001 | 1.070 | 1.05-1.09 | <0.001 |
| PSTR | 4.546 | 1.16-17.8 | .030 | 2.046 | 0.43-9.64 | .365 |
| gender (male as reference) | .932 | 0.63-1.38 | .726 | .920 | 0.62-1.37 | .681 |
| lg(plasma IL6) | 2.858 | 1.45-5.64 | .002 | 2.751 | 1.38-5.49 | .004 |
| lg(peritoneal IL-6 AR) | 1.171 | 0.86-1.59 | .314 | 1.049 | 0.77-1.42 | .760 |
| Comorbidity grade | 2.295 | 1.63-3.22 | <0.001 | 2.167 | 1.53-3.07 | <0.001 |
| peritoneal Pcl | | | | 1.006 | 1-1.01 | .016 |

Pcl, protein clearance; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6; RR, relative risk, CI, confident interval

Table 3-8 Cox regression of patient survival - with and without peritoneal IL-6 and plasma IL-6 as the confounders in the model

| | RR | 95%CI | P value | RR | 95%CI | P value | RR | 95%CI | P value | RR | 95%CI | P value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| age per year | 1.071 | 1.05-1.09 | <0.001 | 1.070 | 1.05-1.09 | <0.001 | 1.070 | 1.05-1.09 | <0.001 | 1.070 | 1.05-1.09 | <0.001 |
| PSTR | 2.442 | 0.54-11.08 | .247 | 2.370 | 0.52-10.85 | .266 | 2.083 | 0.45-9.75 | .351 | 2.046 | 0.43-9.64 | .365 |
| gender (male as reference) | .964 | 0.65-1.42 | .853 | .956 | 0.65-1.41 | .821 | .923 | 0.62-1.37 | .691 | .920 | 0.62-1.37 | .681 |
| lg(plasma IL6) | | | | | | | 2.798 | 1.42-5.53 | .003 | 2.751 | 1.38-5.49 | .004 |
| lg(peritoneal IL-6 AR) | | | | 1.129 | 0.82-1.55 | .457 | | | | 1.049 | 0.77-1.42 | .760 |
| Comorbidity grade | 2.304 | 1.63-3.26 | <0.001 | 2.323 | 1.64-3.29 | <0.001 | 2.158 | 1.52-3.05 | <0.001 | 2.167 | 1.53-3.07 | <0.001 |
| peritoneal Pcl | 1.007 | 1-1.01 | .003 | 1.006 | 1-1.01 | .010 | 1.006 | 1-1.01 | .009 | 1.006 | 1-1.01 | .016 |

Pcl, protein clearance; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6; RR, relative risk; CI, confident interval

Additional models were conducted to clarify the confounding effect of albumin. When add albumin into the model with peritoneal Pcl, plasma albumin replace peritoneal Pcl as an independent predictor for survival. (Table 3-9) Due to the mathematical link between plasma albumin and Pcl, further models with daily peritoneal protein loss rather than Pcl were also conducted. Daily peritoneal protein loss was no longer a significant predictor of survival, especially when plasma albumin was added into the model. As expected, plasma albumin was an independent predictor for survival. (Table 3-10) However, the prediction of daily peritoneal protein loss was different according to the type of comorbidities. When controlled for DM status, rather than the overall severity of comorbidity (comorbidity grade) as shown in Table 3-10, daily peritoneal protein loss and plasma albumin both independently predicted survival. (Table 3-11)

Table 3-9 Cox regression of patient survival - with and without plasma albumin as the confounder in the model

| | RR | 95%CI | P value | RR | 95%CI | P value |
|---|---|---|---|---|---|---|
| age | 1.070 | 1.05-1.09 | <0.001 | 1.074 | 1.05-1.09 | <0.001 |
| PSTR | 2.046 | 0.43-9.64 | .365 | 1.436 | 0.28-7.24 | .661 |
| gender (male as reference) | .920 | 0.62-1.37 | .681 | .970 | 0.65-1.44 | .880 |
| lg(plasma IL6) | 2.751 | 1.38-5.49 | .004 | 2.361 | 1.18-4.74 | .016 |
| lg(peritoneal IL-6 AR) | 1.049 | 0.77-1.42 | .760 | 1.028 | 0.75-1.41 | .866 |
| Comorbidity grade | 2.167 | 1.53-3.07 | <0.001 | 1.917 | 1.34-2.74 | <0.001 |
| peritoneal Pcl | 1.006 | 1-1.01 | .016 | 1.002 | 1-1.01 | .340 |
| albumin | | | | .924 | 0.88-0.97 | .001 |

Pcl, protein clearance; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6; RR, relative risk; CI, confident interval

Table 3-10 Cox regression of patient survival - with and without plasma albumin as the confounder in the model when measuring daily peritoneal protein loss rather than peritoneal Pcl

| | RR | 95%CI | P value | RR | 95%CI | P value |
|---|---|---|---|---|---|---|
| age | 1.069 | 1.05-1.09 | <0.001 | 1.074 | 1.05-1.09 | <0.001 |
| PSTR | 2.703 | 0.6-12.09 | .190 | 1.134 | 0.22-5.85 | .880 |
| gender (male as reference) | 1.101 | 0.74-1.64 | .640 | 1.040 | 0.7-1.55 | .850 |
| log(plasma IL6) | 2.890 | 1.46-5.73 | <0.001 | 2.361 | 1.17-4.75 | .020 |
| log(peritoneal IL-6 AR) | 1.104 | 0.82-1.49 | .520 | 1.011 | 0.74-1.38 | .950 |
| Comorbidity grade | 2.270 | 1.61-3.2 | <0.001 | 1.917 | 1.34-2.75 | <0.001 |
| Daily peritoneal protein loss | 1.070 | 0.99-1.15 | .080 | 1.068 | 0.99-1.15 | .100 |
| albumin | | | | 0.917 | 0.88-0.96 | <0.001 |

Pcl, protein clearance; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6; RR, relative risk; CI, confident interval

Table 3-11 Cox regression of patient survival with different comorbidity status as the confounder in the model – with and without plasma albumin as the confounder in the model

| | RR | 95%CI | P value | RR | 95%CI | P value |
|---|---|---|---|---|---|---|
| age | 1.086 | 1.06-1.11 | <0.001 | 1.088 | 1.07-1.11 | <0.001 |
| PSTR | 1.097 | 0.24-4.98 | .904 | .553 | 0.11-2.85 | .478 |
| gender (male as reference) | .931 | 0.63-1.38 | .721 | .968 | 0.65-1.43 | .872 |
| lg(plasma IL6) | 3.824 | 1.98-7.38 | <0.001 | 2.961 | 1.49-5.86 | .002 |
| lg(peritoneal IL-6 AR) | .953 | 0.69-1.32 | .772 | .892 | 0.64-1.24 | .496 |
| DM | 2.910 | 1.96-4.35 | <0.001 | 2.385 | 1.59-3.57 | <0.001 |
| Daily peritoneal protein loss | 1.110 | 1.03-1.19 | .006 | 1.105 | 1.02-1.19 | .011 |
| albumin | | | | .920 | 0.88-0.96 | <0.001 |
| | RR | 95%CI | P value | RR | 95%CI | P value |
| age | 1.068 | 1.05-1.09 | <0.001 | 1.074 | 1.05-1.09 | <0.001 |
| PSTR | 1.963 | 0.45-8.57 | .370 | .733 | 0.14-3.83 | .713 |
| gender (male as reference) | .903 | 0.61-1.35 | .616 | .996 | 0.67-1.49 | .986 |
| lg(plasma IL6) | 3.181 | 1.68-6.01 | <0.001 | 2.378 | 1.21-4.66 | .012 |
| lg(peritoneal IL-6 AR) | 1.053 | 0.78-1.42 | .736 | .967 | 0.71-1.32 | .833 |
| IHD | 1.241 | 0.79-1.96 | .349 | 1.177 | 0.75-1.85 | .484 |
| Daily peritoneal protein loss | 1.074 | 0.99-1.16 | .076 | 1.069 | 0.99-1.16 | .110 |
| albumin | | | | .900 | 0.86-0.94 | <0.001 |
| | RR | 95%CI | P value | RR | 95%CI | P value |
| age | 1.069 | 1.05-1.09 | <0.001 | 1.075 | 1.06-1.09 | <0.001 |
| PSTR | 1.946 | 0.46-8.29 | .368 | .724 | 0.14-3.66 | .696 |
| gender (male as reference) | .916 | 0.62-1.36 | .663 | 1.003 | 0.67-1.49 | .987 |
| lg(plasma IL6) | 3.283 | 1.75-6.15 | <0.001 | 2.474 | 1.28-4.78 | .007 |
| lg(peritoneal IL-6 AR) | 1.022 | 0.75-1.38 | .889 | .933 | 0.68-1.28 | .667 |
| LVD | 1.576 | 0.91-2.78 | .108 | 1.532 | 0.88-2.63 | .129 |
| Daily peritoneal protein loss | 1.075 | 1-1.16 | .061 | 1.068 | 0.99-1.15 | .103 |
| albumin | | | | .899 | 0.86-0.94 | <0.001 |

DM, diabetes; IHD, ischemic heart disease; LVD, left ventricular dysfunction; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6; RR, relative risk; CI, confident interval

## 3.5 Discussion

The present study confirmed the relationship between peritoneal Pcl and PSTR indicating that membrane area is an important determinant. It also showed that peritoneal Pcl is related to local membrane inflammation and this association is independent to membrane area. The effect of comorbidity on Pcl is partly explained by the association between hypoalbuminaemia and comorbidity, but they are different by type of comorbidity. The association between Pcl and outcome has been repeated in the current cohort. This prediction is not due to the local factors but to its relationship to hypoalbuminaemia and comorbidity. It is again complex and different by type of comorbidity.

### 3.5.1 Uncoupled increase in Pcl and PSTR in local membrane inflammation

Several studies showed that membrane inflammation is associated with high PSTR, primarily a measure of the effective membrane area, specifically of small intercellular pores, in incident patients. [115, 116] However, until now it is not clear how this relates to variability in large pore flux - and membrane inflammation. It is well known that the large pore flux-peritoneal Pcl increases dramatically in peritonitis, i.e. in the presence of severe inflammation that includes an influx of neutrophils, but less clear in the context of stable, non-infected dialysis patients. A study in 40 prevalent CAPD patients showed that the

dialysate appearance of IL-6 correlated with albumin and IgG clearances, which was also related to PSTR at the same time. [118] However it was not clear from this study whether the inflammation associated increase of peritoneal Pcl was coupled with the increase of membrane area. Our result confirmed the association between peritoneal Pcl and local membrane inflammation in the incident cohort and further illustrated that this relationship was independent from but also associated with membrane area. Local IL-6 production may associate with an increase in PSTR through increased effective area. But it is not the only mechanism of increase in Pcl. It may further increase the permeability of peritoneal membrane to protein - large pore area. In other words, there is uncoupled increase in Pcl and PSTR.

### 3.5.2 Peritoneal cavity - a window for systemic vascular damage?

There was also an independent association between peritoneal Pcl and the severity of comorbidity status in this multi-centre incident cohort. It was in keeping with the findings in several single centre studies using different methods to measure the peritoneal protein leak and different definitions of comorbidity.[69 72 71 70 61, 73]

However, we need to be cautious when using Pcl as a marker of systemic vessel damage. Firstly, the permeability of peritoneal membrane to protein also depends on the local factors,

such as local inflammation as demonstrated in this study. Secondly, the calculation of Pcl uses plasma proteins, especially albumin as its denominator and comorbidity may affect plasma concentration independently of the effects on endothelial function. Thirdly, the factors determining permeability of peritoneal membrane to protein may also change with time on PD, as will be demonstrated in the following chapter.

### 3.5.3 Where is the link between Pcl and survival? Local or systemic factors

The current study confirmed a strong relationship between Pcl and survival. This relationship was independent from PSTR or local membrane inflammation as measured by IL-6. This study confirmed the previous finding that Pcl replace PSTR as an independent predictor of survival in a contemporary PD cohort, [61] where APD and polyglucose solutions, such as icodextrin, are widely used to restrict the unfavourable effect of PSTR on fluid balance. One of the important finding of this study is that local membrane inflammation does not predict survival when controlled for other confounders. It is generally in agreement with the clinical observations that although with some controversy, most of the 'biocompatible' solutions actually increase effluent inflammation biomarker level, but there is no evidence that they are related to worse outcome so far, if not better. [152-154]

Due to the mathematical coupling to plasma albumin, there is concern that the Pcl is a surrogate of plasma albumin for the prediction of survival. To further investigate this, the predictive value of daily protein loss on survival was analyzed. Both peritoneal Pcl and daily peritoneal protein loss predicted survival in univariate K-M survival analysis. It is different from Krediet's observation [74] as neither peritoneal albumin clearance nor albumin loss from a 4 hour 3.86% glucose exchange was associated with death in their study. The fact that higher proportion of albumin would go through small pore via convection in 4 hour 3.86% glucose dwells may partially explain their negative finding. The prediction of Pcl, of course, partially relies on, but not solely secondary to, the mathematic correlation to plasma albumin. It also reflects other aspect of the pathophysiological change of the body. From the correlation analysis of this study, it is likely to be a function of comorbidity, with variation of the importance of mechanism according to the different type of comorbidities.

### 3.5.4 Limitations

The findings of the present study must be interpreted in the context of the study design. The strengths of the present study include the multi-centre nature of the study, the large number of patients studied and adjustment for multiple confounding covariates. There are also limitations of the study. Firstly, albumin is in the equation of calculating Pcl. There is mathematical correlation between albumin and Pcl, although extra effort has been made in the data analysis to interpret the physical relationship. However, as Pcl is the most widely

clinically used measurement of membrane permeability to protein, it is more important to understand the clinical meaning of Pcl than a theoretical measure. Secondly, urine protein loss, another route of protein loss that may affect plasma albumin, would ideally be measured. The difference between DM and IHD from the current observation could because of the excessive urine protein loss in DM that is not really related to endothelial permeability in ESRD. Thirdly, a validated correction factor is used to calculate total protein from serum albumin where total plasma protein is required to estimate Pcl. A direct measurement of plasma total protein was not available in this study as well as most of the other studies on peritoneal protein Pcl. It is possible that in extreme systemic inflammation, total serum protein may be underestimated and Pcl overestimated because of an uncoupling between serum albumin and total protein. Albumin is a negative acute-phase protein, and other proteins that are positive acute-phase proteins. The association between systemic inflammation and Pcl may have been amplified in that case. Fourthly, albumin, the predominant plasma and dialysate protein, may also pass through small pores (predominantly by convection). [155] Ideally, a series of proteins at different molecular weights should be measured to precisely dissect out the contribution of the relative pores, a procedure that is beyond the feasibility of a large prospective epidemiological study. Fifthly, multiple models were carried out in the study, but no correction for multiple comparisons was made, which may potentially increase the risk of type I error. The variables selected in

the model were based on scientifically plausible hypothesis rather than every possible combination, which help to limit the risk.

## 3.6 Conclusion

Peritoneal Pcl is a function of PSTR, local membrane inflammation and comorbidity in patients commencing PD. The high peritoneal Pcl predicts worse outcome, which is a combination of hypoalbuminaemia and severity of comorbidity. It remains unclear as to whether this association is due to hypoalbuminaemia caused by reduced protein synthesis or due to systemic endothelial barrier dysfunction or both.

# chapter 4.**Longitudinal dissociation between small solute transport and peritoneal protein clearance (Pcl) in peritoneal dialysis patients**

## 4.1 Summary

### 4.1.1 Purpose

The purpose of the study is to clarify whether the peritoneal protein clearance (Pcl) - a measure of large pore, changes with time on PD or not. If so, it is to further investigate whether this change over time dissociated from the increase in PSTR.

### 4.1.2 Methods

Patients treated continuously for a minimum of four years were extracted from the longitudinal prospective Stoke PD Study. All patients in this cohort commencing PD since 2000 had daily Pcl measured at baseline and 6 monthly intervals, along with peritoneal small solute transport rate (PSTR), ultrafiltration capacity, residual renal function (RRF), comorbid conditions and peritonitis events. Multi-level multi-variate analysis was used to determine associations with Pcl over time taking account of within subject correlations.

### 4.1.3 Results

Of the 279 incident patients, 49 had been on PD for more than 4 years, for whom there were 335 full data sets within the 48 months of follow-up. At baseline Pcl correlated with PSTR whereas over time there was progressive uncoupling of this relationship with increasing PSTR

and stable Pcl. Multi-variate analysis found that age, PSTR, daily ultrafiltration and sodium removal were significant predictors of Pcl when controlled for gender, comorbidity, glucose exposure and RRF. However, the strength of the relationship between PSTR and Pcl was less with time on treatment in keeping with their progressive dissociation. Peritonitis was associated with increased PSTR but a similar pattern of uncoupling.

### 4.1.4 Conclusion

There is an uncoupling of the small (PSTR) and large (Pcl) pore pathways with time on PD which would be in keeping with a switch from local inflammation early on to progressive fibrosis combined with increased vascular surface area. Measuring longitudinal changes Pcl may complement membrane function tests monitoring progressive injury.

## 4.2 Introduction

PD leads to changes in membrane morphology[99] and function[95, 98] over time which in a proportion of patients causes ultrafiltration failure and likely predisposes to the much rarer complication of encapsulating peritoneal sclerosis. Of the functional changes, increasing peritoneal small solute transport rate (PSTR) and a reduction in osmotic conductance (reduced ultrafiltration capacity independent of osmotic gradient) are well established[84, 95]. Longitudinal changes in Pcl, predominantly a reflection of hydrostatic pressure driven leak of plasma proteins through the large pore pathway, are less clear. Cross sectional and longitudinal studies [69, 72] have suggested this does not increase with time on treatment but it is not clear if this represents a real difference from PSTR, partly because Pcl has been found to be associated with age and comorbidity and in some studies is an independent predictor of survival[61, 70-73] leading to informative censoring in cohort studies.

Pcl (large pores) and PSTR (small pores) are known to be correlated at the start of PD treatment and there are at least two reasons for this. First, given that both pore systems are located in the capillary vessel wall it is likely that there will be considerable anatomical coupling. Second, as intra-peritoneal production of the pro-inflammatory cytokine IL-6 is correlated with and likely a key determinant of PSTR, local inflammation would be expected to cause increased numbers of large pores per unit of capillary length. If the increase in PSTR occurring with time on treatment is a function of increasing intra-peritoneal inflammation

then a parallel increase in Pcl would be anticipated. The purpose of this analysis was to test this hypothesis in a cohort of patients treated continuously over four years to avoid the influence of informative censoring by patients discontinuing treatment early with disproportionately increased Pcl due to the associations with age and comorbidity.

## 4.3 Methods

### 4.3.1 Study design and patient population

The Stoke PD study is a long term single centre, longitudinal, prospective observational cohort of consecutive new patients commencing PD. Since April 2000, peritoneal protein losses were measured 6-monthly as part of routinely collected data that includes demography, comorbidity, membrane function (peritoneal equilibration test to include PSTR and ultrafiltration capacity), RRF, dialysis prescription, clearances, achieved ultrafiltration and sodium removal. Data is collected and stored on a validated database (PDDB©) and since 2002 all patients have given their informed consent as one of the recruiting centres for the GLOBAL Fluid Study (MREC:*02/9/14)*. The database was interrogated in July 2011 to identify all patients in whom there were at least four years of continuous data for the purpose of this analysis. During this period the majority of patients (>95%) used conventional glucose fluids (Dianeal) with an increasing proportion using icodextrin (Extraneal).

### 4.3.2 Measurement of membrane function and Pcl

Peritoneal equilibration tests were used to measure PSTR (dialysate:plasma creatinine ratio) and the UF capacity at 4 hours as previously described. The peritoneal dialysate protein loss

was measured by the Biuret method from the collection of 24-hour peritoneal dialysate effluent.

Plasma albumin levels had been measured using the bromocresol green method before 22th Aug 2007 and was switched to bromocresol purple method afterward. An average difference of 5.5g/L between the results of the two methods had been established [149] and this conversion factor was applied. Peritoneal Pcl was calculated using a validated formula: 24 hour dialysate protein loss / (serum albumin/0.4783), and expressed as mL/day.

### 4.3.3 Average glucose exposure

Peritoneal average glucose exposure was calculated by summing the total glucose exposure in each exchange and divided by total volume. For example, a patient using 2*2L exchanges of 1.36% glucose, 1*2L exchanges of 2.27% glucose and icodextrin 2L overnight, the average glucose exposure would be (1.36%*4+2.27%*2)/8=1.25%.

### 4.3.4 Comorbidity and demography

Demography, primary renal disease and comorbidity were recorded at the start of PD. Comorbidity was defined as described previously. Briefly, 7 comorbid domains were considered, including non-cutaneous malignancy, ischemic heart disease (IHD), peripheral vascular disease (PVD), left ventricular dysfunction (LVD), diabetes mellitus (DM), systemic

collagen vascular disease, and any other condition thought to reduce life expectancy. The comorbidity score for each patient was defined as the number of these domains affected. The comorbidity grade was then derived from the comorbidity score. Grade 0 (low risk) was a zero score, grade 1 (medium risk) was a score of 1-2, and grade 2 (high risk) a cumulative score of ≥ 3.

### 4.3.5 Statistical analysis

Continuous data are expressed as means (SD) or medians (interquartile range) according to the distribution. For multivariate models non-parametric data were log or square root transformed to fit a normal distribution. Between-group comparisons used the 2-tailed unpaired t-test, the Mann-Whitney U test, one way ANOVA, independent samples Kruskal Wallis test or the $\chi$-squared test depending on the data type, the number of groups and the distribution. For single-level analysis data was annualised such that if there was more than one set of measurements available the mean was taken for further analysis to limit bias.

Multi-level mixed linear model was applied to determine associations with Pcl and performed using MLwin software (Version 2.22, Centre for multilevel modelling University of Bristol). Repeat observations, e.g. membrane characteristics (level 1) were nested within individuals (level 2). To investigate the long term change of peritoneal membrane, those who were on PD for more than 4 years were extracted from the database. To restrict the survivor's bias,

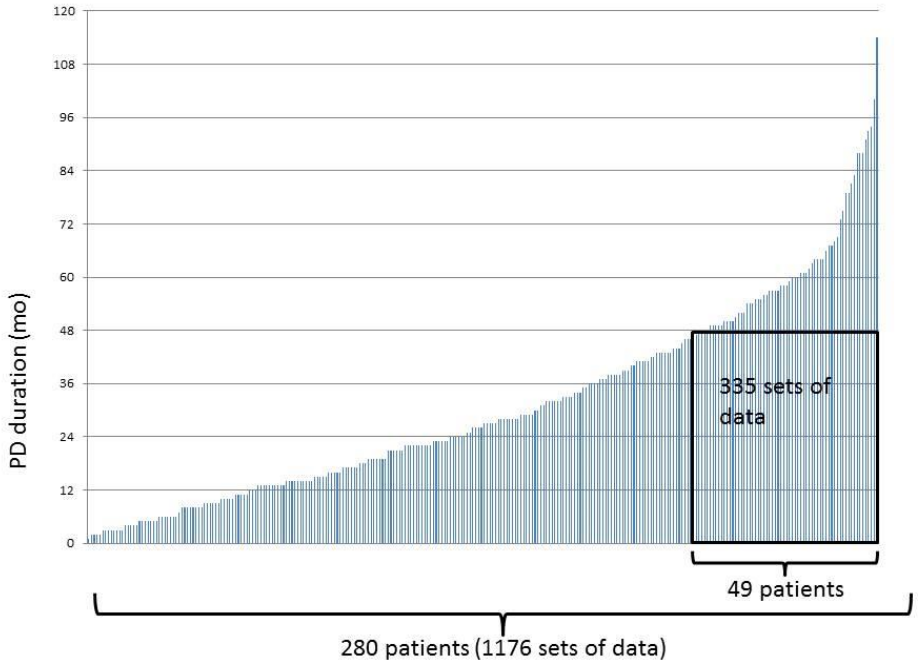
only the first 4 years data from these patients were further analyzed. The dependent variable was the daily protein Pcl and the independent covariates were included in the multivariate model if either they were significantly related to either the dependent variable on the uni-level bivariate correlation or they were plausible explanatory variables according to theoretic model or other studies. Constant covariates, such as gender and comorbidity at baseline were level 2 variables. The intercept was set as random in level 2 to allow for between patient differences. The continuous variables were centered on the mean to facilitate the clinical interpretation of the model.

## 4.4 Results

### 4.4.1 Patient demography and baseline membrane function

1176 sets of membrane function tests and Pcl measurement in 280 patients were available for analysis (see Figure 4-1) from which 335 sets of data in 49 patients for whom four years of continuous data were available were extracted for further analysis. 175 of the 335 observations were in APD and 190 were on icodextrin.

Figure 4-1 Data selection process



The demographic details of the sub-cohort selected for this study are displayed in Table 4-1 and split according to ever or never having peritonitis during the observation period. There was no significant difference between the two groups in terms of demographics or membrane function. Of the 25 patients who had peritonitis, 11 of them had one episode, 8 of them had two episodes and 6 three or more episodes of peritonitis.

Table 4-1 Demography of the sub-cohort selected for this study

| | sub-cohort (n=49) | | | | Whole cohort (n=280) |
|---|---|---|---|---|---|
| | without peritonitis (n=24) | With peritonitis (n=25) | P value[a] | combined (n=49) | |
| Gender (f/m) | 11/13 | 10/15 | 0.68 | 21/28 | 120/160 |
| Diabetic (%) | 29.2 | 32 | 0.83 | 30.6 | 29.6 |
| IHD (%) | 12. 5 | 16 | 0.73 | 14.3 | 21.1 |
| LVD (%) | 4.2 | 4 | 0.98 | 4.1 | 7.5 |
| comorbidity grade (low/medium/high) | 11/12/1 | 9/14/2 | 0.71 | 20/26/3 | 107/146/27 |
| age (years) | 52±17 | 49±19 | 0.51 | 51±18 | 55.3±16.5 |
| total time on PD (mo) | 67±17 | 66±14 | 0.82 | 67±15 | 30.9±22 |
| survival time since PD start (mo) | 85±21 | 80±21 | 0.39 | 83±21 | 56.2±35.1 |
| baseline PSTR | 0.64±0.15 | 0.69±0.11 | 0.21 | 0.66±0.13 | 0.68±0.13 |
| baseline albumin (g/L) | 32.2±3.5 | 30.9±4.5 | 0.28 | 31.5±4.1 | 30.7±4.8 |

a, unpaired t test

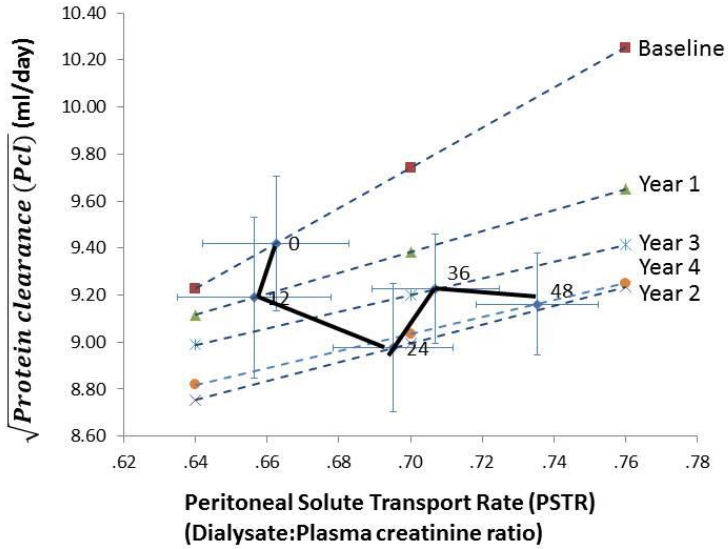
IHD, ischemic heart disease; LVD, left ventricular dysfunction; PSTR, peritoneal solute transport rate; PD, peritonea dialysis

### 4.4.2 Longitudinal relationship between Pcl and PSTR

The changes in PSTR and Pcl with time on treatment are shown in Figure 4-2; PSTR increases over time (baseline, 0.66±0.13, 1st year, 0.66±0.13, 2nd year, 0.70±0.11, 3rd year, 0.71±0.12, to 4th year, 0.74±0.12, P<0.01) whereas Pcl tends to decrease indicating a progressive dissociation of these aspects of membrane function. The mean values of Pcl and PSTR were showed in Figure 4-2. The plasma albumin was stable over the time; baseline: 31.7±3.9g/L, Year 1: 31.0±4.2g/L, Year 2: 31.7±3.4g/L, Year 3: 31.9±2.7g/L, Year 4: 32.1±2.9g/L, respectively.

The relationship between PSTR and Pcl also weakened over time as shown by the changing slope and significance of the regression line, especially after baseline (Figure 4-2). The Pearson correlation coefficient (R) at baseline was: 0.612 (P<0.01), Year 1: 0.28 (P=0.104), Year 2: 0.245 (P=0.113), Year 3: 0.27 (P=0.063), Year 4: 0.282 (P=0.052), respectively.

Figure 4-2 peritoneal protein clearance (Pcl) is positively related to PSTR and this relationship changes with time on PD




- The significant linear regression between Pcl and PSTR in different time point is showed as dash line.
- The actual mean value (SE bars) for protein clearance (Pcl) and PSTR at each time point are shown superimposed upon the linear regression.
- Pcl is positively related to PSTR throughout the therapy.
- However, the slope of the relationship between PSTR and Pcl got smaller with time on PD.

### 4.4.3 Multivariate analysis of the determinants of PCl

Table 4-2 summarizes the initial multi-level mixed linear models constructed to investigate the relationship between Pcl and PSTR. Their positive association persists after adjustment for comorbidity score, gender, age, urine volume and membrane UF capacity, but the time on therapy interaction shows that this is weakened as demonstrated by the significant negative $\beta$ coefficient. Addition of glucose exposure and use of icodextrin to the model (Table 4-2 right) had little overall effect. As there were only a few patients on dry day regime in this cohort (11 of 335 data sets), no sub-analysis on this group was undertaken.

Table 4-2 Multi-level mixed linear model of daily Pcl (square root transformed)-base model and model with added glucose effect

| | Base model | | | with glucose effect | | |
|---|---|---|---|---|---|---|
| | β | standard error | P value | β | standard error | P value |
| constant | 9.57 | 0.3 | <0.01 | 9.44 | 0.34 | <0.01 |
| PSTR (for each 0.1 increase) | 0.39 | 0.11 | <0.01 | 0.43 | 0.11 | <0.01 |
| comorbidity score (for each 1 unit increase) | -0.13 | 0.2 | NS | -0.18 | 0.21 | NS |
| gender (if female) | -0.52 | 0.38 | NS | -0.37 | 0.4 | NS |
| age (year) | 0.016 | 0.011 | NS | 0.019 | 0.011 | 0.1 |
| UF capacity on PET (for each 100ml increase) | 0.04 | 0.03 | NS | 0.04 | 0.04 | NS |
| urine volume (for each 1000ml increase) | 0.2 | 0.1 | NS | 0.2 | 0.2 | NS |
| PSTR *PD duration ($2^{nd}$ compared with $1^{st}$ year for each 0.1 increase in PSTR) | -0.23 | 0.14 | NS | -0.25 | 0.14 | 0.08 |
| PSTR *PD duration ($3^{rd}$ compared with $1^{st}$ year for each 0.1 increase in PSTR) | -0.35 | 0.14 | <0.01 | -0.37 | 0.14 | <0.01 |
| PSTR *PD duration ($4^{th}$ compared with $1^{st}$ year for each 0.1 increase in PSTR) | -0.30 | 0.14 | <0.05 | -0.27 | 0.15 | 0.07 |
| ICO usage (compared with not use) | | | | 0.007 | 0.019 | NS |
| medium glucose exposure (compared with low glucose exposure) (n=51) | | | | 0.02 | 0.02 | NS |
| high glucose exposure (compared with low glucose exposure) (n=13) | | | | 0.03 | 0.03 | NS |

PSTR, peritoneal solute transport rate; UF, ultrafiltration; PET, Peritoneal equilibration tests; ICO, icodextrin; PD, peritoneal dialysis

medium glucose exposure, daily average glucose exposure 1.36%-2.27%

high glucose exposure, daily average glucose exposure >=2.27%

### 4.4.4 The effect of accounting for UF and sodium removal

One potential confounder of this analysis could be the increased requirement over time to obtain more ultrafiltration as a consequence of loss in RRF. Because albumin is able to pass through small pores, increased ultrafiltration rates have the potential to increase the convective removal of albumin and thus increase Pcl. Furthermore, although peritoneal UF and sodium removal are two highly correlated variables, ($r=0.875$, $P<0.01$), by introducing them separately or together in the model it is possible to explore the effect of ultrafiltration via the small pores (sodium coupled) or aquaporins (uncoupled).

Table 4-3 summarises this showing that when either UF or sodium are included separately that Pcl is positively associated with increased fluid or sodium removal. Included together the relationship remains positive with sodium removal (small pore coupled ultrafiltration) but associated negatively with ultrafiltration. For all these more complex models the time dependent relationship between Pcl and PSTR remains unaffected.

Table 4-3 Multi-level mixed linear model of daily Pcl (square root transformed)- investigate the UF and sodium removal effect

| | with daily sodium loss | | | with daily UF | | | with UF and sodium loss | | |
|---|---|---|---|---|---|---|---|---|---|
| | β | SE | P value | β | SE | P value | β | SE | P value |
| Constant | 9.56 | 0.33 | <0.01 | 9.54 | 0.34 | <0.01 | 9.29 | 0.32 | <0.01 |
| PSTR (for each 0.1 increase) | 0.47 | 0.11 | <0.01 | 0.46 | 0.11 | <0.01 | 0.40 | 0.10 | <0.01 |
| comorbidity score (for each 1 unit increase) | -0.13 | 0.2 | NS | -0.16 | 0.21 | NS | -0.14 | 0.19 | NS |
| gender (if female) | -0.18 | 0.38 | NS | -0.31 | 0.4 | NS | -0.14 | 0.37 | NS |
| age (year) | 0.02 | 0.01 | 0.09 | 0.02 | 0.01 | NS | 0.02 | 0.01 | <0.05 |
| UF capacity on PET (for each 100ml increase) | 0.04 | 0.04 | NS | 0.04 | 0.04 | NS | 0.05 | 0.03 | NS |
| urine volume (for each 1000ml increase) | 0.4 | 0.2 | <0.05 | 0.32 | 0.16 | <0.05 | 0.2 | 0.2 | NS |
| PSTR*PD duration ($2^{nd}$ compared with $1^{st}$ year for each 0.1 increase in PSTR) | -0.31 | 0.14 | <0.05 | -0.28 | 0.14 | 0.05 | -0.28 | 0.13 | <0.05 |
| PSTR *PD duration ($3^{rd}$ compared with $1^{st}$ year for each 0.1 increase in PSTR) | -0.41 | 0.13 | <0.01 | -0.40 | 0.14 | <0.01 | -0.37 | 0.13 | <0.01 |
| PSTR *PD duration ($4^{th}$ compared with $1^{st}$ year for each 0.1 increase in PSTR) | -0.31 | 0.15 | <0.05 | -0.28 | 0.15 | 0.06 | -0.32 | 0.14 | <0.05 |
| ICO usage (compared with not use) | -0.03 | 0.02 | NS | -0.01 | 0.02 | NS | -0.03 | 0.21 | NS |
| medium glucose (compared with low glucose) (n=51) | 0.02 | 0.02 | NS | 0.02 | 0.02 | NS | 0.36 | 0.19 | 0.052 |
| high glucose (n=13) | -0.01 | 0.03 | NS | 0.009 | 0.04 | NS | 0.1 | 0.33 | NS |
| daily UF (for each 100ml increase) | | | | 0.00032 | 0.00016 | <0.05 | -0.15 | 0.03 | <0.01 |
| daily sodium loss from dialysate (mmol) | 0.006 | 0.001 | <0.01 | | | | 0.02 | 0.00 | <0.01 |

PSTR, peritoneal solute transport rate; UF, ultrafiltration; PET, Peritoneal equilibration tests; ICO, icodextrin; PD, peritoneal dialysis

medium glucose exposure, daily average glucose exposure 1.36%-2.27%

high glucose exposure, daily average glucose exposure >=2.27%

## 4.4.5 The effect of peritonitis

The longitudinal relationship between Pcl and PSTR in patients who did or did not have peritonitis is shown in Figure 4-3. The pattern of dissociation was seen in both patient groups but PSTR tended to increase more significantly in those who had peritonitis, especially at Year 4. Multi-level modeling by peritonitis subgroup (see Table 4-4) did not alter the previously described associations except for revealing a significant relationship between Pcl and membrane UF capacity in the peritonitis positive group.

Figure 4-3 The uncoupling in both with and without peritonitis cohorts. The dissociation between PSTR and Pcl exists in both patients with and without peritonitis. The PSTR tends to increase more significantly in those with peritonitis.

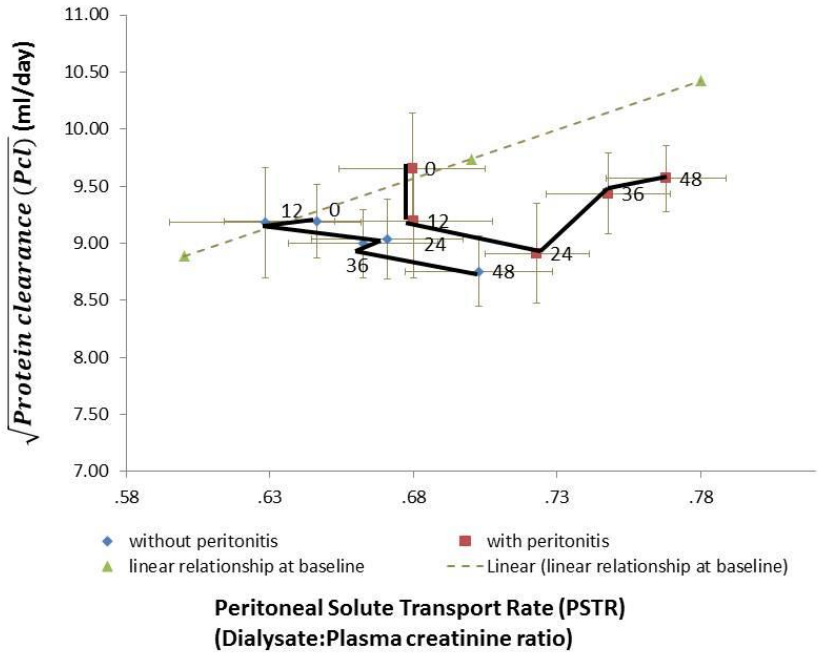


Table 4-4 Multi-level mixed linear model of daily Pcl (square root transformed) subgroup analysis to investigate peritonitis effect

| | without peritonitis (n=171) | | | with peritonitis (n=164) | | |
|---|---|---|---|---|---|---|
| | β | standard error | P value | β | standard error | P value |
| constant | 9.36 | 0.38 | <0.01 | 9.35 | 0.51 | <0.01 |
| PSTR (for each 0.1 increase) | 0.38 | 0.14 | <0.01 | 0.43 | 0.17 | <0.01 |
| comorbidity score (for each 1 unit increase) | -0.12 | 0.28 | NS | -0.28 | 0.28 | NS |
| gender (if female) | -0.54 | 0.43 | NS | 0.3 | 0.6 | NS |
| age (year) | 0.02 | 0.01 | NS | 0.03 | 0.02 | 0.06 |
| UF capacity on PET (for each 100ml increase) | 0.01 | 0.05 | NS | 0.12 | 0.05 | <0.01 |
| urine volume (for each 1000ml increase) | 0.3 | 0.2 | NS | -0.2 | 0.2 | NS |
| PSTR *PD duration (2nd compared with 1st year for each 0.1 increase in PSTR) | -0.19 | 0.16 | NS | -0.46 | 0.23 | <0.05 |
| PSTR *PD duration (3rd compared with 1st year for each 0.1 increase in PSTR) | -0.27 | 0.16 | NS | -0.54 | 0.21 | <0.05 |
| PSTR *PD duration (4th compared with 1st year for each 0.1 increase in PSTR) | -0.44 | 0.19 | <0.01 | -0.29 | 0.22 | NS |
| ICO usage (compared with not used) | -0.14 | 0.31 | NS | 0.1 | 0.27 | NS |
| medium glucose exposure (compared with low glucose exposure) (n=51) | 0.38 | 0.28 | NS | 0.42 | 0.25 | NS |
| high glucose exposure (compared with low glucose exposure) (n=13) | -0.08 | 0.54 | NS | 0.26 | 0.42 | NS |
| daily UF (for each 100ml increase) | -0.11 | 0.04 | <0.01 | -0.21 | 0.05 | <0.01 |
| daily sodium loss from dialysate (mmol) | 0.01 | 0.00 | <0.01 | 0.02 | 0.00 | <0.01 |

PSTR, peritoneal solute transport rate; UF, ultrafiltration; PET, Peritoneal equilibration tests; ICO, icodextrin; PD, peritoneal dialysis

medium glucose exposure, daily average glucose exposure 1.36%-2.27%

high glucose exposure, daily average glucose exposure >=2.27%

## 4.5 Discussion

The intention of this analysis was to determine as cleanly as possible whether longitudinal peritoneal Pcl is coupled to changes in PSTR by studying a continuously treated cohort of patients, uncontaminated by drop-out and adjusted for known clinical and theoretical factors that could influence the association. We demonstrated unequivocally for the first time that these two measures of membrane function dissociate over time and that this is independent of other predictors of Pcl (age, sodium coupled/small pore ultrafiltration) or known drivers of membrane change such as glucose exposure and peritonitis.

The uncoupling of Pcl and PSTR was due to a rise in the PSTR that was not matched by an increase Pcl in line with the relationship seen between these two measures at baseline. Increasing PSTR with time on PD is well established and from a theoretical perspective could be due either to an increase in the anatomical membrane in contact with dialysate, an increase in the density of perfused capillaries (to include neoangiogenesis) or an increase in capillary perfusion rate or any combination of the above. There are good reasons to believe that the clinical variability in PSTR at the start of PD is in large part due to local membrane inflammation and the strong relationship with Pcl at baseline observed in this study supports this. If this had been purely an anatomical coupling this should have remained the case throughout the study whereas in fact an increase in small pore area with a relative decline in the large pore area is observed. This relative reduction in large pore area could reflect a

number of processes including a resolution of the early inflammatory state of the membrane following the start of PD – several studies have also shown that depending on when the initial membrane function tests are done there is also a fall in PSTR in the months after PD is established before the longer term increase. [156-158] Alternatively it could mean that newly formed vessels are abnormally under-represented with large pores (surprising as new vessel formation in diabetic nephropathy is usually associated with increased protein extravasation) or that interstitial and peri-vascular fibrosis, known to occur in PD, is impeding large pore leak or even formation of what considered to be transient structures. Distinguishing these mechanisms is beyond the capacity of this study but it is possible that further development of the three pore membrane/fibre-matrix model, which offered a potential explanation of the observed "uncoupling" of small solute transport (PS) and UF coefficient (LpS) seen in long-term PD[84] might provide mechanistic insights. Measuring Pcl in the clinic is relatively easy and may provide an additional method of identifying membrane injury.

Known drivers of membrane injury, namely glucose and/or glucose degradation products (>95% of patients in thus study were treated with conventional glucose solutions) and peritonitis did not appear to have a marked effect on the progressive uncoupling of PSTR and Pcl. There is more than a suggestion that in the peritonitis group membrane changes were overall more severe over time, especially the increase in PSTR, and in the multivariate analysis of the peritonitis subgroups there was an association between membrane UF

capacity as well as PSTR in those patients who had infections. Cause and effect cannot be inferred from this type of analysis but this would be in line with more severe membrane injury causing reduced osmotic conductance and Pcl through more severe fibrosis.

The failure in this study to see a relationship between Pcl and comorbidity on multivariate analysis deserves comment. There are a number of explanations. First there was a relationship to increasing age as described previously[61, 72] and this could account for some hidden association with comorbid disease. Second this is a selected patient cohort and more serious comorbidity – or specific types such as peripheral vascular disease – were under-represented. Finally only 49 patients were included which may have led to type 2 statistical error in the level 2 component of the mixed-linear model.

It was important to correct for other known theoretical determinants of Pcl that might change with time on PD. Albumin, by far the most abundant protein lost in PD effluent, is of a size that can potentially pass through small as well as large pores. Small pore albumin flux is driven by convection rather than hydrostatic pressure. To determine the contribution of achieved ultrafiltration (small pore convection) to Pcl we included this in the model where it associated with Pcl independent of PSTR without affecting the time on treatment interaction. By including sodium removal which removed predominantly via small pore convection in the model the prediction that Pcl is partly determined by this pathway is confirmed.

As already eluded to this study has a number of limitations. Although our primary hypothesis was refuted it is observational and generates more questions than it answers especially with respect to cause and effect. In dealing with the concern related to informative censoring we had to define a selected patient group so generalisability should be considered carefully. This is a problem that all long-term studies of PD patients suffer from which are by their nature selective and in this sense the present study is no exception and the characteristics of the population studied are typical. For some of the covariates measured, e.g. glucose exposure there may not have been sufficient variability in the population to have sufficient power to detect an effect (only 13 observations used a high glucose regime (mean concentration >2.27%) in our study. The method we used to estimate Pcl is inversely dependent on plasma albumin which in turn co-varies with comorbidity which partly explains the relationship observed in cohort studies between Pcl and survival. This is unlikely to be a confounder in the present analysis both due to lack of drop-out and the fact that throughout the study the plasma albumin was very stable. Finally, a serial of models were carried out in the study, but no correction for multiple comparisons was made, which may potentially increase the risk of type I error. The variables were selected carefully based on scientifically plausible hypothesis rather than every possible combination, which should help to restrict the risk.

## 4.6 Conclusion

There is an uncoupling of the small (PSTR) and large (Pcl) pore pathways with time on PD which would be in keeping with a switch from local inflammation early on to progressive fibrosis combined with increased vascular surface area. Measuring longitudinal changes Pcl may complement membrane function tests monitoring progressive injury.

# chapter 5.**Understanding the ultrafiltration obtained with icodextrin-from theory to bedside**

## 5.1 Summary

### 5.1.1 Purpose

The purpose of the study is to determine whether the ultrafiltration (UF) obtained with icodextrin changes with time on PD. If so, it is to further investigate whether this change over time dissociated from the increase in peritoneal small solute transport rate (PSTR). It is also to test with clinically derived data which other factors are important in determining variations in the UF achieved with icodextrin as predicted from theoretical modelling and thus give a practical explanation of UF obtained with icodextrin for clinical use.

### 5.1.2 Methods

Net UF volumes obtained during long dwells were recorded as well as membrane characteristics and clinical factors every 6 monthly. Multi-level analysis was used to identify the predictor of UF by taking account of within subject correlations. The long dwell with icodextrin was analyzed for primary purpose. The long dwell with glucose solutions were also analyzed to enable comparisons with icodextrin to provide both historical and contemporary controls.

### 5.1.3 Results

2509 long-dwell observations from 613 patients in total were included in the analysis, 690 dwells in 202 patients using icodextrin, 849 dwells in 318 patients using glucose in the icodextrin-contemporary cohort (after 1998) and 970 dwells in 297 patients prior to icodextrin availability (before 1998). Among the 690 icodextrin dwells, 280 were using CAPD (typically 9 hours overnight dwell), 289 were on APD with day fill (typically 15 hours), and 121 were on APD with a short day fill (typically 9 hours day time dwell). In multi-level mixed linear modelling, prolonged time on PD therapy was related with less UF by icodextrin and this was dissociated from the effect of increase in PSTR. The UF negatively correlated to serum albumin, PSTR, UF capacity (UF in PET) and BMI were positively contributed to UF by icodextrin. CAPD was associated with more UF compared with APD using icodextrin or glucose for the long dwells, regardless of dwell length (9 hours or 15 hours). High input volume (2.5L) was related to less UF compared with 2L in both icodextrin and glucose long dwells after 1998.

### 5.1.4 Conclusion

Prolonged time on PD decreases UF obtained with icodextrin. It is uncoupled from the effect of increase in PSTR. It would be in keeping with a progressive fibrosis combined with increased in small pore area. Better UF capacity indicates more UF by icodextrin. The factors

which are likely to affect the oncotic pressure gradient, hydrostatic pressure gradient or icodextrin metabolites concentration (plasma albumin, input volume, patient position, BMI and gender) are more important than the dwell length in explaining UF variability. To achieve better UF obtained with icodextrin, dialysis prescriptions exert high intra peritoneal pressure should be avoided and prolonged dwell length may not be helpful.

## 5.2 Introduction

Growing evidence has shown that peritoneal dialysis (PD) leads to morphological and functional changes in peritoneal membrane over time. Of the functional changes, the "uncoupling" between small solute transport rate (PSTR) and UF capacity has been well established and successfully modeled by an extension of the three pore membrane to include a fiber matrix layer that mimics membrane fibrosis. [84, 95, 103] The work in Chapter 4 further showed there is a progressive uncoupling of the small pore (PSTR) and large pore (Pcl) pathways with time on PD. This would be in keeping with a switch from local inflammation early on to progressive fibrosis in which transcapillary protein leak is impaired combined with increased vascular surface area.

In severe fibrosis, there is "uncoupling" of PSTR from the peritoneal UF coefficient (LpS) or more precisely, the peritoneal osmotic conductance to glucose ($LpS\sigma_g$). [103] It is not clear whether this aspect of membrane function ($LpS\sigma$) can be demonstrated with a non-glucose containing dialysis fluid.

Icodextrin was intentionally chosen in this study. On one hand, it is now widely used nowadays especially when having difficulties with fluid balance. It has been regarded as a standard strategy for patients with high PSTR who tend to achieve less UF with glucose solution. On the other hand, the mechanism of action of icodextrin as a dialysis fluid is quite

different from glucose. Icodextrin achieves UF through oncotic rather than osmotic pressure (it is isotonic with plasma) and this occurs via the small pore system only while glucose solutions obtain UF through both small pore and ultra-small pores (aquaporins).

The purpose of the study is to

(1) Determine whether the UF obtained with icodextrin – which only occurs via the small pore pathway - changes with time on PD.

(2) If so, it is to further investigate whether this change over time dissociated from the increase in PSTR, and thus independent of the change in small pore area.

(3) to test with clinically derived data which other factors are important in determining variations in the UF achieved with icodextrin as predicted from theoretical modelling and thus

(4) give a practical explanation of UF obtained with icodextrin for clinical use.

## 5.3 Methods

### 5.3.1 Study design and patient population

This was a retrospective analysis of a single centre, longitudinal, observational cohort study in which data were prospectively collected from 1990 until censored in Oct 2010. Peritoneal membrane function using peritoneal equilibration test (PET) and dialysis adequacy were routinely measured within the first one or two months of treatment, and then usually at 6 monthly interval unless prevented by acute illness or subject availability. The PD prescription, UF of each exchange and urine volume on the day of dialysis adequacy test was recorded. Baseline data including demography, primary renal disease and comorbidity, were recorded as when they started PD. Data was collected and stored on a validated database (PDDB©).

The long dwells (the longest exchange of the day and no less than 7 hours) was analyzed in this study; typically this was the night dwell in continuous ambulatory peritoneal dialysis (CAPD) and the day dwell in automated peritoneal dialysis (APD), the main difference reflecting the posture of the patient during this period. The long dwell with icodextrin was analyzed as the primary purpose of the study. The long dwell with glucose based solution was also analyzed to compare with icodextrin because there was a major change in clinical policy in June 1998 (see detail below) and because of the potential selective bias related to this, the pre-1998 and post- 1998 observation groups were analyzed separately.

Before June 1998 the vast majority of the patients were treated with CAPD and glucose based solutions. The description of the Stoke cohort before 1998, survival analysis and longitudinal membrane change are described and published previously. [98] The handful exceptions, i.e. those not on CAPD and glucose based solutions before 1998 were excluded from the analysis due to the fact that they were highly selected patients. The clinical policy changed since then. In brief, an increasing proportion of patients were treated with APD, particularly when anuric and with difficulties in achieving a creatinine clearance target of 60L/week/1.73m$^2$, as described in the European Automated Peritoneal Dialysis Outcome Study.[60] Patient choice, mainly related to their life style, was also taken into account in the modality use (APD or CAPD). Icodextrin was more likely to be used in patients with higher PSTR especially when combined with clinical difficulties of fluid management. No more than one bag of icodextrin was used per day.

### 5.3.2 Characteristics of long dwell in different modalities

Three PD modalities with long dwell were applied in our unit:

(1) APD with day fill, short exchanges at night and the long dwell was in day time, typically 15 hours.

(2) APD with short day fill, short exchanges at night and the long dwell was in day time installed in the morning and drained out at about 4pm, typically 9 hours, followed or

not followed by a medium length extra day dwell. This modality was used in patient with difficulties in achieving clearance target even when on APD with day fill or reabsorbing fluid with prolonged dwell length.

(3) CAPD, medium dwells in day time and the long dwell at night, typically 9 hours.

### 5.3.3 UF volume measurement and membrane function test

For APD, the UF volume was measured by the APD device. Net UF volume from CAPD was accessed by weighing the drainage bag, converting the weight into volume by multiplying the specific gravity of 1g/ml and subtracted the expected input volume accounting for the average 'overfill', which had consistently been measured at 200mls in Stoke [159].

We estimated PSTR, through measuring dialysate:plasma ratio of creatinine ($D/P_{creatinine}$) at the end of a standard 2L, 2.27%, 4 hour dwell in peritoneal equilibration test (PET). Net UF from this standard dwell was used as a measure of UF capacity. (Without subtract for overfill)

### 5.3.4 Comorbidity

Comorbidity was documented as described previously [160]. Briefly, 7 comorbid domains were considered, including non-cutaneous malignancy, ischemic heart disease (IHD), peripheral vascular disease (PVD), left ventricular dysfunction (LVD), diabetes mellitus (DM), systemic collagen vascular disease, and any other condition known to reduce life expectancy. The

comorbidity score for each patient was defined as the number of these domains affected. The comorbidity grade was then derived from the comorbidity score. Grade 0 (low risk) was a zero score, grade 1 (medium risk) was a score of 1-2, and grade 2 (high risk) a cumulative score of ≥ 3.

### 5.3.5 Analytical methods

Plasma and dialysate concentrations of creatinine, glucose and plasma albumin were measured by the central lab in the hospital. Plasma albumin levels had been measured using the bromocresol green method before 22th Aug 2007 and was switched to bromocresol purple method afterward. An average difference of 5.5g/L between the results of the two methods had been established by Clase et al and the convert equation derived from them was used to adjust the data [149].

### 5.3.6 Statistical analysis

All data were expressed as means (±SD) unless the distribution was not normal, in which case medians (interquartile range) were shown. Between-group comparisons used the 2-tailed unpaired t-test, the Mann-Whitney U test, one way ANOVA, independent samples Kruskal Wallis test or the chi-squared test depending on the data type, the number of groups and the distribution. Pearson and Spearman correlation were used for bivariate correlation according to the distribution. The majority of the input volumes were 1500ml, 2000ml and

2500ml, with a dozen of exceptions in total, ranging from 500ml to 3000ml. The input volume was re-coded into category variable using the following rules: ≤1750ml→1500ml, 1750ml to 2250ml→2000ml, >2250ml→2500ml.

Multi-level mixed linear model was applied in the study. Repeat observations (level 1) were nested within individuals (level 2). The dependent variable was the UF volume obtained from the long dwell and the independent variables were included in the multivariate model either if they were significantly related to the dependent variables on the uni-level bivariate correlation or they were plausible explanatory variables according to the 3-pore membrane model or known predictors in other studies. Variables that remain constant within patients, such as gender and comorbidity at baseline were put in level 2, whereas those that change within patients over time such as age, time on therapy, membrane characteristics, et al were treated as level 1. The intercept was set as random in level 2 to allow the between patient difference and the other explanatory variables were fixed coefficients. The continuous variables were centered on the mean in the model to facilitate the clinical interpretation. The multi-level modelling were performed using MLwin software (Version 2.22, Centre for multilevel modelling University of Bristol)

## 5.4 Results

### 5.4.1 The patient and membrane characteristics according to different observation groups

2509 long-dwell observations from 613 patients in total were in the analysis, 690 dwells in 202 patients using icodextrin, 849 dwells in 318 patients using glucose in the icodextrin-contemporary cohort (after 1998) and 970 dwells in 297 patients prior to icodextrin availability (before 1998). see Figure 5-1 and Figure 5-2. The medium number of observations per individual was 3, (range 1 to 21).

Figure 5-1 dialysate regime for long dwells over the years

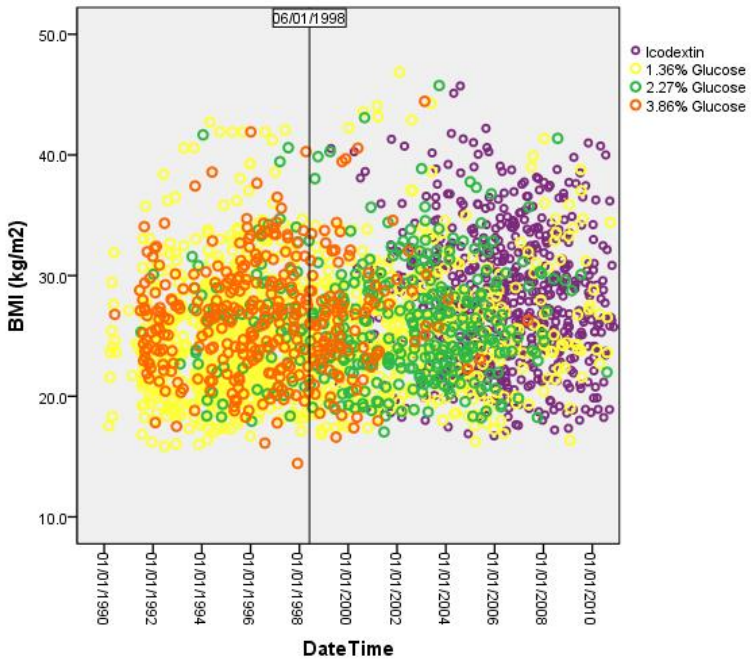
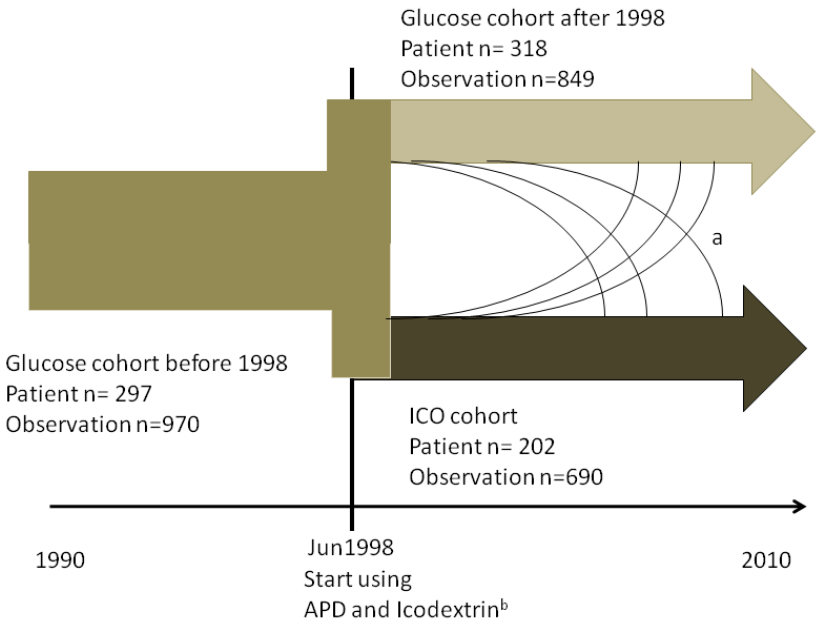


Figure 5-2 sub cohort for statistical analysis according to era and dialysate regime




a. Patients may switch from glucose based long exchange solute to Icodextrin during the time or vice versa.
b. Patients still on the PD therapy at this time may move to after 1998 glucose cohort or switch to icodextrin cohort

The patient and membrane characteristics in the different observation groups were shown in Table 5-1. These reflect the historical change of our PD cohort and clinical practice. In brief, there was more diabetes after 1998 and with higher BMI. The residual renal function, represented by urine volume, was higher after 1998 and patients were on the PD therapy for a longer time. After 1998, patients selectively moved to icodextrin or glucose long dwells according to the new clinical policy described before. As a result, the icodextrin observation

group had higher PSTR, less good UF capacity on PET, less urine output, were on PD for longer time, more likely diabetic and with heavier comorbidity burden.

Table 5-1 Patient characteristics, membrane characteristics and PD prescription in the three observational groups, 1. glucose before 1998, 2. glucose after 1998 and 3. icodextrin (after 1998)

| | Glucose before 1998 n=970 | Glucose after 1998 + icodextrin (after 1998) n=1539 | P value[a, c] | Glucose after 1998 n=849 | Icodextrin (after 1998) n=690 | P value[b, c] |
|---|---|---|---|---|---|---|
| age (year) | 56.4±16.1 | 56.2±16.0 | NS | 55.4±16.5 | 57.1±15.5 | <0.05 |
| gender (M/F)% | 53.0/47.0 | 53.8/46.2 | NS | 49.8/50.2 | 58.7/41.3 | <0.01 |
| BMI (kg/m$^2$) | 25.3±4.9 | 26.7±5.2 | <0.01 | 25.8±4.9 | 27.7±5.4 | <0.01 |
| time on therapy (mo) | 15(6-31) | 19(7-37) | <0.01 | 14(3-34) | 24(13-40) | <0.01 |
| urine volume (ml) | 350(0-930) | 703(197-1335) | <0.01 | 940(319-1572) | 529.5(62-1000) | <0.01 |
| Long dwell input volume % (N)<br>1500ml<br>2000ml<br>2500ml | <br>18.5 (179)<br>73.3 (711)<br>8.2 (80) | <br>20.1(309)<br>70.3 (1082)<br>9.6 (148) | NS | <br>13.4 (114)<br>78.7 (668)<br>7.9 (67) | <br>28.3 (195)<br>60.0 (414)<br>11.7 (81) | <0.01 |
| PSTR | 0.61±0.11 | 0.68±0.14 | <0.01 | 0.63±0.12 | 0.74±0.13 | <0.01 |
| UF capacity on PET (ml) | 419±235 | 423±233 | NS | 451±226 | 388±236 | <0.01 |
| albumin (g/L) | 30.5(27.5-34.5) | 31.5(28-33.5) | NS | 30.7±4.7 | 30.8±4.4 | NS |
| comorbidity grade(Low/Medium/High) | 44.9/47.6/7.4 | 43.7/46.9/9.4 | NS | 50.4/41.3/8.2 | 35.5/53.8/10.7 | <0.01 |
| DM (%) | 13.6 | 23.2 | <0.01 | 17.0 | 39.7 | <0.01 |
| Dialysate type % (N)<br>Icodextrin<br>1.36% Glucose<br>2.27% Glucose<br>3.86% Glucose | <br>0<br>66.7 (647)<br>8.0 (78)<br>25.3 (245) | <br>44.8 (690)<br>30.0 (461)<br>19.0 (293)<br>6.2 (95) | n/a | <br>0<br>54.3 (461)<br>34.5 (293)<br>11.2 (95) | <br>100 (690)<br>0<br>0<br>0 | n/a |
| Modality (N)<br>CAPD<br>APD with day fill<br>APD with short day fill | <br>970<br>0<br>0 | <br>1012<br>378<br>149 | n/a | <br>732<br>89<br>28 | <br>280<br>289<br>121 | <0.01 |

a, Glucose before 1998 vs Glucose after 1998 and icodextrin; b, Glucose after 1998 vs Icodextrin

c, unpaired t-test and one way ANOVA for continuous normal distributed variable, Mann-Whitney U test and independent samples Kruskal Wallis test for non-normal distributed variables, chi-squared test for category variables

NS, not significant; n/a, not applicable; BMI, body mass index; UF, ultrafiltration; PET, Peritoneal equilibration test; DM, diabetes

### 5.4.2 Comparison of patient and membrane characteristics between PD modalities within observation groups

The patient and membrane characteristics of the different modalities within icodextrin and glucose after 1998 observation groups are displayed in Table 5-2. There were fewer patients on CAPD in the icodextrin observation group compared with glucose observation group ($P<0.01$). In both observation groups, CAPD patients were older. The APD with short day fill group was characterized as having less urine output, on PD for longer time, and higher PSTR.

Table 5-2 Comparison of the patient and membrane characteristics between different PD modalities within icodextrin (after 1998) and glucose after 1998 observation groups

| | Icodextrin (after 1998) N=690 | | | | Glucose after 1998 N=849 | | | |
|---|---|---|---|---|---|---|---|---|
| | CAPD (n=280) | APD with day fill (n=289) | APD with short day fill (n=121) | P value[a] | CAPD (n=732) | APD with day fill (n=89) | APD with short day fill (n=28) | P value[a] |
| age (year) | 59.7±13.9 | 54.4±17.6 | 57.6±12.3 | <0.01 | 56.5±16.2 | 48.8±16.3 | 48.3±16.7 | <0.01 |
| gender (M/F)% | 62.9/37.1 | 50.9/49.1 | 67.8/32.2 | <0.01 | 49.5/50.5 | 52.8/47.2 | 50.0/50.0 | 0.84 |
| BMI ($kg/m^2$) | 27.3±5.6 | 27.8±5.7 | 28.4±4.0 | 0.18 | 25.9±5.0 | 24.9±4.6 | 26.6±3.9 | 0.13 |
| time on therapy (mo) | 21.5(12-37.5) | 24(13-39) | 33(20-49) | <0.01 | 13(2-34) | 15(6-33) | 24(17-45) | <0.01 |
| urine volume (ml) | 659(254.5-1131.5) | 597(152-1036) | 0(0-372) | <0.01 | 1006(395-1610.5) | 819(180-1632) | 0(0-140) | <0.01 |
| input volume % (N)<br>1500ml<br>2000ml<br>2500ml | 3.9 (11)<br>78.9 (221)<br>17.1 (48) | 56.1 (162)<br>38.4 (111)<br>5.5 (16) | 18.2 (22)<br>67.8 (82)<br>14.0 (27) | <0.01 | 10.1 (74)<br>82.8 (606)<br>7.1 (52) | 42.7 (38)<br>50.6 (45)<br>6.7 (6) | 7.1 (2)<br>60.7 (17)<br>32.1 (9) | <0.01 |
| PSTR | 0.74±0.11 | 0.74±0.14 | 0.77±0.14 | 0.053 | 0.63±0.12 | 0.66±0.12 | 0.68±0.11 | <0.01 |
| UF capacity on PET (ml) | 432±244 | 380±219 | 309±236 | <0.01 | 451±216 | 465±285 | 397±263 | 0.38 |
| albumin (g/L) | 31.5(29-34) | 31(27.5-33.5) | 31(28.5-33.5) | 0.07 | 31.5(27.5-33.5) | 30.5(28-33) | 30.5(26-36) | 0.72 |
| comorbidity grade (Low/Medium/High) % | 28.9/61.8/9.3 | 38.4/48.8/12.8 | 43.8/47.1/9.1 | <0.01 | 51.2/39.8/9.0 | 42.7/52.8/4.5 | 53.6/46.4/0.0 | <0.01 |
| DM (%) | 47.5 | 37.0 | 28.1 | <0.01 | 16.4 | 15.7 | 3.6 | 0.19 |

a one way ANOVA for continuous normal distributed variable, independent samples Kruskal Wallis test for non-normal distributed variables, chi-squared test for category variables

BMI, body mass index; UF, ultrafiltration; PET, Peritoneal equilibration test; DM, diabetes

### 5.4.3 The multi-level mixed linear model for long-dwell UF according to type of dialysate

The long-dwell UF was different between gender, diabetic status, input volume, comorbidity grade and PD modality in at least one of the three observation groups. Time on therapy, BMI, UF capacity (on PET), PSTR, albumin and urine volume were significantly related to long-dwell UF on uni-level bivariate analysis in one or more observation groups. The detailed between group differences and uni-level bivariate correlations for the UF volume by observation groups is available in Table 5-3. These variables were included in multi-level mixed linear model, see Table 5-4.

Gender, time on PD, PD modality (APD, no matter whole day fill or short day fill, compared with CAPD), input volume, BMI, PSTR, UF capacity on PET and plasma albumin were independent explanatory variables of UF obtained by icodextrin. These explanatory variables explained 21% of the whole variability of UF in icodextrin dwells. Within the unexplained variance, 31% was due to the between patient difference and 69% by within patient difference.

In contrast to the icodextrin dwell, for the glucose long dwell (after 1998), urine volume was an independent covariate ($\beta$=-0.14, P<0.01), while the independent correlation between BMI, time on PD and UF by icodextrin was absent. The correlation between long dwell UF and

PSTR was negative for the glucose dwell and positive for icodextrin dwell. Lower plasma albumin independently correlated with more UF by icodextrin whereas the relationship was the opposite for glucose. Diabetic status did not have an impact on UF using icodextrin, while it correlated to more UF in glucose dwell. 34% of the variance of UF by glucose was explained by the explanatory variables in the model. The glucose concentration on its own explained 15% of the whole variance. 27% of the unexplained variance came from between patient difference and 73% was within patient difference.

Comparing the two glucose observation groups, before and after 1998, urine volume was an independent covariate in both observation groups. Gender, input volume, albumin and diabetic status were not independent covariates previously, but were more recently. Glucose concentration accounted for much more total variance of UF (24% vs 15%) before than after 1998. (Table 5-4)

Table 5-3 between group difference and uni-level bivariate correlation of the UF volume

| | | Glucose before 1998 n=970 | | Glucose after 1998 n=849 | | Icodextrin (after 1998) n=690 | |
|---|---|---|---|---|---|---|---|
| | | mean±SD | P value[a] | mean±SD | P value[a] | mean±SD | P value[a] |
| Gender | Female | -23.4±361.1 | NS | 10.8±382.7 | NS | 317.9±268.9 | <0.01 |
| | Male | -43.7±418.1 | | 3.5±342.8 | | 416.5±322.5 | |
| DM or not | non-DM | -33.9±384.7 | NS | -6.2±349.2 | <0.05 | 342.4±295.2 | <0.01 |
| | DM | -36.0±438.7 | | 72.6±420.4 | | 426.4±313.7 | |
| input volume | 1500ml | -58.9±293.6 | NS | -203.4±337.3 | <0.01 | 283.7±268.9 | <0.01 |
| | 2000ml | -34.3±405.8 | | 35.5±343.5 | | 432.6±311.7 | |
| | 2500ml | 22.8±456.7 | | 83.2±465.3 | | 307.0±288.2 | |
| comorbidity grade | low | -11.9±412.4 | <0.05 | 12.9±360.1 | NS | 338.7±253.1 | NS |
| | medium | -66.0±370.8 | | 3.7±372.4 | | 393.4±336.6 | |
| | high | 35.0±388.3 | | -10.4±338.1 | | 410.1±288.8 | |
| PD modality | CAPD | -34.2±392.3 | n/a | 30.6±354.8 | <0.01 | 489.9±271.5 | <0.01 |
| | APD with day fill | | | -186.0±359.2 | | 287.8±291.4 | |
| | APD with short day fill | | | 8.9±419.0 | | 322.0±328.2 | |
| | | correlation coeffecient[b] | | correlation coeffecient | | correlation coeffecient[b] | |
| age (year) | | 0.01 | 0.78 | 0.04 | 0.26 | 0.04 | 0.27 |
| time on therapy (mo) | | 0.09 | 0.01 | 0.04 | 0.21 | -0.10 | 0.01 |
| BMI (kg/m2) | | 0.04 | 0.24 | 0.06 | 0.08 | 0.15 | <0.01 |
| Weight (kg) | | 0.03 | 0.36 | 0.06 | 0.10 | 0.14 | <0.01 |
| UF capacity on PET (ml) | | 0.09 | <0.01 | 0.14 | <0.01 | 0.13 | <0.01 |
| PSTR | | -0.11 | <0.01 | -0.15 | <0.01 | 0.17 | <0.01 |
| albumin (g/L) | | 0.02 | 0.58 | 0.03 | 0.39 | -0.15 | <0.01 |
| urine volume (ml) | | -0.23 | <0.01 | -0.35 | <0.01 | 0.12 | <0.01 |

BMI, body mass index; UF, ultrafiltration; PET, Peritoneal equilibration test; DM, diabetes

a, unpaired t-test or one way ANOVA according to number of groups

b, Pearson correlation

Table 5-4 Multi-level mixed linear model for long dwell UF obtained by icodextrin or glucose based dialysate (before and after 1998 separately)

| | Glucose before 1998 n=970 | | | Glucose after 1998 n=849 | | | Icodextrin (after 1998) n=690 | | |
|---|---|---|---|---|---|---|---|---|---|
| | β | SE | P value | β | SE | P value | β | SE | P value |
| constant | -159.2 | 26.6 | <0.01 | 108.8 | 34.6 | <0.01 | 472.7 | 32.8 | <0.01 |
| Gender (if female) | -11.3 | 28.8 | NS | -67.5 | 28.0 | <0.05 | -81.4 | 30.9 | <0.01 |
| time on PD (for each month) | 0.73 | 0.61 | NS | 0.80 | 0.52 | NS | -1.17 | 0.54 | <0.05 |
| Urine Volume (for each ml) | -0.08 | 0.02 | <0.01 | -0.14 | 0.02 | <0.01 | -0.02 | 0.02 | NS |
| APD with short day fill (compared with CAPD) | | | | -191.9 | 59.3 | <0.01 | -160.2 | 37.0 | <0.01 |
| APD with day fill (compare with CAPD) | | | | -174.1 | 37.4 | <0.01 | -165.6 | 30.8 | <0.01 |
| input volume 1500ml (compared with 2000ml) | -32.8 | 35.8 | NS | -42.5 | 39.4 | NS | 16.1 | 33.9 | NS |
| input volume 2500ml (compared with 2000ml) | -8.0 | 38.5 | NS | -142.3 | 43.3 | <0.01 | -110.7 | 41.6 | <0.05 |
| BMI (for each 1kg/$m^2$ increase) | -5.1 | 2.9 | NS | 5.1 | 2.8 | NS | 11.4 | 2.7 | <0.01 |
| PSTR (for each 0.1 increase) | -85.1 | 11.7 | <0.01 | -57.35 | 9.71 | <0.01 | 34.5 | 8.56 | <0.01 |
| UF capacity on PET (for each ml increase) | 0.12 | 0.05 | <0.05 | 0.10 | 0.05 | <0.05 | 0.10 | 0.04 | <0.05 |
| Albumin (for each 1g/L increase) | 1.1 | 2.2 | NS | 5.7 | 2.3 | <0.05 | -10.3 | 2.5 | <0.01 |
| Diabetes (if yes) | -27.3 | 39.1 | NS | 75.3 | 34.2 | <0.05 | 4.7 | 31.9 | NS |
| 2.27% Glucose (compared with 1.36% Glucose) | 238.2 | 39.3 | <0.01 | 130.7 | 25.9 | <0.01 | NS | | |
| 3.86% Glucose (compared with 1.36% Glucose) | 520.5 | 26.4 | <0.01 | 402.4 | 37.6 | <0.01 | NS | | |
| Explained variance by the whole model (%) | 36 | | | 34 | | | 21 | | |
| Proportion of whole variance explained by glucose concentration (%) | 24 | | | 15 | | | NA | | |
| Residual variance accounted by between/within patient difference (%) | 25/75 | | | 27/73 | | | 31/69 | | |

BMI, body mass index; UF, ultrafiltration; PET, Peritoneal equilibration test; DM, diabetes

## 5.5 Discussion

This is the first study demonstrated that over time there is less UF obtained using icodextrin in long term PD patients despite the fact that they tend to have a higher PSTR (small pore area). It is also the first study to give such a practical and comprehensive description of factors associated with the variability in UF achieved with icodextrin in a clinical setting. It also provides useful information for the clinician by providing evidence on which to base prescription guidance to get the best UF using icodextrin.

### 5.5.1 The changing membrane

In response to the original research question, the most important findings of this study was the impact of time on therapy and UF capacity in PET on achieved UF obtained with icodextrin; both point to membrane fibrosis and reduced osmotic conductance for glucose. It is likely that one component of the osmotic conductance ($LpS\sigma_g$), namely the liquid membrane permeability (Lp) also affects fluid transport obtained with icodextrin (in effect, $LpS\sigma_g$ is proportional to $LpS\sigma_{ico,}$ although σ is of course very different as will be fluid reabsorption kinetics). It is therefore possible that the observation that UF with icodextrin declined with time on treatment, despite the increase in PSTR, reflects a reduction in $LpS\sigma_{ico}$ due to reduced Lp as observed in severe type 2 UF failure, usually present after prolonged PD use [161]. This is thought to be associated with the development of fibrosis [162] and the cause

the “uncoupling” of PSTR from the peritoneal UF coefficient (LpS) or more precisely, the peritoneal osmotic conductance to glucose ($LpS\sigma_g$) [103].

This study confirmed the previous finding that fast transporters achieve a better UF than slower transporters [163, 164] and also gave a practical estimate of the size of this effect. A patient with PSTR of 0.9 would be expected to have about 100ml more UF than a patient with PSTR 0.6. According to the three pore model, icodextrin draws UF almost exclusively through small pores. High transport status measured by PET corresponds to a bigger effective membrane area and therefore more small pores.

### 5.5.2 Osmotic pressure gradient

The present study was the first clinical study to show that patients with lower albumin levels, who tend in general to have more difficulties in fluid management, have, as predicted by modelling, more UF when using icodextrin compared with those with normal albumin levels. It fits with the clinical observation that patients with poor fluid status gained more UF from icodextrin [165]. According to the three-pore model, icodextrin exerts its main effect by creating colloid osmotic pressure. The computer-simulation based on the three-pore model predicts that plasma colloid osmotic pressure has a major impact on UF in icodextrin dwells [166]. Most of the colloid osmotic pressure is attributable to serum albumin due to its predominant amount in circulation and small molecular weight. A study in a mixed group of healthy

individuals and hospital or clinic patients with various diseases showed a strong correlation between albumin concentration and colloid osmotic pressure, r=0.882 [167]. The reason that previous studies did not find this relationship between UF and albumin is likely due to the high day to day variance in both UF and albumin concentrations. The large sample size and use of a multi-level analytic approach as used here are needed to show this relationship.

The advantage of using icodextrin in diabetes has been noticed and emphasized since it was first established due to its metabolic benefit. Several studies also proved its efficiency in fluid management in dietetic patients [168, 169]. Ahmad, et al, further reported the observation that icodextrin actually produce higher UF in diabetic than in non-diabetic patients [170]. Our study proved their observation in uni-variate analysis. However, when accounting for the other variables in the multi-variate model, especially albumin and transport status, the difference between diabetic and non-diabetic disappear.

### 5.5.3 The effect of dwell volume

The observation that a large input volume was related to less UF is different from the computer stimulation based on the three pore model which predicts similar or, if anything, more UF in large input dwells (2.5L or 3L) [171]. The effect of large input volume in the clinical setting is complicated. Apart from the fact that osmotic pressure maintains longer with large input volume, although this effect is much more predominant in glucose dwells, it increases

the effective membrane area on one hand, and increases intra-peritoneal hydrostatic pressure on the other. According to the present study, it seems that the effect of the longer maintained osmotic pressure and increased efficient membrane area, which potentially increase UF, was over-ridden by the high intra-peritoneal hydrostatic pressure. Input volume has a major impact on intraperitoneal pressure. An elevated intra-peritoneal hydrostatic pressure would favour fluid reabsorption by whatever route (transcapillary or lymphatic). From computer stimulations, we know that an increased rate of macromolecular clearance out of the peritoneal cavity could be related to a decreased UF by icodextrin. There is also clinical data showed the significant effect of hydrostatic pressure on UF with glucose exchanges [172]. A study in children confirmed the negative relationship between intra-peritoneal pressure and net UF achieved by glucose in four hour dwells but found no such relationship in icodextrin [173]. However, these results should be interpreted with caution, especially when extrapolating to long dwells and adults, as observations were limited to 4-hour dwells and it is known that children achieve less UF with icodextrin than seen in adult [174]

### 5.5.4 The UF difference between APD and CAPD

Another important finding of this study was that CAPD achieves more UF than APD in long dwells, regardless icodextrin or glucose solution. The difference in intraperitoneal pressure

between supine and up-right may contribute some of the difference between APD and CAPD long dwells as mentioned before.

But it may not be the only explanation. It is difficult to compare daily achieved UF with APD versus CAPD as it very much depends on the prescription. However, on the one hand, it is widely accepted that CAPD is as good as APD in terms of preserving residual renal function, if not better. On the other hand, higher daily UF in CAPD has been noticed in several studies [175-177]. Neglecting overfills used to be a reason for overestimating UF in CAPD, but awareness of this is now established. [159, 178, 179]. There are still studies like the present one which had clearly accounted for overfill yet still find a difference in UF between APD and CAPD. A follow up study in incident patients showed UF was consistently lower in incident APD patients than in their counterparts undergoing CAPD. Again, unexpectedly, the residual renal function declined faster in the APD group rather than the CAPD group in that study. None of the studies so far, including the present study, have taken into account the difference between volumetric measurement versus weighing of dialysis fluid in assessing the amount of UF. In fact, specific gravity of drained dialysate is greater than 1.0, so using the equivalent values (1g=1ml) in CAPD patients will overestimate UF, whereas it is measured as true volume by the APD device. According to the average specific gravity of drained icodextrin (1.026g/ml) measured in limited number of samples, there is an about 60 ml over estimation of UF in CAPD in a single icodextrin dwell (2L) with 0.4L UF. (More detailed calculation see appendix)

### 5.5.5 The effect of dwell length

UF using icodextrin is expected to increase in a linear fashion for at least until 17 hours according to the computer stimulation base on the "standard" set of parameters of the three-pore model [171]. This analysis suggests that UF from 15 hour dwells is no more than 9 hours dwells on average To address this problem Jeloka at al. designed a study to discover the relationship between UF and dwell length [180] in icodextrin in which Dwell length was increased by one hour every week, from 10 to 14 hours. In keeping with the present observation they found there was no significant increase in UF beyond 10 hours on average but discovered a number of divergent UF patterns, with the peak being achieved at different times in different subjects. By resetting the parameters in three pore model it is possible to partially explain this variability [166]. It is clinically useful to be aware that prolonged dwell time does not always lead to better UF using icodextrin.

### 5.5.6 Effects of the Icodextrin metabolite distribution volume

Another finding of this study that had not been reported before was the positive relationship between UF and BMI, as well as the gender difference in icodextrin UF. Combined with the previous observations that body weight, or more precisely the volume of distribution was the only important predictor of plasma icodextrin metabolite concentrations in both long term patients [181] and in a single exchange kinetics study [182], it raises the possibility that the effects

of BMI and gender are a function of plasma icodextrin metabolite concentrations which then affect achieved UF. It is known that icodextrin use increases the plasma icodextrin metabolite concentrations and the icodextrin metabolites increase plasma osmolality [183-185]. Computer simulations indicate that the osmolar effect of icodextrin metabolites might oppose peritoneal UF [171] and fit with the known phenomenon that patients naïve to icodextrin get more UF than those on long term use. However, the hypothesis that plasma icodextrin metabolites may negatively correlate with UF has not been validated in clinical studies so far. A study done by Ota et al. in 18 patients demonstrated 5 mOsm/L increase in plasma osmolality due to circulating icodextrin metabolites, but the plasma icodextrin metabolite level did not have statistically significant influence on net UF [185]. Taking the relatively small impact of the osmolar effect on UF within the deviation of plasma icodextrin metabolite in PD population, a much bigger sample size such as achieved with the present study would be needed to pick up a the clinically and statistically significant effect on UF. The technical difficulty and expense in measuring icodextrin metabolites make this difficult to carry out such a big study.

Table 5-5 the potential linkage between clinical observation and theoretic parameters

| | Clinical phenomenon | Theoretic parameters |
|---|---|---|
| icodextrin | PSTR↑, UF ↑ | Membrane area (S) |
| glucose | PSTR↑, UF ↓ | |
| icodextrin | Time on therapy ↑, UF ↓ | Hydraulic permeability (Lp)<br>reflection coefficient (σ) |
| | UF capacity in PET ↑ , UF ↑ | |
| glucose | Time on therapy ↑, UF ↔ | |
| | UF capacity in PET ↑ , UF ↑ | |
| icodextrin | Big input volume (2500ml), UF ↓ | hydrostatic pressure gradient |
| | APD less than CAPD in UF | |
| glucose | Big input volume (2500ml), UF ↓ | |
| | APD less than CAPD in UF | |
| icodextrin | Female less than male in UF | Icodextrin metabolite concentration |
| | BMI↑ , UF ↑ | |
| glucose | NA | |
| icodextrin | albumin ↓, UF ↑ | Colloid osmotic pressure gradient |
| glucose | Glucose concentration | crystalloid osmotic pressure gradient |
| icodextrin | 9h vs 15h similar [a] | Dwell length |
| glucose | 9h vs 15h similar [a] | |
| icodextrin | urine volume ↑, UF ↔ | Others: urine volume |
| glucose | urine volume ↑, UF ↓ [b] | |
| icodextrin | nil | Others: comorbidity (diabetes) |
| glucose | nil [c] | |

The selective biases had to bear in mind when interpreting the result.

a, UF in 15 hours dwells was no more than 9 hours, but it could be less. Because one of the reasons to switch from 15 hours to 9 hours dwells was unsatisfied UF.

b, more urine volume related to less UF in glucose dwells. It was likely that those with less UF would be kept on glucose solution only when they had enough urine volumes, otherwise they would have been switched to icodextrin solution or HD.

c, diabetic status was related to more UF in glucose dwells after 1998. Similar argument as b.

### 5.5.7 Limitations

This study had a number of limitations. First, it was not a randomized controlled trial and the selective biases clearly existed in response to the changing prescription policy over 20 years. These selective biases have to be borne in mind when interpreting the result, especially for glucose dwells. For example, more urine volume was related to less UF in glucose dwells in this study. It was likely that those with less UF would be kept on glucose solution only when they had enough urine volumes, otherwise they would have been switched to using the icodextrin solution or HD. Similar arguments may also be applied for the finding that there was no impact of time on therapy in glucose dwells and that diabetic status predicts more UF after 1998. The lack of difference between 9 hour and 15 hour dwells by glucose based solution may again due to the intended switch from long dwells to shorter dwells in patients who were not obtaining enough UF. Secondly, due to the observational nature of the study, the selective biases also came from the disproportional drop out of patients due to technique failure or death. However, the negative correlation between time on PD and UF using icodextrin was not likely to come from this informative censoring as it is unlikely that patients achieving better UF from icodextrin would leave the therapy earlier. Thirdly, the interaction effect between input volume, glucose concentration and PD modality (CAPD/APD) could not been further studied because of the small number of observations in some break down categories. Fourthly, only linear relationships was analysed in detail in the present study,

whereas more complex non-linear relationship may exist as seen in the study by Jeloka[180]. Fifthly, no correction for multiple comparisons was made in the study which may potentially increase the risk of type I error. The variables selected in the model were based on scientifically plausible hypothesis rather than every possible combination, which should limit the risk. Finally, none of the theoretical parameters were directly measured in the study (e.g. plasma oncotic pressure), but rather clinically measured surrogates were used (e.g. plasma albumin).

## 5.6 Conclusion

1. Prolonged time on PD decreases UF obtained with icodextrin.
2. It is uncoupled from the effect of increase in PSTR. It would be in keeping with a progressive fibrosis combined with increased in small pore area.
3. Better UF capacity indicates more UF by icodextrin. The factors which are likely to affect the oncotic pressure gradient, hydrostatic pressure gradient or icodextrin metabolites concentration (plasma albumin, input volume, patient position, BMI and gender) are more important than the dwell length in explaining UF variability.
4. To achieve better UF obtained with icodextrin, dialysis prescriptions excert high intra peritoneal pressure should be avoided and prolonged dwell length may not be helpful.

# chapter 6.**What is the relationship between soluble biomarkers of inflammation and endothelial damage, systemic transcapillary albumin leak and hypoalbuminaemia in PD patients?**

*(the main body of this chapter has published on Nephrol Dial Transplant 2012; 27:4437-4445)*

## 6.1 Summary

### 6.1.1 Purpose

Inflammation, hypoalbuminaemia and Pcl are important predictors of survival in patients treated with PD. We hypothesised that the common link is abnormal endothelial barrier function. To test this, we explored associations between hypoalbuminaemia, systemic albumin leak and soluble markers of systemic inflammation and endothelial injury.

### 6.1.2 Methods

This was a cross-sectional study of 41 prevalent PD patients. Endothelial barrier function was measured as transcapillary escape rate of 125-I albumin ($TER_{alb}$). 17 plasma biomarkers including pro-inflammatory cytokines, endothelial biomarkers and MMPs, were measured. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) were used in this hypothesis generating exploratory study.

### 6.1.3 Results

The mean $TER_{alb}$ was 13.7±8.9 (%/h), higher than in non-uremic subjects 8.22±5.8 (%/h). Three patient clusters were defined from HCA according to their biomarker patterns. Cluster 1 was characterized by inflammation, hypoalbuminaemia, over hydration and intermediate

$TER_{alb}$. Cluster 2 was non-inflamed, preserved muscle mass and more normal $TER_{alb}$. Cluster 3 had highest $TER_{alb}$, platelet activation, preserved plasma albumin and intermediate hs-CRP levels. Two principal components (PCs) were identified from the biomarker matrix, PC1, indicating platelet activation and PC2, pro-inflammatory. $TER_{alb}$ was positively related to PC1 but not PC2. Diabetes and ischemic heart disease were associated with PC1 and PC2 respectively.

### 6.1.4 Conclusions

This exploratory analysis indicates that endothelial barrier function is decreased in PD patients and is associated with diabetic status and markers of platelet activation more than inflammation. In contrast, hypoalbuminaemia is associated more with inflammation and atherosclerotic disease indicating a more complex relationship between systemic endothelial barrier function, inflammation and hypoalbuminaemia which requires further validation.

## 6.2 Introduction

As demonstrated in Chapter 3, Pcl predicts survival of PD patients independent of PSTR and peritoneal membrane inflammation, and its relationship to comorbidity and survival is complex. The association between Pcl and survival is likely to be a combination of hypoalbuminaemia and high vascular permeability due to endothelial dysfunction associated with systemic vascular injury. Inflammation and hypoalbuminaemia are both important predictors of survival in patients treated with dialysis for advanced renal failure [53, 186, 187]. Indeed it may represent the link between increased systemic and/or peritoneal protein leak, inflammation, hypoalbuminaemia and worse survival through its association with reduced capillary barrier function. (See Figure 6-1)

To investigate this relationship between the systemic transcapillary escape rate of albumin ($TER_{alb}$), as an indicator of endothelial barrier function, a panel of biomarkers of endothelial dysfunction and associated clinical phenotypes. Hierarchical clustering (HC) and principle component analysis (PCA) were used to generate patient groups from the patterns of biomarkers observed and then subsequently compared their clinical phenotypes so as to avoid any preconceived assumptions.

Figure 6-1Simplified linear hypothesis linking comorbidity to hypoalbuminaemia invoking reduced endothelial barrier function as the central unifying mechanism

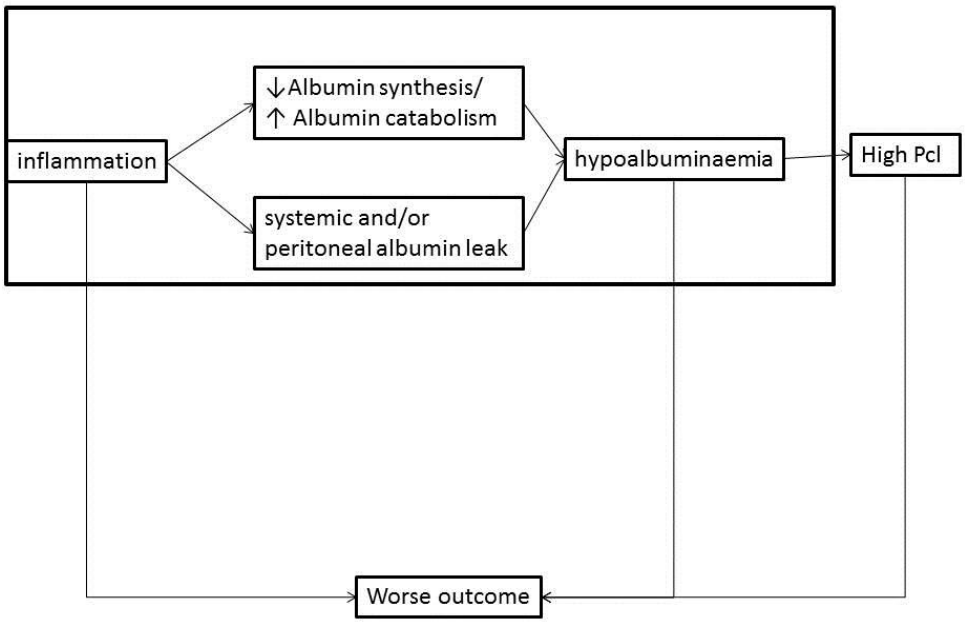
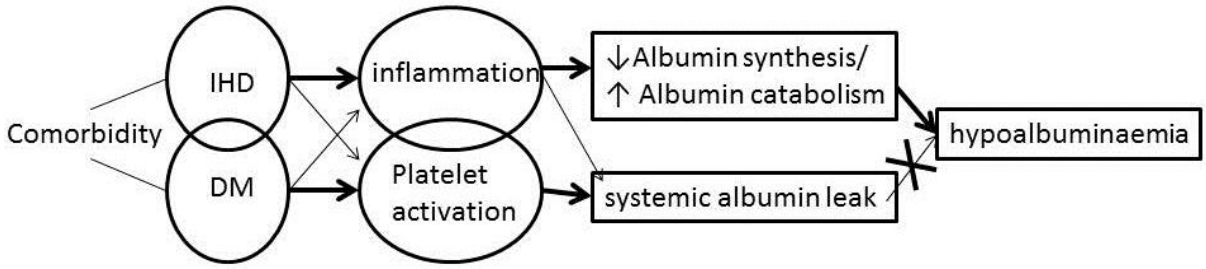



Figure 6-2 Modified relationship between comorbidity, inflammation, systemic protein leak and hypoalbuminaemia in the light of current findings.



## 6.3 Materials and Methods

### 6.3.1 Study design and patient population

This was a cross sectional study of prevalent PD patients in a single centre. Sequential patients were approached to participate and studied while undergoing their routine 6-monthly assessments of peritoneal membrane function and dialysis adequacy tests, provided they were not acutely ill or within 1 month of peritonitis. The study was peer reviewed and approved by the local ethics committee and all patients signed the consent form.

### 6.3.2 $TER_{alb}$ and plasma volume

After a bolus intravenous injection of 10ml 0.185MBq $^{125}$I-human serum albumin (HSA), blood samples were taken at 10, 20, and 30 min at a remote venous site. The isotopic concentration in each case was plotted against time on a semi-logarithmic scale and the best linear fit line was performed from these points. $TER_{alb}$, expressed as percentage loss per hour (%/h) was estimated from the disappearance rate of the $^{125}$I HSA from the gradient of this line. The plasma volume was calculated by extrapolating the line to time zero. The method was also applied for clinical purposes as the measurement of plasma volume in the normalisation of red cell mass and diagnosis of primary polycythaemia. These patients who

were mainly referred from haematologist during the time of this study served as non-uremic contemporaneous 'in house' controls.

### 6.3.3 Biomarker measurement

The plasma sample for biomarker analysis was collected just before $^{125}$I HSA injection on the study day and stored at -20°C. A panel of 17 biomarkers, which are involved in inflammation, endothelial function and tissue remodelling processes, [interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), interleukin-10 (IL-10), monocyte chemotactic protein-1 (MCP-1), inter-cellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), E-selectin, P-selectin, CD40 ligand (CD40-L), vascular endothelial growth factor (VEGF), matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-3 (MMP-3), matrix metalloproteinase-8 (MMP-8), matrix metalloproteinase-9 (MMP-9)], was measured on a Luminex suspension array system (BioPlex 200TM platform, Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) using commercially available multi-analyte cytokine kits (Milliplex MAP, Millipore (UK) Ltd, Walford, Hertfordshire, UK) or Fluorokine multi-analyte profiling kits for MMPs (R&D Systems, Abingdon, UK). Assays were carried out according to the manufacturer's instructions. A brief description of the biomarkers measured was summarised in Table 6-1.

Table 6-1 A brief summarize of the biomarkers measured in the study [15, 188]

| biomarkers | Main sources | Main biological function |
|---|---|---|
| CD 40 ligand | Activated platelets | Bound to CD40, promotes leukocyte recruitment and transmigration on endothelial cells. Enhances plaque thrombogenicity through mediating tissue factor expression. |
| Platelet factor 4 | Activated platelets | Promotes blood coagulation and leukocyte attraction |
| P-selectin | Activated platelets, endothelial cells | Mediates endothelial cell/platelet-leukocyte interaction |
| E-selectin | Endothelial cells | Promotes leukocyte rolling |
| VEGF | Endothelial cells, leukocytes, activated platelets | prime regulator of angiogenesis and contributes to vascular permeability, regulated by growth factors and cytokines |
| ICAM-1 | Endothelial cells, leukocytes | Promotes leukocyte adherence and migration |
| VCAM-1 | Endothelial cells | Promotes leukocyte adherence |
| IL-6 | Endothelial cells, macrophages | Stimulate acute phase proteins and antibody production, activate cytotoxic lymphacytes |
| TNF-α | macrophages | Promotes expression of adhesion molecules and inflammatory cell recruitment |
| IFN-γ | leukocytes | Immunoregulatory cytokine |
| IL-10 | leukocytes | Down-regulation of adhesion molecules and proinflammatory cytokines |
| IL-1β | macrophages | Promotes expression of adhesion molecules and inflammatory cell recruitment |
| MCP-1 | Endothelial cells | Recruits monocytes to sites of vascular injury or inflammation. Contributes to thrombin generation and thrombus formation. |
| MMPs | macrophages | Tissue remodeling, contribute to plaque instability |

VEGF, vascular endothelial growth factor; ICAM-1, inter-cellular adhesion molecule 1; VCAM-1, vascular cell adhesion protein 1; IL , interleukin; TNF-α, tumour necrosis factor-alpha; IFN-γ, interferon-gamma; MCP-1, monocyte chemotactic protein-1; MMP, matrix metalloproteinase

### 6.3.4 Body composition, solute clearance, membrane function and blood biochemistry

Estimated total body water (TBW) and extra-cellular water (ECW) were evaluated by bioimpedance (BIA, multi-frequency Xitron Hydra device, Model 4200, Xitron Technologies, San Diego, CA). ECW/TBW was calculated and took as a measure of hydration status.[189, 190] At the same time, absolute TBW was measured by deuterium ($D_2O$) dilution technique. The detailed methodologies have been described previously [191]. In brief, a baseline and an equilibrated blood samples were taken before and 2.5 hours after an oral dose of 99.8% $D_2O$ (Cambridge Isotope Laboratories, USA). The difference between the headspace HDO abundance of the two samples, measured by flowing afterglow mass spectrometry (FA-MS), was used to determine $TBW_D$ after accounting for equilibration with dialysate and 4% D exchange with H in body proteins. The difference between BIA estimated ($TBW_{BIA}$) and D dilution measured TBW ($TBW_D$) reflects tissue over hydration [144, 191].

The dialysis dose and residual renal function were calculated as the weekly $Kt/V_{urea}$ from the 24-hour urinary and dialysate clearance by direct measurement of urea in urine and dialysate. Peritoneal dialysate protein loss was measured from the collection of 24-hour dialysate effluent. A validated equation was used for the calculation of protein clearance, (Pcl), =24 hour dialysate protein loss/(serum albumin/0.4783).

Solute transport was measured by standard 4-hour PET test with 2.27% glucose concentration 2-L exchange. The dialysate:plasma ratio of creatinine at the completion of the 4-hour dwell period (D/P creatinine) was used to estimate PSTR.

Plasma albumin was estimated using the bromocresol purple colorimetric method, peritoneal dialysate and urine protein by the Biuret method. C-reactive protein was measured using a latex enhanced immunoturbidimetric method.

### 6.3.5 Statistical analysis

One way ANOVA and unpaired t test were used to examine differences in normally distributed continuous data among groups, chi-square for categorical data. Univariate correlations were examined by Pearson correlation coefficient. Variables not normally distributed were $\log_2$ transformed for further analysis. Significance was considered at P values <0.05. Statistical analyses were performed using IBM SPSS Statistics version 19 except for the HCA.

#### 6.3.5.1 Hierarchical cluster analysis (HCA)

The biomarker levels were first converted to $\text{Log}_2$ and expressed relative to the mean value for normalization. These measurements were used to generate heat map using Genesis software (version 1.7.2, Alexander Sturn, Institute for Genomics and Bioinformatics, Graz

University of Technology). The Genesis programme uses a two-dimensional HC method, using the average linkage clustering agglomerative rule that enables groups of variables with similar expression levels to be clustered together as well as grouping together patient samples with similar expression patterns.

#### 6.3.5.2 Principal component analysis (PCA)

To decrease the dimensionality of the biomarker data set while retaining as much of the variance as possible, an exploratory PCA was employed. Principal components (PCs) were extracted using varimax rotation, with the factor selection based on an eigen value cut-off of 1.0. Because of limitations of the sample size, 8 biomarkers only were processed in the final analysis. These biomarkers were selected from the first two PCs, which contributed the most variance of the whole matrix. Those biomarker variables not normally distributed after log transformation were converted to binary data.

## 6.4 Results

A total of 41 prevalent patients (M/F 22/19, mean age 61±17 years, median time on PD 20 (9-33) months, diabetics 29%) were studied. The $TER_{alb}$ was higher in the PD patients 13.74±8.8 (%/h) than the non-uremic comparator group 8.22±5.8 (%/h) (n=13, P<0.05).

Three clusters and one outlier were defined from the HCA (Figure 6-3). Patient characteristics, peritoneal membrane function and $TER_{alb}$ (Table 6-2) and absolute biomarker concentrations (Table 6-3) are shown by cluster. Cluster 1 was characterized by an inflammatory profile, associated with higher hs-CRP, lower albumin, intermediate $TER_{alb}$ and over hydration indicated by higher $ECW/TBW_{BIA}$ and the big discrepancy between measured TBW ($TBW_D$) and estimated ($TBW_{BIA}$) – a more sophisticated measure of over hydration. (Figure 6-4, Figure 6-5) Cluster 2 was non-inflamed according to the lowest CRP levels, had the lowest $TER_{alb}$ and the most normal body composition as evidenced by the highest body water by body weight but good agreement between D dilution and BIA measures, indicating well preserved muscle mass but least over hydration. Cluster 3 was characterised by biomarkers consistent with platelet activation, had the highest $TER_{alb}$ but only moderate CRP levels, less inflammation and less over hydration than Cluster 1 patients.

Figure 6-3 Plasma concentration profiles of 17 biomarkers in 41 patients expressed as a hierarchical clustering analysis (HCA) heat map.

Plasma concentrations of biomarker close to, higher and lower than the mean values are represented by black, red and green colours, respectively. Three patient clusters, Cluster 1 (blue bar), Cluster 2 (green bar), Cluster 3 (red bar) and one outlier are generated by HCA.

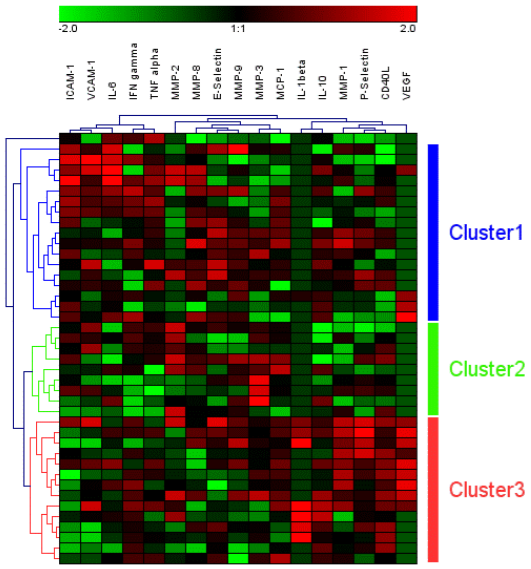


Table 6-2Characteristics of the three patient clusters derived from hierarchical clustering analysis

| | Cluster 1 (n=17) | Cluster 2 (N=9) | Cluster 3 (n=14) | Single Outlier |
|---|---|---|---|---|
| $TER_{alb}$ (%/h) | 13.88±9.23 | 9.12±5.29[a] | 17.29±9.11 | 3 |
| Age (yr) | 62.1±17.2 | 61.4±19.2 | 60.3±15.7 | 60 |
| Gender (M%) | 65 | 56 | 36 | M |
| BMI ($kg/m^2$) | 26.1±5.7 | 25.8±3.9 | 28.3±5.1 | 25.1 |
| PD duration (mo) | 22.9±20.3 | 27.6±21.0 | 26.8±21.2 | 10.5 |
| PSTR | 0.81±0.13 | 0.78±0.12 | 0.74±0.17 | 0.7 |
| Albumin (g/L) | 29.1±4.9[b] | 31.3±3.3 | 33.2±4.1 | 33 |
| CRP(mg/L) | 14.3(4.8-21)[c] | 1.5(0.6-4) | 4.6(0.8-9.5) | 16.8 |
| Peritoneal Pcl (ml/day) | 95.6±48.3 | 67.3±21.7 | 69.0±24.7 | 64.04 |
| Daily peritoneal protein loss (g/24h) | 6.3±3.2 | 4.9±1.6 | 5.0±1.8 | 4.28 |
| Urine protein loss (g/24h) | 0.58±0.5 | 0.88±0.85 | 0.74±0.51 | 1.12 |
| Total daily protein loss (g/24h) | 7.11±3.00 | 6.06±2.06 | 6.02±2.07 | 5.4 |
| Urine volume (ml) | 709±631 | 1128±626 | 1059±759 | 997 |
| Adjusted PV ($ml/m^2$) | 1439±300 | 1441±173 | 1446±197 | 2158 |
| $TBW_D$/Weight | 0.51±0.04 | 0.53±0.07[a] | 0.47±0.06 | 0.52 |
| $TBW_{BIA}$/Weight | 0.45±0.05 | 0.53±0.08[c,d] | 0.45±0.06 | 0.56 |
| $TBW_D$/Weight - $TBW_{BIA}$/Weight | 0.049±0.041 | 0.006±0.025[e] | 0.023±0.055 | -0.046 |
| $ECW/TBW_{BIA}$ | 0.50±0.04 | 0.47±0.03[f] | 0.48±0.04 | 0.44 |
| Ever smoked (%) | 44 | 22 | 23 | yes |
| IHD (%) | 41 | 22 | 29 | No |
| Diabetes (%) | 17 | 22 | 50 | No |

a, Cluster 2 vs Cluster 3, P<0.05; b, Cluster 1 vs Cluster 3, P<0.05; c, Cluster 1 vs Cluster 2, P<0.01; d, Cluster 2 vs Cluster 3, P<0.01; e, Cluster 1 vs Cluster 2, P<0.05; f, Cluster 1 vs Cluster 2, P=0.085. (one way ANOVA post hoc test (Fisher's least significant difference (LSD))

TERalb , transcapillary escape rate of albumin; Pcl, protein clearance; BMI, body mass index; Adjusted PV, plasma volume adjusted by body surface area; TBWD/Weight, Total body water (TBW) normalized to body weight as determined from deuterium (D) dilution; TBWBIA/Weight, Total body water (TBW) normalized to body weight as estimated by BIA; IHD, ischemia heart disease.

Table 6-3 biomarker concentration of the three patient clusters derived from hierarchical clustering analysis

| | cluster 1 | cluster 2 | cluster 3 | single outlier | P value [a] |
|---|---|---|---|---|---|
| ICAM-1 (ug/ml) | 0.49±0.09 | 0.4±0.07 | 0.34±0.08 | 0.41 | <0.01 |
| VCAM-1 (ug/ml) | 0.83±0.18 | 0.74±0.16 | 0.72±0.2 | 0.52 | NS |
| E-Selectin (ng/ml) | 68.21±31.07 | 44.16±18.08 | 56.23±27.51 | 26.13 | NS |
| P-Selectin (ng/ml) | 94.63±31.68 | 79.94±27.23 | 126.52±46.95 | 48.53 | <0.05 |
| MMP-1 (pg/ml) | 33.4(15-104.8) | 10.1(1-60.8) | 345(35.9-821.8) | 1 | <0.05 |
| MMP-2 (ug/ml) | 0.24(0.17-0.28) | 0.76(0.34-1) | 0.29(0.23-0.44) | 0.24 | P=0.081 |
| MMP-3 (ng/ml) | 11.5±5 | 22.76±12.76 | 13.52±4.49 | 9.04 | <0.01 |
| MMP-8 (ng/ml) | 3.69±1.66 | 2.26±0.8 | 2.04±1.15 | 0.33 | <0.01 |
| MMP-9 (ng/ml) | 67.66±38.23 | 55.54±26.02 | 53.13±30.4 | 30.7 | NS |
| IFN gamma (pg/ml) | 1.4(0-4.6) | 0.5(0-1.4) | 1.7(1.1-2.3) | 2.1 | NS |
| IL-1beta (pg/ml) | 0.1(0.1-0.1) | 0.1(0.1-0.1) | 0.4(0.1-4.1) | 0.1 | <0.01 |
| IL-6 (pg/ml) | 5.4(3-11.4) | 2.2(1-4.2) | 3.5(2.8-5.1) | 8.7 | <0.05 |
| IL-10 (pg/ml) | 2.7±2 | 1.4±1.9 | 4.5±4 | 1.4 | <0.05 |
| MCP-1 (pg/ml) | 357.8±156.9 | 377.5±175.2 | 417.1±131.2 | 61.7 | NS |
| CD40L (ng/ml) | 3.1±2.3 | 4.16±2.58 | 7.85±2.61 | 1.16 | <0.01 |
| TNF alpha (pg/ml) | 12.1±4.9 | 6.4±4 | 8.9±3 | 19.5 | <0.01 |
| VEGF (pg/ml) | 3(3-3) | 3(3-3) | 50.9(3-135.7) | 3 | <0.01 |

a, one way ANOVA for continuous normal distributed variable, independent samples Kruskal Wallis test for non-normal distributed variables

Figure 6-4 ECW/TBW (estimated by BIA) grouped by clusters determined by hierarchical clustering analysis (HCA).

ECW/TBW (estimated by BIA) is positively associated with over hydration and related to outcome. [190] Cluster 2 patients show relatively lower ECW/TBW compared with Cluster 1 and the variance between patients is less.

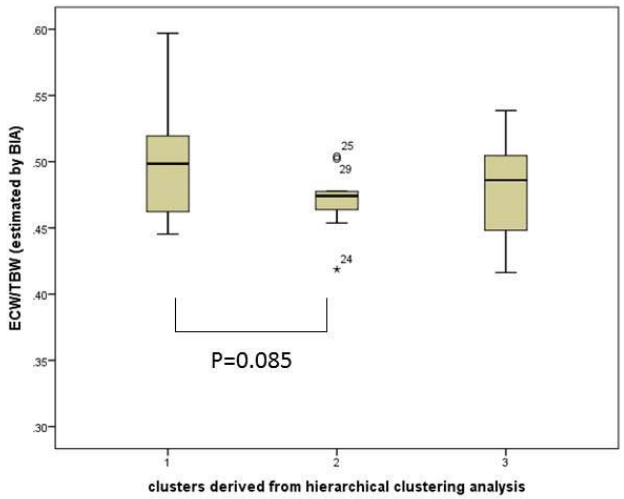
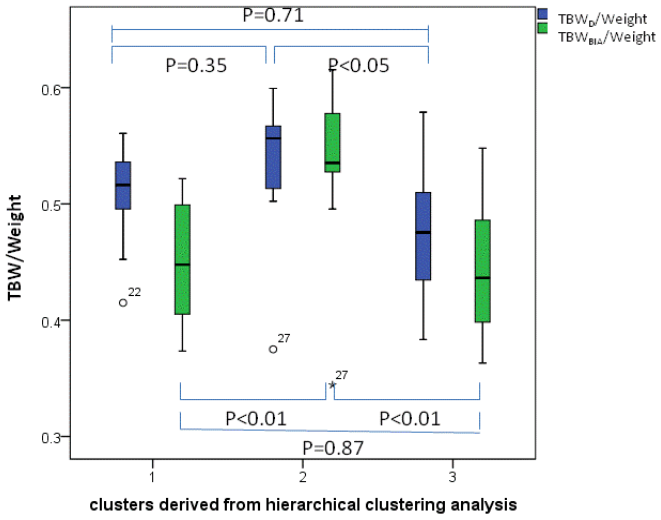


Figure 6-5 Total body water (TBW) normalized to body weight as determined from deuterium (D2O) dilution (TBWD/Weight) (blue) and estimated by BIA (TBWBIA/Weight) (green) grouped by clusters determined by hierarchical clustering analysis (HCA).

Excess TBW determined from BIA compared with D dilution is associated with over hydration [144, 191]. Cluster 2 patients show good agreement between the methods and have the highest normalised body water indicating their well preserved muscle mass. Patients in Cluster 1 and to a lesser extent Cluster 3 have higher measured body water than estimated by BIA indicating their over hydration.




The PCA was first carried out in the whole panel of the 17 biomarkers. Seven principal components (PCs) were identified, explaining 74.9% of the total variance, Figure 6-6. The eight biomarkers that composed the two strongest PCs, contributing the majority of the variance, were selected for the final PCA and are shown with their eigen values (> 1) in Figure

6-7. The first PC (PC1-platelet activation) was composed mainly of MMP-1, P-selectin, CD40L and VEGF. The second PC (PC2 pro-inflammation) featured an inverse relationship between MMP-3, and positive association with IFN-γ, TNF-α and VCAM-1. This component showed a positive relationship with CRP (r=0.31, P=0.047) and was negatively correlated with plasma albumin (r=-0.50, P=0.001).

Figure 6-6 Principal component analysis (PCA) from the whole panel of the 17 biomarkers

Eigen values and percentage contributing to the total sample variance of each of the 7 principal components (PCs) is presented. Individual composition feature is showed along with their rotated loading coefficients, a measure of the importance of each biomarker to the factor, displayed as a bar chart.

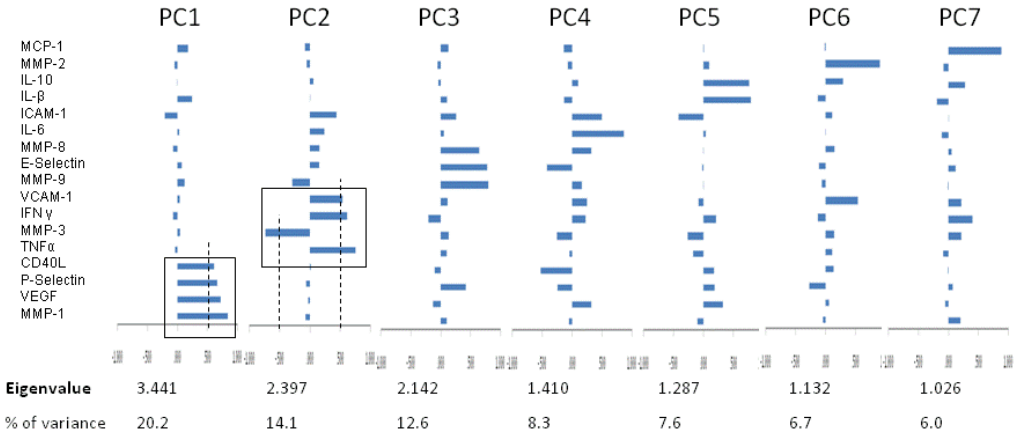
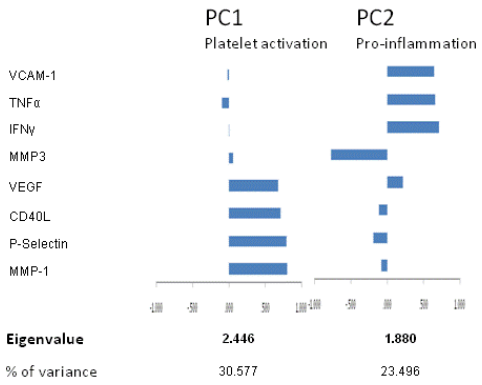


Figure 6-7 Principal component analysis (PCA) from reduced number of biomarkers

The 8 biomarkers that compose the two strongest principal components (PCs), contributing the majority of the total sample variance are selected for the final PCA. The composition of the 2 PCs derived from the final model is displayed along with their rotated loading coefficients expressed as a bar chart.




The relationship between the HCA and PCA is shown graphically in Figure 6-8. It can be seen that despite differing dimensions that these two analytical techniques give patterns that are in agreement. Patients with different comorbidities also showed differences in PCs such that patients with IHD and DM have different, albeit overlapping patterns of biomarkers (Figure 6-9). Diabetes had higher PC1, while PC2 was higher in ischemic heart disease (IHD). Peripheral vascular disease (PVD) showed no difference in PC1 and PC2, (Table 6-4).

Figure 6-8 Combined graphical representation of the hierarchical clustering analysis (HCA) (centroid plot) and principal component analysis (PCA) (dimensionless weighted scores).

There is good agreement between the methods showing association between cluster 3 and increased platelet activation, cluster 1 with inflammation and cluster 2 with the absence of markers of endothelial dysfunction.

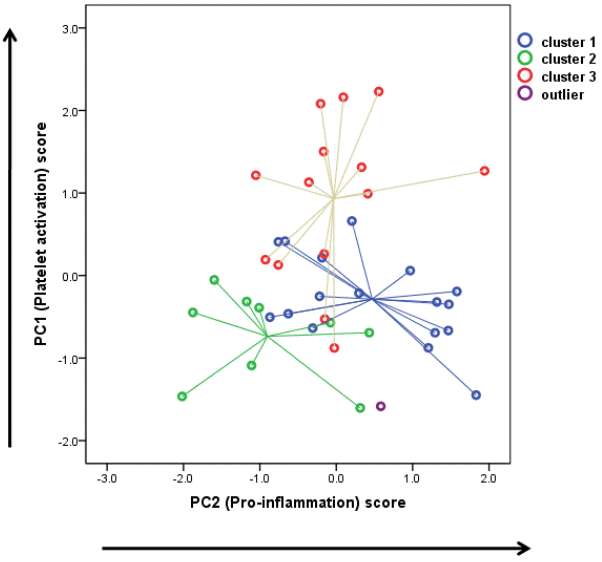


Figure 6-9 Combined graphical representation of PC scores and comorbidity; heart disease (IHD) and diabetes (DM) are clustered according to their different pattern of PC scores.

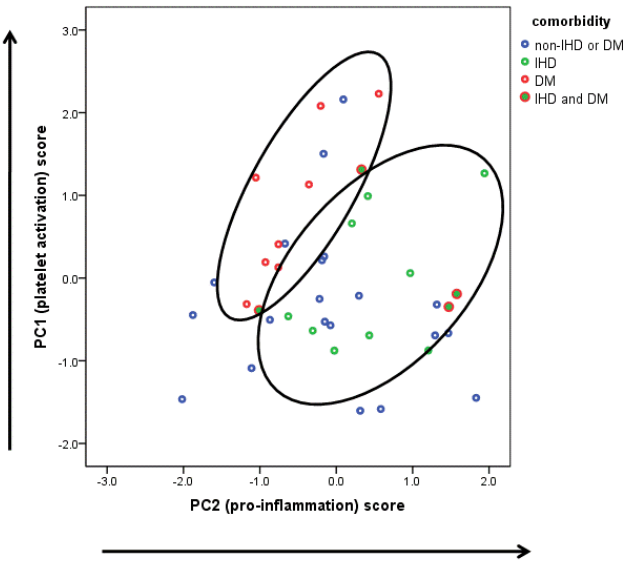


Table 6-4 Principal components (PCs) in different comorbidity status

| | IHD (n=13) | Non-IHD (n=28) | P value[a] | DM (n=12) | Non-DM (n=29) | P value[a] | PVD (n=12) | Non-PVD (n=29) | P value[a] |
|---|---|---|---|---|---|---|---|---|---|
| PC1 | -0.01±0.80 | 0.01±1.09 | 0.951 | 0.62±0.94 | -0.26±0.92 | 0.009** | -0.01±0.76 | 0.01±1.10 | 0.96 |
| PC2 | 0.51±0.89 | -0.23±0.51 | 0.025* | -0.19±0.97 | 0.08±1.02 | 0.436 | -0.06±0.85 | 0.02±1.07 | 0.814 |

PC, principal component; IHD, ischemia heart disease; DM, diabetes mellitus; PVD, Peripheral vascular disease

a, Unpaired t-test

*, P<0.05

**, P<0.01

To further explore the relationship between clinical factors and biomarker profiles with our primary endpoint, $TER_{alb}$, univariate regression analysis was undertaken. None of the clinical measures, including demographics, membrane function, PD duration, CRP or systemic dynamic factors (pulse pressure, mean blood pressure), showed significant correlation to $TER_{alb}$ whereas the biomarker derived PC1 score (platelet activation) showed a positive relationship (r=0.33 P=0.03). (Table 6-5) Multivariate analysis found this relationship to be independent of age, gender and PD duration.

Table 6-5 Univariate correlation of transcapillary albumin escape rate ($TER_{alb}$)

| | Correlation coefficient | P value[a] |
|---|---|---|
| Age (yr) | -0.21 | 0.19 |
| BMI (kg/m2) | -0.09 | 0.57 |
| PD duration (mo) | 0.26 | 0.11 |
| Pulse pressure (mmHg) | 0.10 | 0.54 |
| Albumin (g/L) | -0.09 | 0.56 |
| $Log_2$CRP(mg/L) | -0.04 | 0.81 |
| PSTR | 0.12 | 0.46 |
| UF capacity (ml) | -0.03 | 0.88 |
| Daily peritoneal protein loss (g/24h) | 0.23 | 0.16 |
| Peritoneal Pcl (ml/day) | 0.15 | 0.36 |
| urine protein loss (g/24h) | -0.16 | 0.34 |
| Total protein loss (g/24h) | 0.20 | 0.23 |
| Urine volume (ml) | -0.22 | 0.17 |
| Gender | | 0.1 |
| Ever smoking | | 0.28 |
| IHD | | 0.39 |
| Diabetes | | 0.19 |
| PVD | | 0.71 |
| Principal Component 1 | 0.33 | 0.03* |
| Principal Component 2 | 0.07 | 0.69 |

Pcl, protein clearance; IHD, ischemia heart disease; PVD, Peripheral vascular disease; BMI, body mass index; PSTR, peritoneal solute transport rate

a, Pearson or Spearman correlation for continuous variables as appropriate according to distribution and unpaired t test for between group difference

*, $P<0.05$

## 6.5 Discussion

This is the first study to measure systemic endothelial barrier function in patients with chronic kidney disease (CKD) stage 5 and relate this to a wide panel of circulating biomarkers of inflammation and endothelial dysfunction. It confirms that endothelial barrier function is abnormally decreased and suggests that this is not solely related to inflammation but more so to platelet activation in this clinically stable cross-sectional cohort. Furthermore, patients with IHD and DM have different, albeit overlapping patterns of biomarkers, although both exhibit endothelial dysfunction.

The $TER_{alb}$ is a widely accepted measurement of endothelial barrier function, first described as early as 1973 [192, 193]. $TER_{alb}$ is increased in several conditions associated with endothelial dysfunction such as diabetes, hypertension and atherosclerosis [29-32] and also sepsis where it is clearly associated with marked inflammation. However endothelial barrier function has been less well investigated in end stage renal disease. This study found that mean $TER_{alb}$ is higher in CKD stage 5 patients than in non-uremic controls that likely had other pathologies as they were undergoing investigation for polycythemia. A previous study of 29 non-diabetic CKD patients (GFR (11-44)ml/min*1.73$m^2$) whose other markers of endothelial function (e.g. von Willebrand factor) were normal unless subjects were current smokers [194] showed no difference between CKD patients and normal controls. This suggests that severe renal failure is associated with more severe endothelial dysfunction and is in broad agreement with other

rather small previously published studies in dialysis patients. $TER_{alb}$ was increased in 11 diabetics treated with PD (but not in non-diabetics) [195] and 9 pre-dialysis haemodialysis patients [196]. This is supported by in vivo experiments in which human uremic plasma increased frog mesenteric micro- vascular permeability to both water and protein, [197] subsequently confirmed in other studies. [22] One of the strengths of our study, which demonstrated a relatively high median $TER_{alb}$ was that we recruited sequential patients undergoing routine assessment of their therapy with <5% of patients declining investigation, so reducing selection bias.

Although a relationship between $TER_{alb}$ and blood pressure was seen in some early studies in hypertension patients [192, 198, 199] we did not observe this to be the case in this cross-sectional study. The relationship may be more complex in this dialysis cohort with multiple comorbidities and concomitant medication. A study comparing $TER_{alb}$ according to the angiotensin-converting enzyme (ACE) gene polymorphism in essential hypertension showed that ACE D/D homozygosity, which is associated with high risk of atherosclerotic vascular disease is related to higher $TER_{alb}$ despite identical 24 hour blood pressure readings [33]. Furthermore, endothelial function can be influenced by medications used in the patients with related effect on $TER_{alb}$. High-dose simvastatin reduces low-density lipoprotein cholesterol by 39%, and is reported to normalize $TER_{alb}$ [34]. No clear relationship between medications and $TER_{alb}$ were observed in this study.

The measurement of a wide range of biomarkers combined with hierarchical cluster and PCA allowed us to investigate the overall pattern of their association with endothelial barrier dysfunction. It is important to note that these analyses are exploratory in nature but allow the generation of hypotheses for further testing. Using this approach we were able to identify three main patient clusters and there was good agreement between these and the two main components identified through PCA; additional PCs were identified that warrant further investigation in a larger validation cohort but were excluded from further analysis here to avoid type 1 statistical error. The advantage to this approach is that while biomarkers are likely to correlate to each other as they all reflect endothelial function it enables identification of possible multiple biomarker functions or different metabolic or signalling pathways involved in endothelial dysfunction. For example, CD40L is expressed in lymph cells, epithelial cells, fibroblasts, endothelial cells and platelets in response to pro-inflammatory cytokines, platelet activators and nitric oxide signalling. After combining with CD40, it subsequently up-regulates the pro-inflammatory and pro-atherogenic genes [17] Although results from our PCA should be taken as exploratory, we find that PC1 (HC3 phenotype) is composed primarily of MMP-1, P-selectin, CD40L and VEGF, all of which are released in significant amounts from platelets, and reflect platelet activation [200]. PC2, composed by IFN-γ, TNF-α and VCAM-1 and negatively contributed by MMP-3, is mainly involved in pro-inflammatory pathways and defined the inflamed, overhydrated and hypoalbuminaemia HC1 phenotype. HC2 was a relatively healthy phenotype with most normal body composition and

least abnormal $TER_{alb}$. PC1 and PC2 could explain a significant proportion (34.3%) of the variance in the whole biomarker matrix.

One important finding of this study is demonstration that the link between platelet activation and increased endothelial permeability may not be just through inflammation. The links between inflammation as a trigger for platelet activation in vascular lesions and between inflammation, atherosclerosis and endothelial dysfunction in uraemia are well established [201]. These pathways may however act either independently or sequentially; activated platelets can induce secretion of chemokines for monocyte recruitment [128] whereas single microvessel perfusion with TNF-$\alpha$ alone, without platelet activation, does not alter the endothelial permeability[129]. In an aseptic animal injury model, systemic depletion of neutrophils with antibody failed to prevent the increase in vascular permeability, whereas antiplatelet pre-treatment reduced this by 25% [130]. These findings suggest that platelet activation may have a critical role in endothelial hyper permeability that may not necessarily be through inflammation, especially in the low grade inflammatory status seen in uremia.

This study found that DM and IHD tend to cluster with different patterns of biomarkers associated with endothelial dysfunction and in keeping with other studies, we found that diabetics more commonly display an increase in platelet reactivity. [202] Worse cardiovascular outcomes in diabetics may reflect inadequate responses to anti-platelet therapy [203]. Another important finding of this study is that the systemic leak of albumin from the circulation to

extravascular space is not correlated to hypoalbuminaemia and thus unlikely to be causally related. It is well established that the single strongest predictor of plasma albumin concentration in PD patients is the daily peritoneal protein loss [66, 204], in turn strongly correlated to the rate of PSTR, an indicator of the effective vascular area in contact with dialysate. There is, however, residual variability in peritoneal protein losses that has now been shown in several studies to be an independent predictor of patient survival[61, 72]. This link between peritoneal Pcl and survival can at least in part be explained by an association with increasing age and cardiovascular comorbidity, raising the possibility that some of the variability may reflect endothelial dysfunction. We did not see any clear relationship between systemic protein leak and peritoneal protein losses in this study probably because the latter is dominated by effective peritoneal vascular area in contact with dialysate. It is also possible, given that this was a study of prevalent patients, that some had acquired membrane changes that could influence protein losses. The reason that plasma albumin is more strongly associated with peritoneal protein losses than with systemic leak is that the former represents net daily losses (typically 5-10 g/day) whereas the latter reflects the re-circulation between intra and extra-vascular pools of up to 10 times this amount daily. Net plasma albumin concentration will also be determined by the balance of synthesis and catabolism, and the former in relatively fit PD patients has been shown to be increased above normal.[66] The patients with the lowest albumin in this study were those with inflammation compared

to markers of platelet activation, in keeping with previous observations that hypoalbuminaemia in inflamed patients is associated with reduced synthesis.

This study has a number of limitations. First, the number of patients would ideally be larger to enable a more extensive correlation of the PCA with clinical phenotype. Despite this, it remains the largest study of $TER_{alb}$ in dialysis patients to date and the numbers were limited by the ethical permission which was primarily obtained to measure plasma volume in an adequately powered study of fluid status and hypoalbuminaemia [191]. Secondly, the biomarker patterns identified, while good evidence that the relationship in more complex that originally envisaged can only be considered as hypothesis generating at this stage. Further validation in a larger patient cohort of selected biomarkers is planned. Thirdly, we did not have ethics permission to study normal subjects, so limiting our comparison to a non-uremic control group under clinical investigation, which might be expected to have worse endothelial function than normal subjects [205]. As acknowledged above, the cross-sectional design means that there may be confounding of the data by factors known and unknown that change with time on treatment.

## 6.6 Conclusion

In conclusion, this study elaborates a more complex relationship between abnormal endothelial dysfunction and clinical phenotype in patients on PD, emphasising the importance of platelet activation as well as inflammation (Figure 6-1, Figure 6-2). In addition, it further clarifies our understanding of the mechanisms of hypoalbuminaemia, an important predictor of survival in patients on PD. It remains unclear how this endothelial dysfunction in relation to hydration status. This would be discussed in Chapter 7.

# chapter 7.**What is the relationship between soluble endothelial biomarker patterns, hydration status and hypoalbuminaemia in PD patients?**

## 7.1 Summary

### 7.1.1 Purpose

In the previous pilot study, we demonstrated that the systemic endothelial barrier function was decreased in PD patients, especially diabetics. But it was not associated with hypoalbuminaemia or overhydration. To independently validate this and further investigate the determinate of fluid redistribution in PD patients, we explored the associations between hypoalbuminaemia, endothelial biomarkers and hydration status in a multi-centre cohort.

### 7.1.2 Method

This was a multi-centre cross sectional study of 149 prevalent PD patients. Hydration status was measured as ECW/TBW by a single frequency (50Hz) BIA device. A panel of 14 plasma endothelial biomarkers were measured. The selection of the biomarkers in the current study was mainly based on the pilot study (chapter 6) with further emphasis on platelet activation, which was found to relate to systemic endothelial permeability. Principal component analysis (PCA) was again used to identify the patterns in endothelial dysfunction.

### 7.1.3 Results

Five principal components (PCs) were identified from the biomarker matrix. PC1, mainly composed of PF 4, P-selectin, MMP-1 and CD40L, indicating platelet activation, was related to diabetes. PC2, featured a positive association with VEGF, IFN-γ, TNF-α and IL-6, prone to pro-inflammatory, tended to be related to ischaemic heart disease (IHD). The combination of the PCs and the association to comorbidities were consistent with the pilot study. ECW/TBW was higher in patients who were female, with severe comorbidities and increase with age. It was negatively related to albumin and positively to PC 4 (adhesion molecular) in univariate analysis, while gender, comorbidity and albumin remained significant in multivariate model.

### 7.1.4 Conclusion

Platelet activation and inflammation are the two important processes contribute to endothelial dysfunction in PD patients. Endothelial dysfunction in different comorbidity is not universal but with different predominant mechanism, so that specific strategy may be applied. Hydration status is related plasma albumin and it may through its colloid effect.

## 7.2 Introduction

In the previous pilot study, we demonstrated that the systemic endothelial barrier function was decreased in PD patients, especially diabetics. But it was not associated with hypoalbuminaemia or overhydration.[206] It indicates that reduced endothelial barrier function resulted in a higher albumin turnover rate between intravascular and extravascular space but does not directly cause low albumin levels in the blood stream.

On the other hand, fluid redistribution plays an important role in overhydration in PD patients. [191] In patients with hypoalbuminaemia, there was tissue overhydration even without an increase in plasma volume. The attempt to normalize the hydration status in hypoalbuminamic patients may lead to hypovolemia and in the risk of loss of residual renal function. It is not clear whether this fluid redistribution is a direct result of hypoalbuminaemia or the change of endothelial permeability.

Bioimpedance (BIA) is simple to perform and gives highly reproducible measurements of two components of body composition: TBW and ECW. Intervention studies have demonstrated its ability to identify changes in fluid status in response to changes in therapy[189]. ECW:TBW ratio is a powerful predictor of patient survival[190] and accepted as a measure of hydration status.

The purpose of this study was to independently validate the previous finding in the pilot study and further investigate the determinate of fluid redistribution. We explore the

associations between hypoalbuminaemia, endothelial biomarkers and hydration status in a multi-centre cohort.

## 7.3 Methods

### 7.3.1 Study design and patient population

This was a multi-centre cross sectional study of prevalent PD patients. The patient population was part of the BIA study (Developing Bioimpedance (BIA) as a tool for fluid management in Peritoneal Dialysis Patients: A validation Study ClinicalTrials.gov NO: NCT00801112.) See appendix for the detailed BIA study design. There were three UK centres in the BIA study, the University Hospital of North Staffordshire, St. James's University Hospital and Sheffield Kidney Institute, Northern General Hospital. The patients were more than 16 years old and had been stably established on PD for more than 3 months Patients with acute inflammation, e.g. peritonitis within one month were excluded. Clinically determined optimal fluid status was achieved on the sampling day. If not, extra effort would be taken to achieve optimal fluid status before taking the sample and BIA measurement. The patient was excluded from the study if clinical optimal fluid status could not be achieved. Clinical optimal fluid status was defined as the minimal body weight that patient can achieve without adverse effect, eg, hypotension.

The clinical characteristics, including membrane function, PD regime, biochemistry profiles and comorbidity were collected locally. The plasma samples for CRP and biomarker measurement were mostly collected on the same day of BIA measurement and stored in the local centre before sent to the central lab (Stoke). The local storage temperature was -20°C. The storage duration was 652.4±105.3, 404.3±120 and 294.4±69.1 ($P<0.01$) days, for Stoke, Sheffield and Leeds, respectively. Apart from the clinical recorded albumin, it was also measured centrally in the stored samples.

### 7.3.2 Clinical characteristics

Comorbidity was recorded at the time of enrollment by local consultant. Stoke comorbidity score were used in the study. In brief, 7 comorbid comorbidity domains were considered, including non-cutaneous malignancy, ischemic heart disease (IHD), peripheral vascular disease (PVD), left ventricular dysfunction (LVD), diabetes mellitus (DM), systemic collagen vascular disease, and any other condition thought to reduce life expectancy. The comorbidity score was the number of these domains affected. The comorbidity grade was then derived from the comorbidity score. Grade 0 (low risk) was a zero score, grade 1 (medium risk) was a score of 1-2, and grade 2 (high risk) a cumulative score of ≥ 3.

Membrane function was evaluated by standard peritoneal equilibration test (2.27% PET) in all three centres. The clinical routine biochemistry profiles were measured locally as part of clinical practice. The sampling date of the biochemistry profile including albumin may or may not be on the same day as BIA measurement or samples for biomarker. The interval in between was less than one month.

Plasma albumin was measured by the Bromocreosol Purple colorimetric method in Stoke during the study period. It had been using Bromocreosol Green colorimetric method in Sheffield before switch to Bromocreosol Purple colorimetric method at Feb 2011. Bromocreosol Green colorimetric method was used in Leeds over the study period. An average difference of 5.5g/L between the results of the two methods had been established by Clase et al and the converting equation derived from their work was used to adjust the data to Bromocreosol Purple colorimetric method.[149]

### 7.3.3 BIA measurement

BIA parameters were measured by a single frequency (50Hz) BIA device (Akern BIA 101 Anniversary) in the study. The total body resistance and reactance were measured according to the operation manual for further calculation of total body water (TBW) and extra-cellular water (ECW). (See appendix for calculation detail)

### 7.3.4 Biomarker measurement

A panel of 14 endothelial biomarkers, which involved in inflammation, platelet activation and tissue remodelling processes, were measured in the study. It was mainly based on the pilot study with further emphasis on platelet activation, which was found to relate to systemic endothelial permeability in the pilot study.Luminex suspension array system (Bio-Rad) was used to measure interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), CD40 ligand (CD40-L) and vascular endothelial growth factor (VEGF) (Human cytokine/chemokine MAP multiplex kit (Millipore corporation, USA)); inter-cellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), E-selectin and P-selectin (Human adhesion molecule Fluorokine MAP multiplex kit (R&D Systems, Minneapolis, USA)); matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-3 (MMP-3) (Human MMP Fluorokine MAP multiplex kit (R&D Systems, Minneapolis, USA). ELISA was applied to measure matrix metalloproteinase-8 (MMP-8), matrix metalloproteinase-9 (MMP-9) and platelet factor 4 (Duoset Human ELISA kits (R&D Systems, Minneapolis, USA). Assays were carried out according to the manufacturer's instructions. Albumin and CRP were also centrally measured in Stoke.

### 7.3.5 Statistical analysis

Continuous data were expressed as mean values ± SD or median (inter quartile range), as appropriate. One way ANOVA was used to examine differences in normal distributed continuous data, and chi-square for categorical data. For those not normal distributed, Kruskal Wallis H test was applied to test the difference between groups. Unpaired t test were applied to compare difference between two independent samples. The univariate correlation was examined by Pearson correlation coefficient or Spearman correlation coefficient as appropriate according to distribution. Multi-level analysis was applied for the multivariate analysis and the constant in the model was allowed to be different between centre to account for centre effect.

To decrease the dimensionality of the biomarker data set and retain as much of the variance as possible, an exploratory PCA was employed. Principal components (PCs) were extracted using varimax rotation, with the factor selection based on an Eigen value cut-off of 1.0.

Significance was considered at P values <0.05. Multi-variant analysis was done by MLwin. Other statistical analyses were performed using IBM SPSS Statistics version 20.

## 7.4 Results

### 7.4.1 Demography, patient characteristics and hydration status

A total of 149 prevalent patients (f/m 61/88, mean age 58.59±14.76 years, median time on PD 23 (8-36) months, diabetics 37/149) in three centres were studied. (Table 7-1) There were significant centre differences in residual renal function and hydration status. The PSTR, PET UF and the use of bio-compatible solutions were also different. The distribution of comorbidity did not reach statistical significant difference among centres.

Table 7-1 Demography, patient characteristics and hydration status by centre

| | Stoke (n=57) | Sheffield (n=40) | Leeds (n=52) | p value[a] | Total (n=149) |
|---|---|---|---|---|---|
| gender (f/m) | 21/36 | 20/20 | 19/33 | .345 | 60/89 |
| IHD (N/Y) | 46/11 | 34/6 | 45/7 | .685 | 125/24 |
| PVD (N/Y) | 48/9 | 36/4 | 45/7 | .705 | 129/20 |
| DM (N/Y) | 47/10 | 28/12 | 37/15 | .255 | 112/37 |
| comorbidity grade (low/medium/high) | 23/29/5 | 20/16/4 | 15/35/2 | .112 | 58/80/11 |
| age (year) | 58.21±15.81 | 59.4±13.77 | 58.38±14.56 | .920 | 58.59±14.76 |
| urine volume (ml) | 1194.5(739-1829) | 1398(786-2077.5) | 635.5(207.5-1063) | <0.001 | 940(531-1683) |
| PD duration (mo) | 23(5-44) | 22(9.5-32) | 24.5(9-40) | .527 | 23(8-36) |
| DPCr | 0.66±0.12 | 0.76±0.13 | 0.7±0.09 | .001 | 0.69±0.12 |
| PET UF (ml) | 322.7±207 | 53.9±207.5 | 144.9±260.2 | <0.001 | 205.1±250.7 |
| use of biocompitible solution | 3/57 | 23/40 | 4/52 | <0.001 | 30/149 |
| albumin (g/L) Cotemporary local adjusted method | 32.5±4.6 | 33.1±3.7 | 34.5±3.4 | .043 | 33.4±4.1 |
| albumin (g/L) Stored central measurement | 26.7±4 | 32.7±4.4 | 30.5±3.5 | <0.001 | 29.6±4.6 |
| Hgb (g/dl) | 11.7±1.4 | 11.8±0.9 | 11.4±1.6 | .354 | 11.6±1.4 |
| Height (cm) | 169.26±9.72 | 168.83±9.87 | 166.33±10.44 | .275 | 168.12±10.04 |
| weight (kg) | 79.95±17.13 | 77.95±16.65 | 72.53±17 | .069 | 76.81±17.16 |
| TBW (L) | 41.83±7.73 | 43.33±9.32 | 39.21±7.88 | .055 | 41.29±8.33 |
| ECW (L) | 19.14±3.77 | 20.14±4.21 | 16.34±4 | <0.001 | 18.41±4.25 |
| ECW/TBW | 0.46±0.06 | 0.47±0.05 | 0.42±0.08 | .001 | 0.45±0.07 |
| TBW/weight | 0.53±0.07 | 0.56±0.08 | 0.55±0.07 | .216 | 0.54±0.07 |
| $H^2/R$ ($cm^2/\Omega$) | 58.18±12.02 | 62.38±15.14 | 54.51±12.49 | .019 | 58±13.34 |
| $H^2/Xc$ ($cm^2/\Omega$) | 567.32±152.82 | 613.38±170.95 | 462.05±152.81 | <0.001 | 542.47±168.56 |

IHD, ischemia heart disease; PVD, peripheral vascular disease

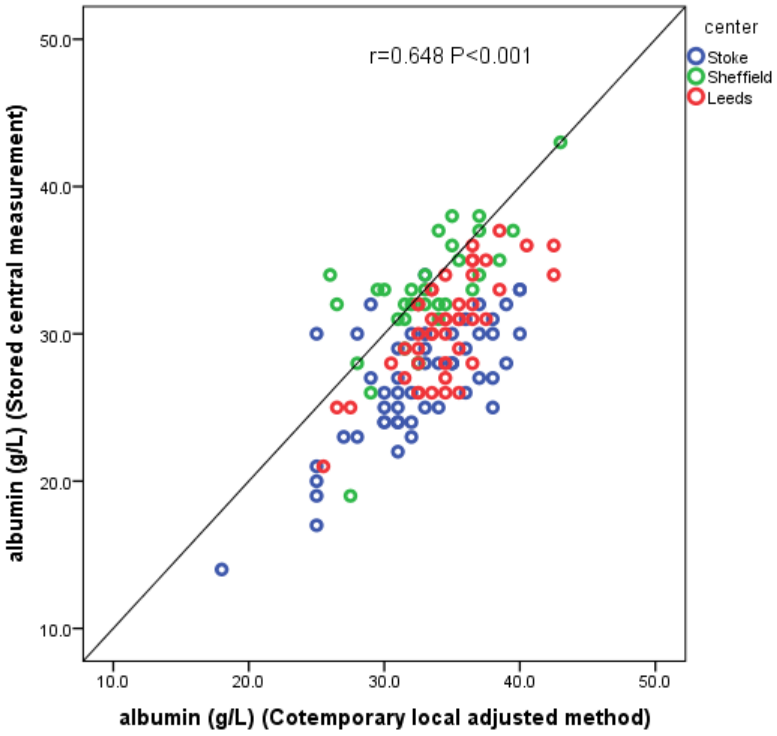
TBW, Total body water; ECW, extracellular water

a. One way ANOVA for normal distributed continuous data and Kruskal Wallis H test for those not normal distributed. Chi-square for categorical data

## 7.4.2 Albumin measurement

As stated in the methods section there were two sets of albumin measurements. One was the clinical locally measured albumin, adjusted according to the different methodologies. The other was the stored central measurement. Both were significantly different among centres. (Table 7-1) The blood taken for central measurement was the same for the biomarker assays, including CRP, obtained on the same day as BIA measurement and within one month of the local albumin measurement. The correlation between the two albumin measurements was 0.648 ($p<0.001$) (Figure 7-1)

Figure 7-1 The correlation between the two sets of albumin measurement



### 7.4.3 Endothelial biomarkers

There were 12 patients who did not have biomarker profiles measured due to the sample availability or poor quality. One patient was confirmed to have acute inflammation at sampling so was excluded from the PC analysis.

The correlation between CRP and endothelial biomarkers are shown in Table 7-2. Five principal components (PCs) were identified, explaining 68.3% of the total variance. (Figure 7-2) The first PC (PC1-platelet activation) was composed mainly of PF 4, P-selectin, MMP-1 and CD40L. The second PC (PC2 pro-inflammation) featured a positive association with VEGF, IFN-γ, TNF-α and IL-6. The third PC (PC3-MMPs) was mainly the combination of MMP 8 and MMP 9. PC 4 (–adhesion) was contributed mostly by the two adhesion molecular VACM-1 and ICAM-1. PC 5 was negatively related to MMP-3, and positively to E-selectin. The combination of the PCs was rather similar to the pilot study. (Figure 6-6, Figure 6-7)

Table 7-2 CRP and biomarkers matrix

| | | CRP | IFN-γ | IL-6 | CD40L | TNFα | VEGF | ICAM-1 | VCAM | E-Selectin | P-Selectin | MMP-1 | MMP-3 | MMP-8 | MMP-9 | PF-4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CRP | r [a] | 1.000 | -.040 | **.195** | **.171** | .160 | -.068 | **.169** | .107 | .045 | .135 | .034 | .020 | **.315** | .108 | .061 |
| | P value | | .641 | **.021** | **.044** | .059 | .423 | **.042** | .200 | .587 | .105 | .688 | .811 | **<0.01** | .193 | .463 |
| IFN-γ | r | | 1.000 | **.468** | **.463** | **.341** | **.618** | .064 | -.057 | .023 | **.458** | **.339** | .050 | .025 | -.061 | **.572** |
| | P value | | | **<0.01** | **<0.01** | **<0.01** | **<0.01** | .458 | .510 | .786 | **<0.01** | **<0.01** | .558 | .773 | .477 | **<0.01** |
| IL-6 | r | | | 1.000 | **.364** | **.395** | **.418** | .114 | .108 | .158 | **.413** | **.268** | .035 | .112 | .083 | **.331** |
| | P value | | | | **<0.01** | **<0.01** | **<0.01** | .184 | .207 | .064 | **<0.01** | **.001** | .681 | .189 | .333 | **<0.01** |
| CD40L | r | | | | 1.000 | **.316** | **.313** | .088 | -.126 | .083 | **.669** | **.488** | .089 | **.295** | .065 | **.784** |
| | P value | | | | | **<0.01** | **<0.01** | .305 | .141 | .335 | **<0.01** | **<0.01** | .298 | **<0.01** | .448 | **<0.01** |
| TNFα | r | | | | | 1.000 | **.488** | .117 | **.256** | .105 | .163 | -.116 | -.150 | .076 | .136 | .151 |
| | P value | | | | | | **<0.01** | .171 | **.002** | .221 | .056 | .174 | .079 | .375 | .110 | .077 |
| VEGF | r | | | | | | 1.000 | .068 | .089 | .026 | **.266** | .155 | -.135 | .021 | .073 | **.348** |
| | P value | | | | | | | .427 | .301 | .762 | **.002** | .070 | .116 | .803 | .391 | **.000** |
| ICAM-1 | r | | | | | | | 1.000 | **.356** | **.257** | **.357** | .117 | .088 | .044 | **.246** | .064 |
| | P value | | | | | | | | **<0.01** | **.002** | **.000** | .161 | .292 | .601 | **.003** | .444 |
| VCAM | r | | | | | | | | 1.000 | -.075 | .019 | .027 | .084 | .089 | .070 | -.129 |
| | P value | | | | | | | | | .367 | .818 | .747 | .315 | .285 | .403 | .120 |
| E-Selectin | r | | | | | | | | | 1.000 | **.310** | .009 | **-.170** | .117 | **.239** | .080 |
| | P value | | | | | | | | | | **<0.01** | .913 | **.041** | .159 | **.004** | .338 |
| P-Select | r | | | | | | | | | | 1.000 | **.572** | **.196** | **.213** | .033 | **.771** |
| | P value | | | | | | | | | | | **<0.01** | **.018** | **.010** | .694 | **<0.01** |

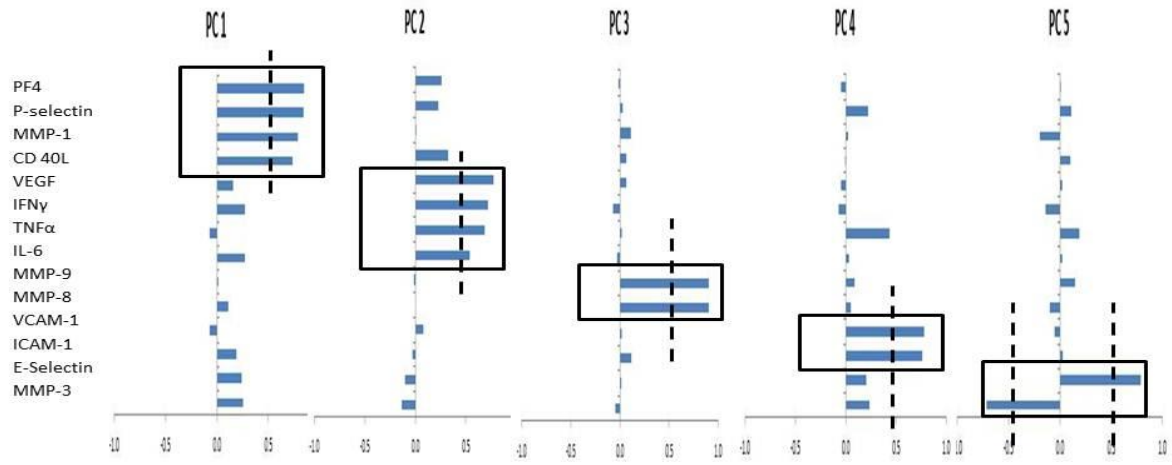
| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| in | | | | | | | | | | | | | | | | |
| MMP-1 | r | | | | | | | | | | | 1.000 | **.218** | .084 | -.060 | **.623** |
| | P value | | | | | | | | | | | | **.008** | .313 | .476 | **<0.01** |
| MMP-3 | r | | | | | | | | | | | | 1.000 | .107 | -.072 | .116 |
| | P value | | | | | | | | | | | | | .200 | .391 | .164 |
| MMP-8 | r | | | | | | | | | | | | | 1.000 | **.446** | .132 |
| | P value | | | | | | | | | | | | | | **<0.01** | .113 |
| MMP-9 | r | | | | | | | | | | | | | | 1.000 | -.083 |
| | P value | | | | | | | | | | | | | | | .321 |
| PF4 | r | | | | | | | | | | | | | | | 1.000 |
| | P value | | | | | | | | | | | | | | | |

a, Spearman correlation,

IL-6,interleukin-6, TNF-α, tumour necrosis factor-alpha, IFN-γ, interferon-gamma, CD40-L, CD40 ligand and VEGF, vascular endothelial growth factor; ICAM-1, inter-cellular adhesion molecule 1, VCAM-1, vascular cell adhesion protein 1, E-selectin and P-selectin; MMP, matrix metalloproteinase; PF4, platelet factor 4

Figure 7-2 Principal component matrix.

Eigen values and percentage contributing to the total sample variance of each of the 5 principal components (PCs) is presented. Individual composition feature is showed along with their rotated loading coefficients, a measure of the importance of each biomarker to the factor, displayed as a bar chart.




| | | | | | |
|---|---|---|---|---|---|
| Eigenvalue | 3.730 | 1.801 | 1.548 | 1.301 | 1.185 |
| % of variance | 26.644 | 12.863 | 11.059 | 9.293 | 8.464 |

The correlation between albumin and PCs tended to be stronger for the stored central measurement. There was a positive relationship between PC 1 and albumin (stored central measurement) and generally negatively related to all the other PCs. There was no significant correlation between albumin (contemporary local adjusted method) and PCs, apart from PC4. (Table 7-3)

Table 7-3 correlation between albumin, CRP and principal components (PCs)

| | albumin (Stored central measurement) | P value [a] | albumin (Cotemporary Local adjusted method) | P value [a] |
|---|---|---|---|---|
| albumin (stored sample) | | | .648** | **<0.001** |
| PC1 | .229** | 0.007 | .135 | .138 |
| PC2 | -.160 | 0.063 | -.036 | .692 |
| PC3 | -.161 | 0.062 | -.094 | .302 |
| PC4 | -.297** | **<0.001** | -.299** | .001 |
| PC5 | -.231** | 0.007 | .019 | .837 |
| CRP | -.034 | .685 | -.060 | .491 |

PC, principal component

a, Pearson correlation coefficient or Spearman correlation coefficient as appropriate according to distribution

*, P<0.05

**, P<0.01

### 7.4.4 Biomarker pattern in different comorbidity

Patients with different comorbidity (DM vs IHD) showed different, albeit overlapping biomarker patterns. Diabetes had higher PC1 (platelet activation), while PC2 (inflammation) tended to be higher in ischemic heart disease (IHD). (Table 7-4) It reproduced the biomarker pattern in different comorbidity found in the pilot study. (Figure 7-3)

Table 7-4 biomarker pattern in different comorbidities

| | None (N=90) | DM (N=33) | IHD, without DM (N=13) | P value[a] |
|---|---|---|---|---|
| PC 1 | -0.43(-0.89-0.45) | 0.32(-0.52-1.1) | -0.42(-0.68-0.48) | .029 |
| PC 2 | -0.29(-0.74-0.34) | -0.34(-0.78-0.39) | 0.12(-0.18-0.94) | .202 |
| PC 3 | -0.24(-0.47-0.21) | -0.13(-0.5-0.13) | -0.36(-0.57-0.47) | .928 |
| PC 4 | -0.01±1.05 | 0.03±0.95 | 0±0.85 | .975 |
| PC 5 | -0.03±0.93 | 0.21±1.05 | -0.33±1.3 | .230 |

PC, principal component

IHD, ischemia heart disease; DM, diabetes mellitus

None - without DM or IHD

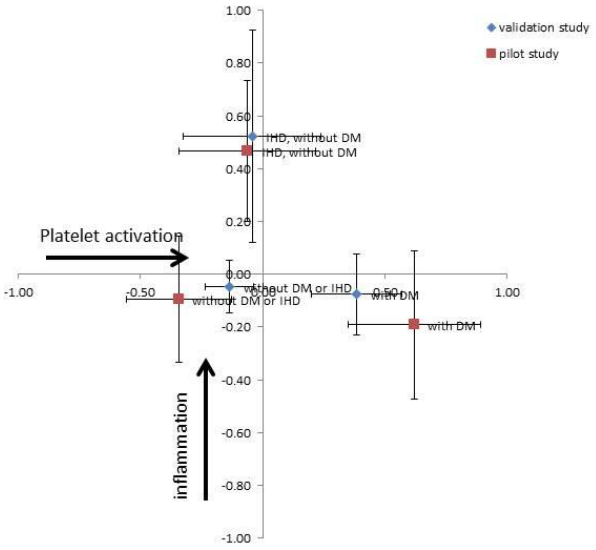
DM – with DM, may or may not have IHD

IHD without DM – IHD and without DM

a, One way ANOVA for normal distributed continuous data and Kruskal Wallis H test for those not normal distributed

Figure 7-3 Combined graphical representation of PC scores and comorbidity; heart disease (IHD) and diabetes (DM) are clustered according to their different pattern of PC scores

The data points represent the mean values (±SE) for the patient with (a) diabetes (DM), (b) ischemic heart disease (IHD) but without DM and (c) without DM or IHD. Diabetes has higher PC1 (platelet activation), while PC2 (inflammation) tends to be higher in ischemic heart disease (IHD). It reproduces the patterns found in the pilot study.




## 7.4.5 Correlations to hydration status (ECW/TBW)

ECW/TBW, as a measure of hydration status, was positively correlated to age and comorbidity grade. Female had a higher ECW/TBW as measured by BIA. Albumin was negatively correlated to ECW/TBW. (Figure 7-4, Figure 7-5) Among the PCs, PC 4 was

significantly correlated to ECW/TBW in univariate analysis. (Table 7-5) Gender, comorbidity status and albumin level remained significant (either contemporary local adjusted method or stored central measurement) in multivariate model. (Table 7-6)

Figure 7-4 The relationship between albumin (stored central measurement) and hydration status, as measured by extracellular water (ECW)/total body water (TBW)

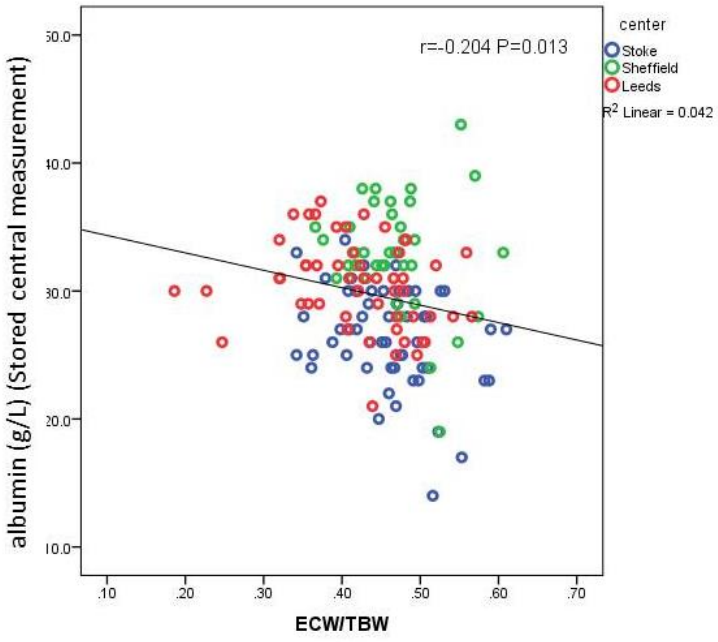


Figure 7-5 The relationship between albumin (cotemporary local adjusted method) and hydration status, as measured by extracellular water (ECW)/total body water (TBW)

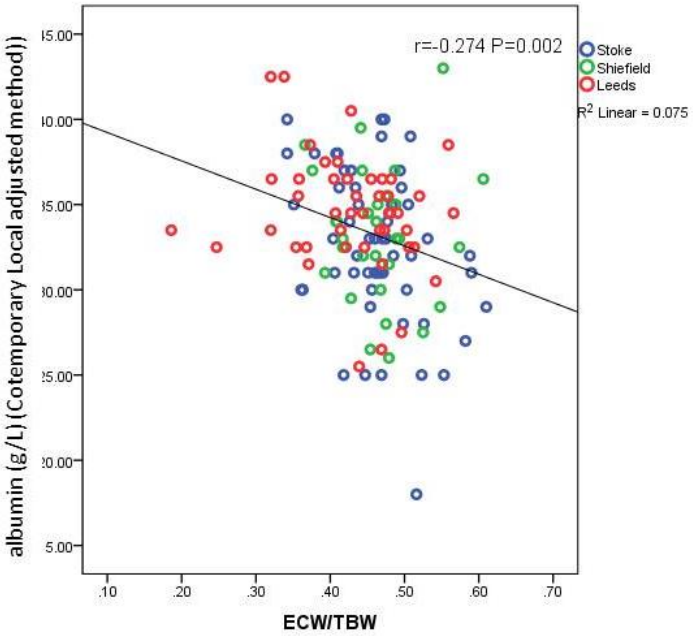


Table 7-5 Univariate correlation between hydration status (ECW/TBW) and clinical characteristics and principal components (PCs)

| | ECW/TBW | P value [a] |
|---|---|---|
| gender (f/m) | 0.47±0.07/0.43±0.07 | .003 |
| comorbidity (low/medium/high) | 0.43±0.06/0.45±0.07/0.50±0.09 | .003 |
| age (year) | .231** | .005 |
| PSTR | .130 | .176 |
| Albumin (g/L) (Cotemporary Local adjusted method) | -.274** | .002 |
| Albumin (g/L) (Stored central measurement) | -.204* | .013 |
| PD duration (mo) | 0.032 | .699 |
| urine volume (ml) | -0.1 | .254 |
| Daily fluid removal (ml) | -.126 | .148 |
| PC1 | -.058 | .505 |
| PC2 | .092 | .289 |
| PC3 | .077 | .377 |
| PC4 | .214* | .013 |
| PC5 | -.088 | .313 |
| CRP (mg/l) | .072 | .392 |

PC, principal component; PSTR, peritoneal solute transport rate

a, Pearson correlation coefficient or Spearman correlation coefficient as appropriate according to distribution, t test or ANOVA to compare groups

Table 7-6 multivariate model for hydration status (ECW/TBW)

| | albumin (Cotemporary Local adjusted method) | | | albumin (Stored central measurement) | | |
|---|---|---|---|---|---|---|
| ECW/TBW | β | standard error | P value | β | standard error | P value |
| constant | 0.45646 | 0.0095 | <0.01 | 0.46071 | 0.01119 | <0.01 |
| gender (if male) | -0.02795 | 0.01011 | <0.05 | -0.03761 | 0.01017 | <0.01 |
| comorbidity medium (compared with low) | 0.0174 | 0.01086 | NS | 0.01616 | 0.01087 | NS |
| comorbidity high (compared with low) | 0.08869 | 0.01893 | <0.01 | 0.08002 | 0.01996 | <0.01 |
| age (year) | 0.00044 | 0.00035 | NS | 0.00059 | 0.00035 | NS |
| albumin (for each 1g/L increase) | -0.00374 | 0.00124 | <0.05 | -0.00241 | 0.00126 | 0.055 |
| lgCRP (mg/l) | 0.01102 | 0.01654 | NS | 0.00036 | 0.01511 | NS |
| PC1 | -0.00554 | 0.00509 | NS | -0.00383 | 0.00558 | NS |
| PC2 | 0.00411 | 0.0048 | NS | 0.00068 | 0.00487 | NS |
| PC3 | 0.00593 | 0.00476 | NS | 0.00664 | 0.00495 | NS |
| PC4 | 0.00449 | 0.00535 | NS | 0.00742 | 0.00534 | NS |
| PC5 | -0.00066 | 0.0052 | NS | -0.00632 | 0.00545 | NS |

PC, principal component; PSTR, peritoneal solute transport rate; ECW, extracellar water; TBW, total body water

## 7.5 Discussion

This study independently validated the previous finding in the pilot study that patients with IHD and DM had different, albeit overlapping patterns of soluble endothelial biomarkers, although both exhibit endothelial dysfunction. The reproducible combination of PCs indicated that inflammation and platelet activation were two independent although highly related processes in endothelial dysfunction. Hydration status (as measured by ECW/TBW) was negatively related to albumin, while its relationship to soluble endothelial biomarkers lost significance when controlled for albumin concentration. A plausible explanation for this is that the relationship between albumin and hydration status is through its colloid effect rather than as a surrogate of endothelial dysfunction.

### 7.5.1 Reproducibility of PC combinations and biomarker patterns according to different comorbidities

A large panel of endothelial biomarkers was measured in the study. The selection of the biomarkers was based on the pilot study with further emphasis on the platelet activation, the process identified from the pilot study to be related to endothelial permeability. Principle component analysis was applied again in this study to identify possible different pathway involved in endothelial dysfunction. The PCs identified from the current study had good agreement with the previous pilot study. [206] PC1- composed primarily of biomarkers highly

related to platelet activation. These were platelet factor 4, P-selectin, MMP-1 and CD40L, all of which were released in significant amounts from platelets and/or reflect platelet activation. [188] PC2, composed of VEGF, IFN-γ, TNF-α and IL-6, was mainly involved in the pro-inflammatory pathway. The main difference in the combination of PCs between the pilot study and the current study is VEGF, which was combined in PC 1, and now in PC 2. It may reflect the fact that VEGF, a central regulator of angiogenesis and well-known circulating permeability factor [207], is involved in both pathways. This reproducible combination of PCs reflected that inflammation and platelet activation are two important mechanisms in endothelial dysfunction in PD patients.

PC1-platelet activation factor, was higher in DM. PC2-inflammation factor, tended to be higher in IHD. It again reproduced the previous finding in the pilot study. [206] It indicated that the mechanism of endothelial dysfunction underlying DM and IHD may be different. It is important to notice this as different treatment strategy may need to emphasize.

### 7.5.2 Endothelial dysfunction, albumin and hydration status

Plasma albumin is known to relate to hydration status in PD patients. [208, 209] A relationship is also found between hydration status and inflammation and other endothelial dysfunction evidence.[209] In the current multi-variant analysis, albumin and comorbidity were the two independent variables related to hydration status as measured by ECW/TBW, while the

endothelial biomarker PCs lost significance in multi-variant analysis. Hydration status is not just the balance between fluid input and output but also a result of fluid re-distribution between different components in PD patients.[191] As showed in the pilot study, endothelial permeability, measured by TERalb, was not related to hydration status.[206] It is, therefore more likely that albumin determines hydration status through its colloid effects rather than as a surrogate of inflammation or endothelial injury.

### 7.5.3 The centre effect

A significant centre effect was noticed in the study. Leeds showed significantly lower residual renal function and lower ECW/TBW. As this is a cross sectional analysis, we were not in a position to investigate the causation. The plausible thought raised here would be that less good residual renal function was not necessarily related to worse hydration status in a selective cohort, while the possibility that patients has less residual function as a consequence of being less overhydrated needs to be considered. Consideration needs to be taken about the balance between residual renal function and hydration status. Taking the observation from the current study that overhydration was partially a result of hypoalbuminaemia, attempt to correct the hydration status in hypoalbuminaemic patient by increasing UF may not be effective and potentially put at risk the loss of residual renal function.

For the centre differences in membrane function (PSTR and PET UF capacity), apart from the real physiological difference, variance for other reasons need to be considered. More patients were on bio-compatible solution in Sheffield, which is known to relate to less UF. Potentially, different method of measuring UF, plasma and dialysate creatinine may also contribute to the centre effect, e.g. overfill.

### 7.5.4 Limitations

This study has a number of limitations. Firstly, two different sets of albumin were measured in the study. The albumin) measured locally was done by different methods and the samples were not taken exactly on the same day of BIA measurement and the samples for biomarker measurement. On the other hand, the samples for albumin measured centrally were stored for significant period of time. The stability of albumin beyond 4 months is not clear.[210] It may explain the absence of the classic inverse relationship between albumin and inflammation. Possibly due to the close relationship between albumin levels on two different days, within one month in apart, we were still able to pick the relationship between albumin and hydration status.

Secondly, no direct measurement of osmotic pressure was taken due to clinical feasibility. Plasma albumin was measured as a surrogate. Thirdly, detailed fluid balance (fluid in and out) was not recorded. However, extra effort had been made to achieve clinical optimal fluid

status. The possibility of worse fluid status due to inappropriate dialysis regime or excess fluid intake was limited as much as we can. The majority of the variability of fluid status should come from the patient related factors. Fourthly, hydration status was measured as ECW/TBW by BIA. It is not able to differentiate worse fluid status and increased muscle mass by BIA measurement although the latter was much less likely in PD patients. Finally, the different biomarker pattern between DM and IHD observed in the current study was based on PD patients. It is not clear whether it can be generalized to non PD populations. Further studies in common populations need to be done.

## 7.6 Conclusion

In conclusion, platelet activation and inflammation are the two important processes contribute to endothelial dysfunction in PD patients. Endothelial dysfunction in different comorbidity is not universal but with different predominant mechanism. Specific strategy may be applied accordingly. Plasma albumin and hydration status are related and may through the colloid effect of albumin.

# chapter 8.**General discussion**

## 8.1 The journey of the project so far

The observation that Pcl is related to patient survival in PD has been repeated in several cohorts. This key observation was the primary stimulus for the work presented in this thesis: the main purpose was to investigate the reasons why Pcl is related to survival. The centrally stated hypothesis was that peritoneal Pcl is linked to survival because it reflects a systemic abnormality of the capillary circulation, specifically at the level of the endothelium, rather than being evidence of local membrane injury. The latter is complex because membrane injury is believed to change with time on dialysis. A series of connected studies, using different methods and patients cohorts were undertaken to address this hypothesis from different perspectives.

**Chapter 3: Is peritoneal protein clearance (Pcl) related to comorbidity, local peritoneal membrane inflammation or both at the commencement of PD? Which of these are predictors of patient survival?**

It was demonstrated that Pcl is determined by both local and systemic factors. Locally, both membrane area, determined form the PSTR and local membrane inflammation as evidenced by intra-peritoneal IL-6 production, both have independent effects on Pcl. Given that capillary protein leak is one of the cardinal features of inflammation, this would suggest that for a given anatomical membrane area (small pores) there is a greater representation of

large pores. Systemically, comorbidity, which is not associated with local peritoneal inflammation, also affects Pcl and this is partly explained by the known association between hypoalbuminaemia and comorbidity, with plasma albumin acting as a 'reverse' acute phase protein. However, this association is different by type of comorbidity (eg. The effect of diabetic status on Pcl relies much on hypoalbuminaemia while to less extent for IHD.)

The observation that Pcl predicts survival was again repeated in the current project providing further external validation of this association. It has been further clarified that this prediction is not due to the local factors, namely, PSTR or membrane inflammation. The predictive value of Pcl on survival is related to its relationship to hypoalbuminaemia and comorbidity. It is again complex and different by type of comorbidity.

Neither the cross sectional associations nor the prediction of survival in the follow-up study of Pcl found in the incident cohort was found to exist in prevalent patients. This raises the possibility that the nature of Pcl in prevalent patients is different from incident patients.

**Chapter 4: Does peritoneal Pcl change with time on PD? If so, is this change over time coupled with the known increase in PSTR?**

From the longitudinal analysis of the Stoke cohort, it was shown that peritoneal Pcl was initially proportional to membrane area (PSTR) but that it decreased with time on PD treatment. In fact there is an uncoupling of the small pore (PSTR) and large pore (Pcl)

pathways with time on PD. This would be in keeping with a switch from local inflammation early on to progressive fibrosis in which transcapillary protein leak is impaired combined with increased vascular surface area.

This is in keeping with other studies which have demonstrated the uncoupling between PET UF capacity and PSTR.[95] This has also been successfully modeled by an extension of the three pore membrane to include a fiber matrix layer that mimics membrane fibrosis. [103] The current work gives the clinical evidence of synchronous similar effect of fibrosis on large pore flow and it would be of great interest to see if this is also predicted by this adapted 3-pore/fibre matrix model.

In severe fibrosis, there is "uncoupling" of PSTR from the peritoneal UF coefficient (LpS) or more precisely, the peritoneal osmotic conductance to glucose ($LpS\sigma_g$). The question arises as to whether this aspect of membrane function (LpSσ) can be demonstrated with a non-glucose containing dialysis fluid, specifically icodextrin which achieves ultrafiltration through oncotic rather than osmotic pressure, but for which the liquid permeability component (Lp) of fluid conductance is similarly affected by fibrosis. This was the reason for exploring the determinants of UF using icodextrin and investigating whether this is also "uncoupled" from PSTR over time on PD, further validating the three pore membrane/fiber matrix hypothesis.

**Chapter 5: Does the ultrafiltration obtained with icodextrin – which only occurs via the**

**small pore pathway, change with time on PD? If so, is this change over time dissociated from the increase in PSTR?**

Again, using data collected prospectively for the longitudinal observations of patients in the Stoke cohort, it was demonstrated that prolonged time on PD decreases UF and it is uncoupled from the effect of increase in PSTR, which would otherwise be expected to improve the efficiency of this glucose polymer. As already argued, this would be in keeping with a progressive fibrosis combined with increased vascular surface area.

The analysis while also confirming the expected impact of certain membrane characteristics on UF such as high PSTR and better UF capacity, also demonstrated that factors which are likely to affect the oncotic pressure gradient, hydrostatic pressure gradient or icodextrin metabolites concentration are more important than the dwell length in explaining UF variability.

These observations fit with the theoretical modeling and have clear implications for dialysis prescription.

**Chapter 6: What is the relationship between soluble biomarkers of inflammation and endothelial damage, systemic transcapillary albumin leak and hypoalbuminaemia in PD patients?**

As demonstrated in Chapter 3, Pcl predicts survival of PD patients independent of PSTR and peritoneal membrane inflammation and its relationship to comorbidity and survival is complex. The association between Pcl and survival is likely to be a combination of hypoalbuminaemia (to which it is mathematically coupled) and possibly high vascular permeability due to impaired endothelial barrier function associated with systemic vascular injury. To clarify the association between systemic vascular permeability, hypoalbuminaemia and comorbidity, this pilot biomarker study was conducted.

It was demonstrated that endothelial permeability is increased in PD patients and it is different in different type of comorbidity and different patterns of biomarkers. DM is more related to increased endothelial permeability compared with IHD. DM tended to be associated with biomarkers known to represent platelet activation whereas cardiovascular comorbidity, especially ischemic heart disease associated with inflammation. As this was an exploratory analysis, using pattern recognition methodology such as principle component analysis, it required further validation.

Recent mechanistic studies have shown that hypoalbuminaemia and comorbidity are strongly related to overhydration in PD patients [191] and that this is because of increased extravascular tissue hydration presumed to be a consequence of reduced plasma oncotic pressure. This pilot study also suggested that the increased endothelial permeability is not

associated with hypoalbuminaemia or over hydration. This raises the hypothesis that endothelial hyper-permeability is not the main cause of fluid redistribution in PD patients.

**Chapter 7: What is the relationship between soluble endothelial biomarker patterns, hydration status and hypoalbuminaemia in PD patients?**

The validation of the pilot study (Chapter 6) was undertaken in a different patient cohort – a multicentre centre clinical trial designed to investigate the value of bioimpedance in the management of fluid status in PD patients. One of the reasons for selecting these patients on entry to the trial was the possibility of relating these biomarker and comorbidity patterns to hydration status.

This study found that endothelial biomarker patterns associated with the different types of comorbidity were reproducible in this independent cohort. However the hydration status was related plasma albumin, age and comorbidity rather than any independent association with endothelial dysfunction suggesting that the association is mediated through its colloid effect.

## 8.2 Implications and Further thoughts

### 8.2.1 Should we measure Pcl in clinical setting?

Combined with PSTR, Pcl measurements could help to distinguish an anatomically large membrane from an inflamed membrane and/or systemic comorbidity, at least in incident patients. However, there is argument that the local inflamed membrane by itself may not necessary predict worse survival. (according to current project and unpublished data from the GLOBAL fluid study) By measuring Pcl is not able to separate the effect of local inflammation and systemic comorbidity. Longitudinal monitoring of Pcl may give information about membrane fibrosis. The drawback of this is that it would be difficult to interpret on individual level, as it is confounded by the change of comorbidity, plasma albumin level and membrane inflammation. It therefore seems that this is unlikely to become a routine method of assessing injury which might be achieved better by measuring specific biomarkers.

### 8.2.2 Do we need a “Peritoneal Equilibration Test (PET)” for icodextrin?

Currently, all membrane function tests are based on glucose solution. As showed in the present project, the UF achieved by icodextrin is related to the UF from a standard 4 hours 2.27% dwell, but a lot more variance is beyond the UF capacity from a standard PET test. A

"PET" for icodextrin will give a better prediction of UF for icodextrin dwells, adding extra information about the peritoneal membrane.

For the purpose of comparison, the changeable variables that may affect icodextrin UF as determined in the current project should be controlled, namely, the position of the patient, dwell length, input volume. The test should not be conducted until the circulation level of icodextrin metabolites is stable. Combine these, an overnight, 8 hours dwell with 2L input volume would likely to be the most practical "standard icodextrin dwell".

### 8.2.3 What determines the plasma albumin concentration in PD patient? What strategies would increase plasma albumin level?

Albumin level is an important predictor of survival not just in PD population but also in a more general population. Factors that may contribute to albumin concentration include synthesis rate, catabolic rate, exogenous loss (predominantly to urine and peritoneal cavity in the case of PD), redistribution to the interstitial space and plasma volume.

Albumin synthesis is controlled in part by nutrition. However, malnutrition alone does not usually cause hypoalbuminaemia, unless in extreme case. Patients with anorexia nervosa have essentially normal albumin level. Actually, inflammation plays a much more important

role in determining albumin levels, especially those with multiple chronic conditions, both by decreasing synthesis and increasing catabolic rate.

Exogenous loss, predominantly to urine and the peritoneal cavity in PD, is the most important determinant of hypoalbuminaemia in PD patients. However, it has been shown in healthy PD patient, even in the 'nephrotic' range protein loss through peritoneal cavity, plasma albumin level would be maintained by increase synthesis and reduced catabolic rate. [211] We now know from the current project, that the relationship between peritoneal protein loss and hypoalbuminaemia is more complex due to its link through comorbidity.

From the present project, increased TERalb, which may indicate a high turnover rate with recirculation back to the plasma compartment via lymphatics, does not change plasma albumin level significantly. This is different from what is seen in acute sepsis, where albumin redistributes to extra-vascular space in short time period.

However, plasma volume expansion in normal subject should not change plasma albumin level as seen when we move to high altitude. [212] However, there is paradoxical result in dialysis population. Plasma volume expansion was shown to be related to low albumin concentration in a HD cohort ,[213] but not the case in another PD cohort. [191] Taking account of the current finding that plasma albumin level relates to hydration status of PD patient and it is independent to endothelial dysfunction, the cause and result relationship between plasma volume and albumin level needs to be reconsidered.

Taken together, the best ways to maintain plasma albumin level is either to suppress the factors that may reduce synthesis and increase catabolism, eg. Inflammation, or decrease exogenous protein loss, both from urine and peritoneal cavity. The former can be achieved with ACE inhibition and/or ARBs, whereas the latter would require an adaptation of the PD process in which dialysate is re-circulated after removal of fluid and toxic metabolites. Portable absorbent cartridges could be developed to do this. Of course, infection would be the main concern of this therapy. In principle, more 'biocompatible' dialysis solutions that attract less membrane inflammation may also help to reduce albumin loss from peritoneal cavity. However, so far, there is no such dialysis fluid showing convincing evidence of causing less membrane inflammation. [154]

### 8.2.4 What is the difference between IHD and DM?

Another interesting finding from this thesis is the differences observed between DM and IHD. Firstly, IHD and DM have different impact on Pcl. Compared with IHD, Pcl in DM is more a function of hypoalbuminaemia, while DM patients are systemically leakier (higher TERalb). One possible explanation of these findings may due to the fact incident DM patient on PD have more protein loss from urine. Excessive urine protein loss is a clear source of hypoalbuminaemia. This hypoalbuminaemia would then relate to a higher Pcl than it would be expected from systemic vascular damage. Secondly, DM and IHD have different endothelial biomarker pattern, with DM more associated with endothelial barrier injury, IHD

more inflammatory. This is in keeping with the different response of therapy in DM patients compared with non-DM. This points to different therapeutic strategies needing to be developed according to the predominant pathology process that may be helpful in the future. All these findings are based on PD cohorts in the current study, the difference between DM and IHD need to be studied in common populations.

# chapter 9.**Appendix**

## 9.1 Measuring ultrafiltration (UF) -more complicated than I thought

### 9.1.1 How do we measure UF in clinical practice and in research?

To measure UF in PD is clearly extremely important for both clinical and research purpose. It is thought to be easy to measure. The detailed methodology of measuring UF is not even always mentioned in the literature. The 'routine' way is weighting the drainage bag and subtracting the labelled input volume in CAPD. In some carefully designed clinical trials, both drainage and input bags are weighted and UF are calculated as the difference between the two bags. The overfill issue has been raised for some years. [159, 178] Some studies start to account for overfill when measuring UF. For APD, the machine gives the UF reading based on volume measurement.

### 9.1.2 The controversy findings around UF

The UF difference between APD and CAPD is difficult to understand. It is widely accepted that CAPD is as good as APD in terms of preserving residual renal function, if not better. On the other hand, a favoured 24 hour UF in CAPD has been noticed in several studies [175-177].

Neglecting overfill used to be one of the reasons for over estimating UF in CAPD. However, there are studies like us which have clearly accounted for overfill still find difference in UF between APD and CAPD. A follow up study in incident patients [177] showed UF were consistently lower in incident APD patients than in their counterparts on CAPD. Controversially, the residual renal function declined faster in the APD group rather than the CAPD group in that study.

### 9.1.3 The impact of specific gravity

The fact that dialysate weight slightly heavier than water was believed to be neglectable by almost the whole PD society. We evaluated the impact of specific gravity in measuring UF volume in this study.

The specific gravity in dialysate drainage was measured from sequential patients came for their routine dialysis clearance test. 9 drainage dialysate samples from different patients were collected. Three were from icodextrin long dwells, three were from 1.36% glucose 4 hour dwells and the other three were from 2.27% glucose 4 hour dwells. Weight and volume were measured and specific gravity was calculated as weight/volume. Fresh dialysate and water were served as controls. The measurement was repeated for three times in each sample. (Table 9-1)

Table 9-1 specific gravity of different type of dialysate

| | | Specific gravity (g/ml) mean±SD | P value[a] (compared with water) |
|---|---|---|---|
| water | | 1.001±0.002 | |
| icodextrin | fresh | 1.037±0.002 | <0.01 |
| | Long dwell drainage | 1.026±0.006 | <0.01 |
| Glucose (1.36%) | fresh | 1.017±0.002 | <0.01 |
| | 4 hour drainage | 1.021±0.001 | <0.01 |
| Glucose (2.27%) | fresh | 1.017±0.001 | <0.01 |
| | 4 hour drainage | 1.011±0.003 | 0.016 |

a, one sample t test

Taking the average specific gravity from our measurement in limited number of samples, the potential over estimation of UF in CAPD in a single icodextrin dwell (2L) with 0.4L UF can be calculated as following.

Reported UF (L, misleading by kg)

= [2L (input volume) + 0.4L (UF)] * 1.026 (g/ml) - 2L (input volume)

= 0.461(L, misleading by kg) (over estimate for 0.061L)

The specific gravity should be different between patients and dwells and potentially depend on the UF and transport status. Our calculation should only be taken as a rough estimation. However, it is worth to keep in mind that for a patient with typical CAPD regime (eg. 1.36% glucose 4L+2.27% glucose 2L+Icodextrin 2L), the specific gravity issue may account for about 100ml overestimation of UF.

### 9.1.4 Conclusions

The specific gravity difference may lead to measureable overestimation of UF in CAPD. It is important to keep this in mind in both clinical and research setting.

## 9.2 What do we learn from the methodology upgrade of plasma creatinine measurement in a peritoneal dialysis centre?

### 9.2.1 Background

The difference of plasma creatinine measurement by different methodology has been noticed for some years in early stage chronic kidney disease (CKD), due to its critical impact on estimating glomerular filtration rate (GFR). The National Kidney Disease Education Program (NKDEP) of National Institutes of Health recommends that an estimated GFR should be calculated from plasma creatinine and reported to physicians to assist early identification of patients with renal disease. The NKDEP Laboratory Working Group has done a great amount of work to establish standardization and improve accuracy of plasma creatinine measurements in clinical laboratories worldwide. [214]

However, the variability of creatinine measurement in end stage renal disease (ESRD) has been regarded as neglectable for long time. It is somehow reasonable in the context of measuring estimated GFR. The estimated GFR is 7ml/min/1.73m$^2$ for a 60 year old non African American male when plasma creatinine is 700umol/L and 6 ml/min/1.73m$^2$ if the plasma creatinine is reported as 770umol/L according to the MDRD equation. [215] However, a variety of plasma creatinine from 100umol/L to 110umol/L is related to a 7ml/min/1.73m$^2$ difference in estimated GFR in a subject with same gender, race and age.

Presumably because of this, the available Standard Reference Materials (SRMs) of creatinine, which are supposed to be used to evaluate the accuracy of clinical methods and to validate working or secondary reference materials, are no more than 467.4umol/L. [216] In another word, the accuracy and commutability of the creatinine measurement in higher level can be unreliable.

The local lab upgraded the creatinine measurement method during the project. This study was to investigate whether this change in creatinine measurement had any effect on dialysate/plasma (D/P) creatinine, the most important parameter in peritoneal dialysis (PD) patients for both clinical and research purpose.

## 9.2.2 Methods

### 9.2.2.1 The methodology change of plasma creatinine measurement in the clinical laboratory

The clinical laboratory upgraded the methodology for plasma creatinine from the O'leary modified kinetic Jaffé method to creatininase based enzymatic method on 29th Oct 2008. Advia analyser (Siemens Healthcare Diagnostics Inc, Newbury, UK) was used both before and after. There was no change in dialysate creatinine measurement - Beckman Synchron reagents (Kinetic Jaffé method) on DxC800 analyser (Beckman Coulter UK Ltd, High Wycombe, UK).

#### 9.2.2.2 Patient population

It was a single centre retrospective observational study. The baseline demography, residual renal function, peritoneal solute clearance, and membrane function measured by peritoneal equilibration test (PET), were routinely recorded within the first one or two month of treatment, and then usually at 6 monthly intervals in all patients as part of the clinical routine in our centre. The patients who had creatinine measured both 12 months before and 12 months after 29$^{th}$ Oct 2008 were included in the analysis. For the patients had more than one creatinine measurement during the 12 months, the one closest to the switch date was analysed.

29-10-08 was the real switch time point. 29-10-07 and 29-10-09 were selected artificially to serve as controls. Paired creatinine results around 29$^{th}$ Oct 2007 and 29$^{th}$ Oct 2009 were selected in the same way as for 29-10-08. The three sub-cohorts were analysis in the study.

The clinical policy did not change and no new dialysate was introduced into the centre during the study period of time.

#### 9.2.2.3 Statistical analysis

Paired t-test was performed to compare the difference before and after the switch day. Delta of each pair of the data was calculated as a function of difference before and after the switch. The difference between different switch points, one real switch point and two artificial

switch points, was compared by one-way analysis of variance (ANOVA). Statistical analysis was performed using SPSS version 18 (Chicago, IL, USA).

### 9.2.3 Results

No significant difference in age, gender, PD duration and time interval between the two measurement dates was found in the three sub-cohorts. (Table 9-2)

Table 9-2 demography of the three sub-cohorts around the different switch time point

| | 29-10-07 (n=66) | 29-10-08 (n=60) | 29-10-09 (n=55) | P value[a] |
|---|---|---|---|---|
| Age (years)[b] | 56±17 | 59±16 | 61±16 | NS |
| Gender (M/F) | 28/38 | 27/33 | 21/34 | NS |
| PD duration (mo) [b] | 27±23 | 29±23 | 30±28 | NS |
| Interval between two measures (days) | 198±32 | 198±41 | 200±55 | NS |

29-10-08 was the real switch time point. 29-10-07 and 29-10-09 were the two time points artificially selected to serve as controls.

a, ANOVA or chi square P value

b, PD duration and age were until the earlier measurement of creatinine within the comparison

The increases of plasma creatinine before and after the two artificial switch time points were not significant (29-10-2007, from 657.5±248.2umol/L to 682.5±275.8umol/L, P=0.135; 29-10-2009, from 688.0±289.4umol/L to 707.0±273.0umol/L, P=0.458). There was a significant

increase after the real switch time point, 29-10-2008, from 610.7±260.2umol/L to 697.0±285.5umol/L (P<0.001).

D/P creatinine was stable before and after the two artificially selected cut points (29-10-2007, before 0.79±0.15, after 0.81±0.14, P=0.282; 29-10-2009, before 0.66±0.12, after 0.67±0.13, P=0.734). If anything, there was a small increase of D/P creatinine in these two sub-cohorts. The average D/P creatinine levels significantly decreased from 0.78±0.13 to 0.72±0.12 after the lab switch the methodology (P<0.001). The difference of the delta D/P creatinine before and after the switch time points across the three sub-cohorts was shown in Figure 9-1.

No statistics correlation was found between the delta of D/P creatinine and absolute D/P creatinine level. (Figure 9-2) A 0.06±0.02 mean decrease after the methodology switch was constant through the observed D/P creatinine range.

Figure 9-1 delta D/P creatinine before and after the switch time point, 29-10-2008, and the two artificially selected switch time points, 29-10-2007 and 29-10-2009

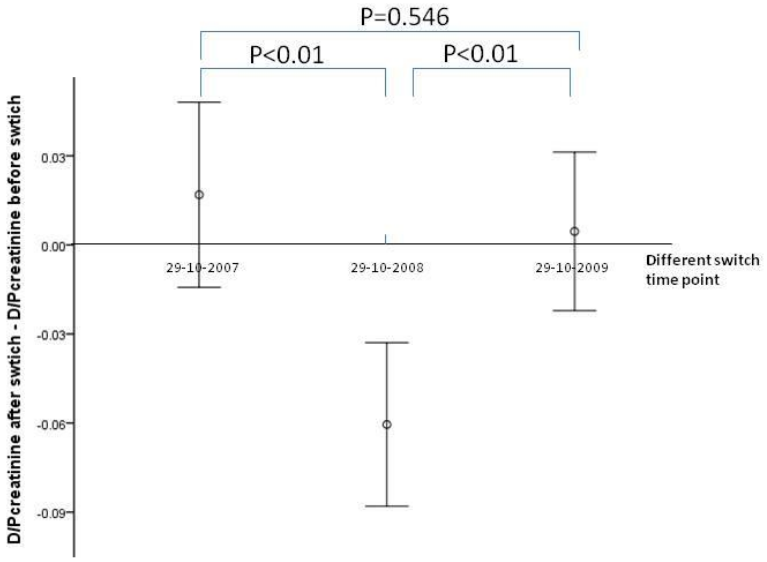
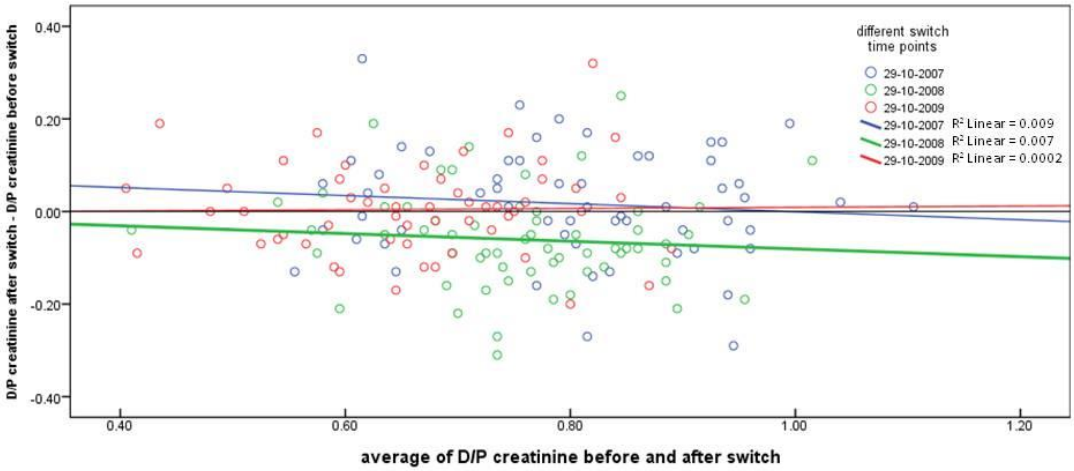


Figure 9-2 correlation between delta of D/P creatinine before and after the switch time points and absolute D/P creatinine

There was no significant correlation between delta of D/P creatinine before and after the switch time points and absolute D/P creatinine. A 0.06±0.02 mean decrease after the methodology switch was constant through the observed D/P creatinine range.




## 9.2.4 Discussion

D/P creatinine at four hours is the most important parameter in managing PD patients. Firstly, it is related to survival. Patients with a high D/P creatinine at baseline have increased mortality and technique failure, at least historically.[51, 55] Secondly, it gives the guidance for original dialysis regime, [217] although clinician would modify it according to clinical response.

Thirdly, it is also recommended to be used in monitoring patient longitudinally and in diagnosing ultrafiltration failure. [217]

#### 9.2.4.1 The impact on longitudinal observation

The first time we noticed the impact of this upgrade was during a longitudinal analysis of Stoke cohort. There was a significant decrease in D/P creatinine, since the end of 2008. It has been well described that the D/P creatinine ratio steadily increases during the year on the therapy. According to the previous study in our centre, the mean D/P creatinine was about 0.65 in the incident PD cohort and gradually increased to 0.74 after spending seven years on the therapy. [95] For those two artificial cut points in the current analysis, D/P creatinine ratio was somehow increased but far from significant different in the 6 month interval. On the background of the slow creep of D/P creatinine, the drop of D/P creatinine, from 0.78±0.13 to 0.72±0.12 before and after the methodology change was remarkable. It was not likely that any novel clinical revolution improved the membrane function dramatically.

The methodologies of routine clinical measurements need to be upgraded from time to time with the development of laboratory technique. The new methods are generally more precise and reliable. But the commutability between the new and old methods should always be aware of by both clinicians and labs.

### 9.2.4.2 The impact on multi centre study

The centre effect of membrane function has been noticed for long time, as we found in the current project. The reported proportions of high transporters range from about 17% [218] to approximately 50%.[217, 219] Race, age, gender and diabetes mellitus or comorbidity burden, which have been proved to have effect on membrane function, are thought to contribute to centre effect. [160, 220, 221] However, the centre effect has never been fully understood. The different methodology of creatinine measurement might have a silent but important contributor to the centre effect.

It would be technically difficult to compare the results from different centres because of the lack of the SRMs in high level. SRMs and a global traceable system would be the solution for this problem.

### 9.2.4.3 The impact on the classification of PSTR

The widely accepted classification of different PSTR is based on 4 hours D/P creatinine ratios as first described in 1987. [222] Twardowski et al did the study in 86 patients with 18 diabetic nephropathies. Mean, mean-1SD, mean+1SD levels of D/P creatinine were used as the cut off to divide the whole cohort into high (H), high average (HA), low average (LA), and low (L) peritoneal transporters. Mean, mean-1SD, mean+1SD levels of D/P creatinine in that specific cohort were 0.65, 0.50, and 0.80, the cut off points we are familiar nowadays.

The exact values are now used to classify transport status in different centres instead of using the centre specific means and mean+-SDs. There is advantage of this strategy. It facilitates the comparison between different cohorts. However, to use the same exact value as criteria we need to make sure that the D/P creatinine measurement is reliable and commutable, more specifically, commutable to Twardowski's lab, in the first place. More precisely, it is to the lab 25 years ago. To set up a classic classification of PSTR, a standard methodology of D/P creatinine measurement is important. It should be traceable to different centres and traceable to future methodologies.

### 9.2.5 Summary

The methodology upgrade in plasma creatinine measurement had effect on dialysate/plasma (D/P) creatinine. It is important to aware of this and its further impact. Standardization in high range of plasma creatinine is needed.

## 9.3 Bioimpedance as a Tool for Fluid Management in Peritoneal Dialysis (PD) Patients-study protocol

NCT00801112 http://clinicaltrials.gov

### 9.3.1 Background

Low peritoneal ultrafiltration, and by inference low sodium removal, is associated with worse outcomes in PD. Equally, excessive fluid removal is a risk factor for dehydration and loss of residual renal function. Current guidelines have advocated a daily UF volume of 1litre. However, their blunt application could lead to either inappropriate early loss of residual renal function or modality transfer. Body composition changes spontaneously with time on PD. Short term changes in hydration (specifically extracellular fluid volume, ECFv) combined with medium term changes in muscle and fat make it difficult for the clinician to be sure if fluid status is stable. There is a significant need for evidence on how to best manage fluid status in PD patients, both in terms of an appropriate clinical strategy and also a simple and reproducible tool to guide clinicians to apply the strategy.

Bioimpedance (BIA) gives highly reproducible measurements of two components of body composition: it measures the resistance of the body to an electrical current, inversely proportional to the total amount of fluid in the body (total body water-TBW), and the storage

(reactance) of the current, proportional to the cell membrane mass, which then proportional to intracellular water (ICW). It is simple to perform. Intervention studies have demonstrated its ability to identify changes in fluid status in response to changes in therapy[189] and the ECW:TBW ratio is a powerful predictor of patient survival.[190] It is likely that BIA will become the standard tool to assist clinicians in assessing fluid status.

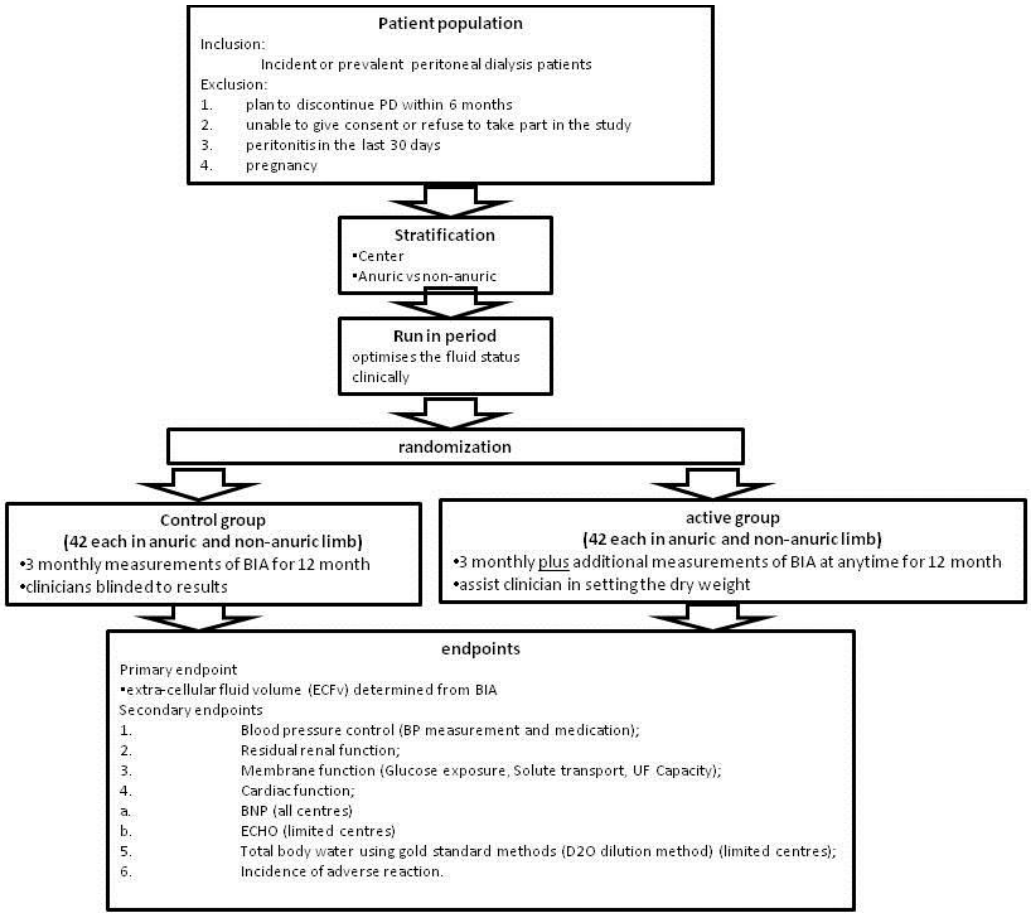
We hypothesize that regular monitoring of BIA adds value to the management of fluid status in PD patients.

## 9.3.2 Method

### 9.3.2.1 Study design

It is a prospective, randomized controlled trial. Following a 2-3 month run-in period, during which fluid status is optimized – (if necessary). Patients are then randomized 1:1 with stratification for centre and urine volume (anuric or non-anuric). BIA measurements are taken in both groups 3 monthly in the 12 months study period and in addition at any time of clinical need in active group. The BIA information will be available to the clinician and assist to set the dry weight in active group, while the clinicians will be blinded to the results in the control group. Figure 9-3

Figure 9-3 The BIA study design




### 9.3.2.2 Study setting

Three UK centres and one centre in Shanghai are involved in the study. They are University Hospital of North Staffordshire, St. James's University Hospital, Sheffield Kidney Institute Northern General Hospital and Shanghai Renji Hospital. All of them are University hospitals with well established PD programmes. The planned recruiting patient number is more than half of their whole PD population.

The samples will be sent to University Hospital of North Staffordshire shortly after being collected for further analysis in the three UK centres. The samples in Shanghai Renji Hospital will be stored and analyzed locally.

#### 9.3.2.3 Patient population

The patient who are more than 16 years old and has been stably established on PD for more than 3 months in the study centres are approaching for the study.

##### 9.3.2.3.1 Exclusion criteria:

1. plan to discontinue PD within 6 months
2. unable to give consent or refuse to take part in the study
3. peritonitis in the last 30 days
4. pregnancy
5. implanted electronic devices

##### 9.3.2.3.2 Withdraw

Patients permanently leave the PD therapy (transfer to haemodialysis, renal transplantation, spontaneous recovery of renal function), or loss to follow-up.

#### 9.3.2.3.3 Sample size calculation

We hypothesize that the control group will have a drift upward in ECW while the active group will remain stable (no change from baseline). Based on historical data, we expect the standard deviation of mean change from baseline is about 1.22 (this is the standard deviation of the differences in ECV over an 8 month period in a group of 27 patients). 38 patients per group will have adequate statistical power to detect a 0.8kg increase in control group, assuming type I error of 5% and 80% power. 42 patients in each group is planned to enroll with the assumption of 15% of dropout rate.

### 9.3.2.4 BIA measurement

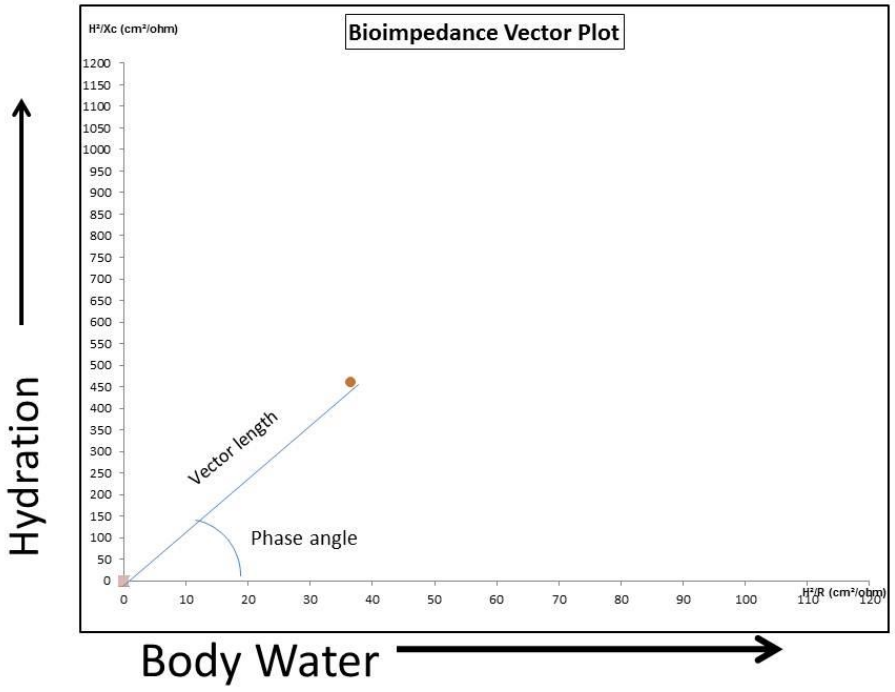
The single frequency (50Hz) BIA device (Akern BIA 101 Anniversary) is used in the study. The total body resistance and reactance are measured according to the operating manual. The patient may with or without PD fluid in the peritoneal cavity when do the measurement but it should be consistent during the follow up.

### 9.3.2.5 Interpretation of the BIA information

In active group, hight$^2$/resistance, inversely proportional to TBW, and hight$^2$/reactance, inversely proportional to cell membrane mass, will be plotted. The baseline result from each patient will be used as reference for the individual patient. The follow up measurement will be plotted and chased up. No intention will be made to normalize the patient to their age

and gender matched ‘normal reference range’ from the general population. Generally speaking, prolonged vector length and increased phase angle indicate the increase of TBW and hydration status. (Figure 9-4) Both clinical and BIA information will be taken into account when making decision of target weight. When the BIA result is opposite to the clinical symptoms and signs, the clinician can make the decision whether go with the clinical or the BIA information. In control group, the BIA result will not be plotted out and the clinician will make the decision based on pure clinical information.

Figure 9-4 BIA vector




### 9.3.2.6 Intervention

The difference between the control and active group is at the stage of setting target weight. The BIA information is available to the clinician to help in setting target weight in active group while in control group the BIA information is not available to clinician.

In terms of achieving target weight, it is left to the clinical team to use whatever tools at their disposal in both groups. These will include more intensive advice on dietary salt and fluid intake, increased use of hypertonic solutions, icodextrin, modality change (APD v. CAPD), or most likely a combination of these. It is also important to notice that the clinical fluid

management policy in different centre can be different. (E.g. icodextrin and APD are not available in Shanghai) It will be documented and taken into account for secondary analysis.

#### 9.3.2.7 Outcome measures

Primary outcome measure is ECW determined from BIA.

Secondary outcome measures include

1. Blood pressure control (BP measurement and medication);
2. Residual renal function;
3. Membrane function (Glucose exposure, PSTR, UF Capacity);
4. Cardiac function;
    a. BNP (all centres)
    b. ECHO (limited centres)
5. Total body water using gold standard methods (D2O dilution method) (limited centres);
6. Incidence of adverse reaction.

#### 9.3.2.8 Sample handling and storage

Blood samples will be collected and centrifuged at 3000rpm on site before being stored at -20 degree until further analysis.

#### 9.3.2.9 Data analysis plan

Paired t-test will be used for the primary endpoint and secondary endpoint, comparing the results before and after treatment. Differences in proportions will be evaluated by chi-square of Fisher's exact tests, as appropriate. Multi level regression model will be applied to control covariates. Repeated measures (level 1) will be nested within individual (level 2). The centre effect will also be addressed by using multi level strategy by nesting individual within centre (level 3). A further level will be considered regarding the big difference between Shanghai and the three UK centres (level 4).

## 9.4 How Akern BIA 101 Anniversary transform the resistance and reactance to TBW and ECW?

### 9.4.1 Purpose

To investigate how Akern BIA 101 Anniversary transform the resistance and reactance measurements to TBW and ECW.

### 9.4.2 Methods

172 sets of patient information (including gender, height and body weight) and resistance and reactance were put into the software of Akern BIA 101 Anniversary. The TBW and ECW output from the software were recorded. Based on reference, [223] the first 100 records was used to generate a set of formulas to calculate TBW and ECW from height, body weight, gender and raw resistance and reactance readings. The formulas were then applied to calculate TBW and ECW for all records.

### 9.4.3 Results

The formulas generated were as following.

For TBW, If female,

$$TBW = 0.511 \times \frac{\text{hight}^{\frac{4}{3}} \times \text{weight}^{\frac{1}{3}}}{\text{resistance}^{\frac{2}{3}}} + 6.87$$

For TBW, if male

$$TBW = 0.608 \times \frac{\text{hight}^{\frac{4}{3}} \times \text{weight}^{\frac{1}{3}}}{\text{resistance}^{\frac{2}{3}}} + 3.37$$

For ECW

$$\text{ECW} = \frac{\text{TBW}}{1 + 22.907576(\frac{\text{resistance} + \text{reactance}}{\text{reactance}})^{-1.255081}}$$

The correlations between the TBW and ECW derived from the Akern BIA Anniversary software and the TBW and ECW calculated from the formulas above were show in Figure 9-5 and Figure 9-6. The difference between the two methods was not related to the absolute values Figure 9-7 and Figure 9-8.

Figure 9-5 the correlation between the TBW from the machine (derived from the Akern BIA Anniversary software) and the TBW calculated from the formula

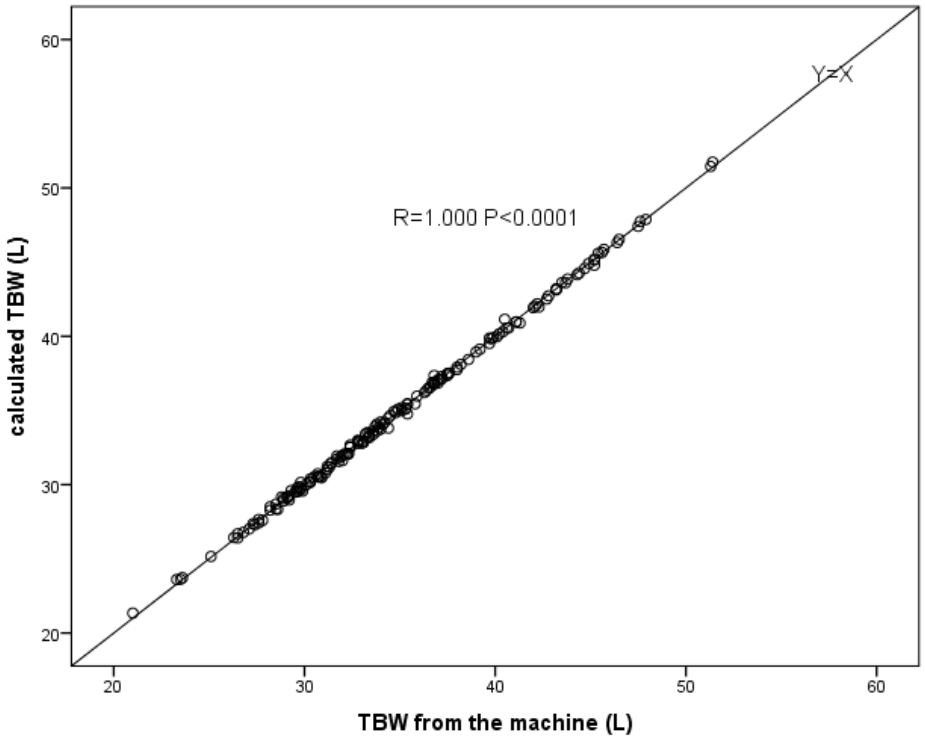


Figure 9-6 the correlation between the ECW from the machine (derived from the Akern BIA Anniversary software) and the ECW calculated from the formula

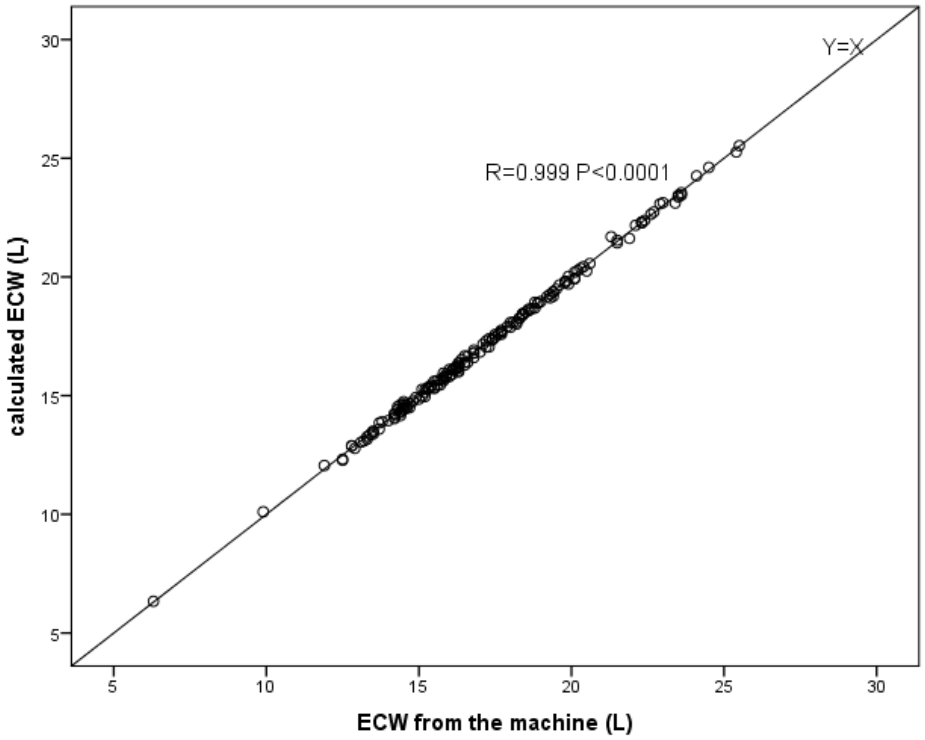


Figure 9-7 the correlation between the difference of the calculated TBW from the formula and from the machine and the absolute value of TBW

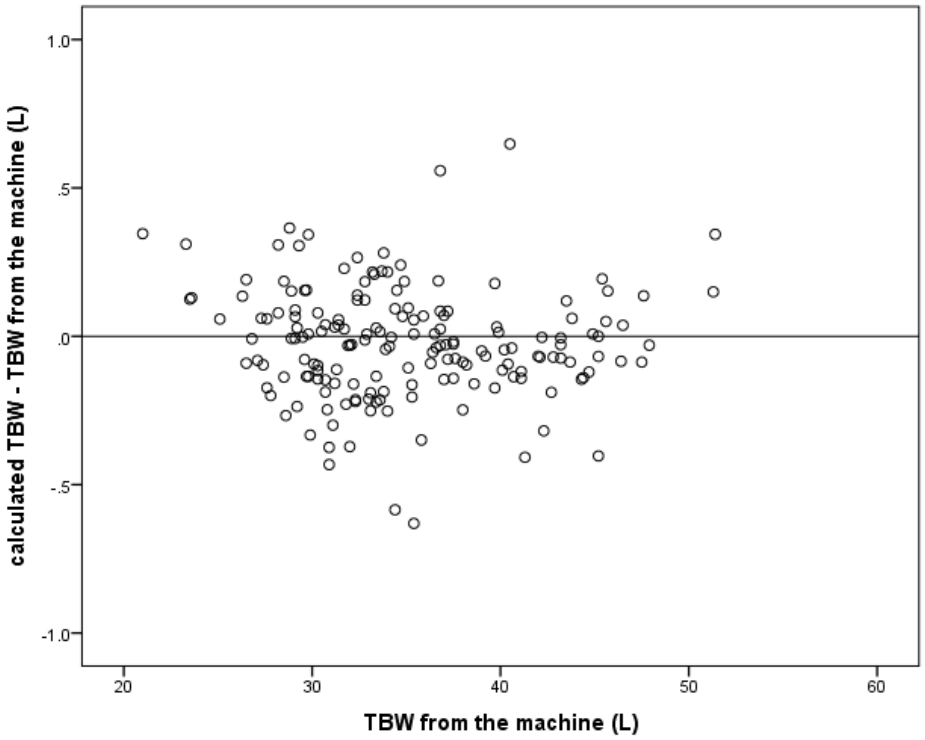
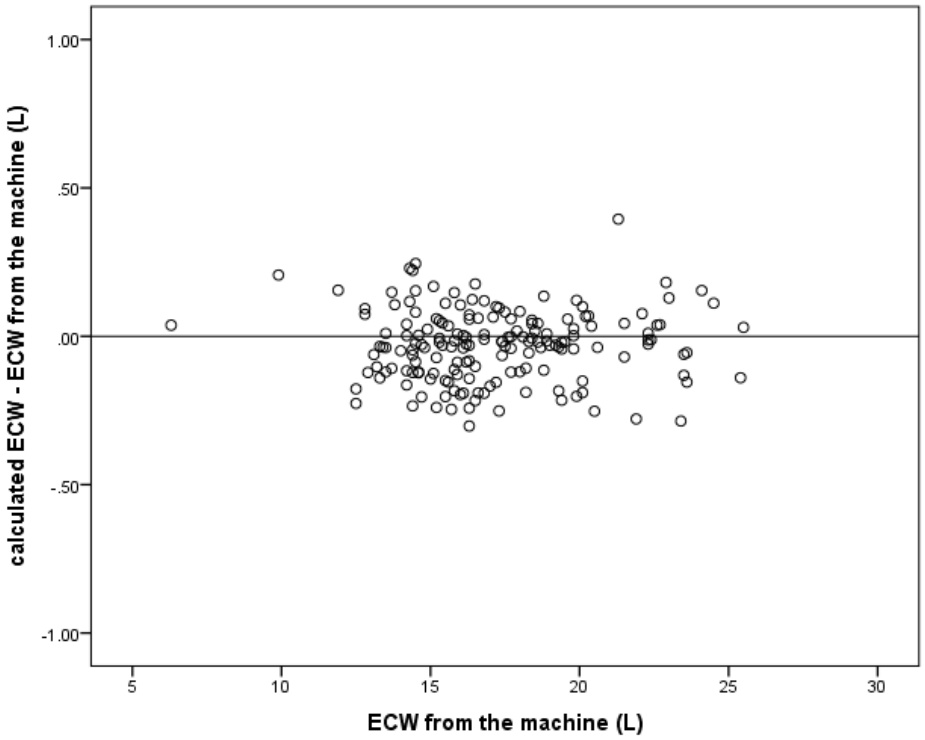


Figure 9-8 the correlation between the difference of the calculated ECW from the formula and the ECW from the machine and the absolute value of ECW




## 9.4.4 Conclusion

The formulas generated in this analysis have good agreement with the manufacturer's algorithms (Akern BIA 101 Anniversary BIA device). TBW and ECW output from the BIA device are calculated from resistance, reactance, height, weight and gender using universal algorithms, without accounting for age or comorbidities.

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Multi-Centre Research Ethics Committee for Wales

MREC for WALES

Pwyllgor Ymchwil Ethegau Aml-Ganolfan yng Nghymru

Chairman/Cardeirydd: Dr John Saunders

Administrator/Gweinyddes: Corinne Scott

Temple of Peace and Health, Cathays Park, Cardiff CF10 3NW
Teml Heddwch ac Iechyd, Parc Cathays, Caerdydd CF10 3NW

WHTN 0 1809 Telephone enquiries to: 029 2040 2455 Fax No. 029 2040 2504

MREC website: http://ds.dial.pipex.com/mrec
e-mail : corinne.scott@bro-taf-ha.wales.nhs.uk

Dr. Nicholas Topley,
Institute of Nephrology,
University of Wales College of Medicine,
Heath Park,
Cardiff CF14 4XN

April 16th 2002

Dear Dr. Topley,

**Research Protocol MREC 02/9/14** **(Please quote this in all correspondence)**
**Longitudinal evaluation of peritoneal membrane function, inflammation and structural integrity in peritoneal dialysis**

I have reviewed the documents submitted in response to the MREC for Wales decision made at its meeting held on April 16th 2002, and set out in our letter dated April 16th 2002.

The documents reviewed were as follows:
*(By full Committee)*
- Application Form including Annexe C
- Full Protocol and references
- ~~Patient Information Sheet, version 1.1 dated March 16th 2002~~ **Superseded**
- ~~Patient Consent Form~~ **Superseded**
- GP letter
- Curriculum Vitae for Principal Researcher, Dr. Nicholas Topley

*(By Chairman)*
- Patient Information Sheet and Consent Form, version 1.2 dated April 8th 2002

As Chairman, acting under delegated authority, I am satisfied that these accord with the decision of the Committee and agree that there is no objection on ethical grounds to the proposed study. I am, therefore, happy to give you our approval on the understanding that you will follow the conditions of approval set out below. A full record of the review undertaken by the MREC is contained in the attached Response Form. The project must be started within three years of the date on which MREC approval is given.

- You must follow the protocol agreed and any changes to the protocol will require prior MREC approval.

- If projects are approved before funding is received, the MREC must see, and approve, any major changes made by the funding body. The MREC would expect to see a copy of the final questionnaire before it is used.

- You must promptly inform the MREC of:

(i) deviations from or changes to the protocol which are made to eliminate immediate hazards to the research subjects;
(ii) any changes that increase the risk to subjects and/or affect significantly the conduct of the research;
(iii) all adverse drug reactions that are both serious and unexpected;
(iv) new information that may affect adversely the safety of the subjects or the conduct of the trial.

- You must complete and return the standard progress report form to the MREC one year from the date on this letter and thereafter on an annual basis. This form should also be used to notify the MREC when your research is completed.

While the MREC has given approval for the study on ethical grounds, it is still necessary for you to obtain management approval from the relevant Clinical Directors and/or Chief Executive of the Trusts (or Health Boards/HAs) in which the work will be done.

**LREC Review**

When undertaking the review of your project the MREC observed that this study falls under the Supplementary Operational Guidelines for NHS Research Ethics Committees, published in November 2000. This study is classed as Category D research, and therefore does not require LREC review.

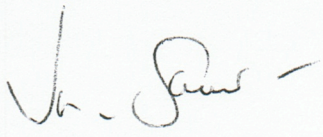
For this reason you are asked to only inform the appropriate LREC of the project by sending a copy of this letter and also **giving the name and contact details of the local clinician involved**. If (unusually) the LREC has any reason to doubt that the local clinician is competent to carry out the tasks required, it will inform the clinician and the MREC that gave ethical approval giving full reasons.

You are not required to wait for confirmation from the LREC before starting your research.

Whilst the MREC would like as much information as possible about local sites at the time you apply for ethical approval it is understood that this is not always possible. You are asked, however, to send details of local sites as soon as a researcher has been recruited. This is essential to enable the MREC to monitor the research it approves.

The MRECs are fully compliant with the International Conference on Harmonisation/Good Clinical Practice (ICH GCP) Guidelines for the Conduct of Trials Involving the Participation of Human Subjects as they relate to the responsibilities, composition, function, operations and records of an Independent Ethics Committee/Independent Review Board. To this end it undertakes to adhere as far as is consistent with its Constitution, to the relevant clauses of the ICH Harmonised Tripartite Guideline for Good Clinical Practice, adopted by the Commission of the European Union on 17 January 1997. The Standing Orders and a Statement of Compliance were included on the computer disk containing the guidelines and application form and are available on request or on the Internet at http://dspace.dial.pipex.com/mrec.

Yours sincerely,



**Dr. John Saunders**
**Chairman**
**MREC for Wales**

*ENCS : MREC Response Form and Attendance List for MREC Meeting of April 11th 2002.*

# MULTI-CENTRE RESEARCH ETHICS COMMITTEE FOR WALES

## RESPONSE FORM

| | |
|---|---|
| 1 | **Details of Applicant** |
| | Dr. Nicholas Topley,<br>Institute of Nephrology,<br>University of Wales College of Medicine,<br>Heath Park,<br>Cardiff CF14 4XN |
| | |
| 2 | **Title of Project** |
| | Longitudinal evaluation of peritoneal membrane function, inflammation and structural integrity in peritoneal dialysis |
| | |
| 3 | **Name of Sponsor** |
| | None |
| | |
| 4 | **Details of MREC** |
| | MREC for Wales,<br>Temple of Peace & Health, Cathays Park, Cardiff, CF10 3NW. |
| | |
| 5 | **MREC Reference Number** |
| | 02/9/14 |
| | |
| | **YOUR APPLICATION HAS BEEN CONSIDERED BY THE MREC FOR WALES WHO MADE THE FOLLOWING COMMENTS :** |
| 1 | **Qualifications of the Applicant** |
| | No comments |
| | |
| 2 | **Scientific Value and Validity of the Proposal** |
| | No comments |
| 3 | **The Welfare of the Research Subject** |
| | No comments |

| | |
|---|---|
| **4** | **Patient Information Sheet** |
| | The PIS should state the following :<br>1) That the subject is free to withdraw from the study at any time<br>2) the subject will not benefit from participation in the study<br>3) the subject's GP will be contacted to inform them of their participation in the study<br>4) there should be some comment regarding the anonymisation of the individual's data<br><br>Consent form should :<br>1) give permission for the subject's GP to be contacted<br><br>***The revised Patient Information Sheet was received on April 16th 2002. This has been reviewed and approved by the Chairman of the MREC for Wales, Dr. John Saunders.*** |
| | |
| **5** | **Confidentiality** |
| | No comments |
| | |
| **6** | **General Comments** |
| | The need for LREC review<br>This research falls under Category D of the Guidelines for Epidemiological Research and no LREC approval is therefore required. |
| | |

**REVIEW BY THE MREC**

**The following items have been reviewed by the MREC for Wales in connection with the above study to be conducted by the above researcher :**

| | |
|---|---|
| Protocol | ✓ |
| Investigators Drug Brochure | n/a |
| Patient Information Sheet and Consent Form, version 1.2 dated April 8th 2002 | ✓ |
| GP letter | ✓ |
| CTX | n/a |
| Protocol amendment | n/a |
| Methods of initial recruitment to study | ✓ |
| Compensation arrangements for subjects | n/a |
| Payments to researcher | n/a |
| Provision of expenses for subjects | n/a |

**Your application has been approved.**

**Date of review : April 11th 2002**
**Date of approval : April 16th 2002**

**Signature of Chairman** ..... **Date** 16-4-02

## MREC FOR WALES

## Attendance List for the MREC for Wales' meeting on April 11th 2002

| | | |
|---|---|---|
| Dr John Saunders | Chairman | Professional (Hospital Consultant) |
| Dr. Gordon Taylor | Vice Chairman | Lay member |
| Dr. Barbara Bale | | Professional (Midwife) |
| Dr. Peter Beck | | Professional (Hospital Consultant) |
| Dr. Alison George | | Lay member |
| Mrs. Phillipa Herbert | | Lay member |
| Dr. Mohammad Obaidullah | | Professional (GP) |
| Mr. Simon Rivers | | Professional (Pharmacist) |
| Dr. Paul Wainwright | | Professional (Nurse) |

National Research Ethics Service
North Staffordshire Local Research Ethics Committee
North Staffordshire Medical Institute
Hartshill Road
Hartshill
Stoke-on-Trent
Staffs
ST4 7NY

Telephone: 01782 714935

30 March 2009

Professor Simon J Davies
Professor of Nephrology and Dialysis Medicine
University Hospital of North Staffordshire
Princes Road
Hartshill
Stoke-on-Trent, Staffordshire
ST4 7LN

Dear Professor Davies

**Full title of study:** **Developing Bioimpedance (BIA) as a tool for fluid management in Peritoneal Dialysis Patients: A validation study.**

**REC reference number:** **09/H1204/19**

Thank you for your letter of 10 March 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

**Ethical review of research sites**

The favourable opinion applies to the research sites listed on the attached form

**Conditions of the favourable opinion**

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

**North Staffordshire Local Research Ethics Committee**
**LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION**

*For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.*

| | | | | | |
|---|---|---|---|---|---|
| **REC reference number:** | 09/H1204/19 | **Issue number:** | 1 | **Date of issue:** | 30 March 2009 |
| **Chief Investigator:** | Professor Simon J Davies | | | | |
| **Full title of study:** | Developing Bioimpedance (BIA) as a tool for fluid management in Peritoneal Dialysis Patients: A validation study. | | | | |

*This study was given a favourable ethical opinion by North Staffordshire Local Research Ethics Committee on 30 March 2009. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.*

| *Principal Investigator* | *Post* | *Research site* | *Site assessor* | *Date of favourable opinion for this site* | *Notes* (1) |
|---|---|---|---|---|---|
| Professor Simon J Davies | Professor of Nephrology | University Hospital of North Staffordshire | North Staffordshire Local Research Ethics Committee | 30/03/2009 | |

Approved by the Chair on behalf of the REC:

Janet Clarke ..... (Signature of ~~Chair~~/Co-ordinator)
(delete as applicable)

JANET CLARKE ..... (Name)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

| 09/H1204/19 | **Please quote this number on all correspondence** |
|---|---|

With the Committee's best wishes for the success of this project

Yours sincerely

Janet Clarke

**Dr Mark Gunning** pp
**Vice Chair**

Email: Janet.Clarke@uhns.nhs.uk

*Enclosures:* "After ethical review – guidance for researchers"
*Site approval form*

*Copy to:* Dr D Clements, R&D Manager, UHNS

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| *Document* | *Version* | *Date* |
|---|---|---|
| Response to Request for Further Information | | 10 March 2009 |
| Participant Consent Form | 2.0 | 10 March 2009 |
| Participant Information Sheet | 2.0 | 10 March 2009 |
| Statistician Comments | | 09 January 2009 |
| Peer Review | from funder | 27 November 2008 |
| Covering Letter | | 12 January 2009 |
| Protocol | 1 | 15 October 2008 |
| Investigator CV | | |
| Application | | 14 January 2009 |

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

**After ethical review**

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review –guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

**North Staffordshire Local Research Ethics Committee**

**Attendance at Sub-Committee of the REC meeting on 30 April 2008**

| | | | |
|---|---|---|---|
| **Present:** | Miss N Brooks | (Chair) | Lay |
| | Professor C Cullen | Psychologist | Expert |
| | Professor M Spiteri | Physician | Expert |

**R&D approval**

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

| **06/Q2604/186:** | **Please quote this number on all correspondence** |
|---|---|

Yours sincerely

Janet Clarke

**Janet Clarke**
**Committee Co-ordinator**

E-mail: Janet.Clarke@ssh-tr.nhs.uk

Enclosures — List of names and professions of members who were present at the meeting and those who submitted written comments

*Copy to:* — Dr D Clements, R&D Manager UHNS

NHS
National Research Ethics Service
North Staffordshire Local Research Ethics Committee
Mellor House
Corporation Street
Stafford
Staffordshire
ST16 3SR

Tel: 01785 257888 ext 5941

06 May 2008

PROFESSOR SIMON DAVIES
CONSULTANT NEPHROLOGIST
ROYAL INFIRMARY
PRINCES ROAD,
HARSTHILL
STOKE ON TRENT
ST4 7LN

Dear PROFESSOR DAVIES

**Study title:** **Is there an association between fluid distribution within the extracellular compartment, inflammatory status and treatment with icodextrin in peritoneal dialysis patients?**
**REC reference:** **06/Q2604/186**
**Amendment number:** **2**
**Amendment date:** **18 April 2008**

The above amendment was reviewed at the meeting of the Sub-Committee of the REC held on 30 April 2008.

**Ethical opinion**

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

**Approved documents**

The documents reviewed and approved at the meeting were:

| Document | Version | Date |
|---|---|---|
| Participant Information Sheet | 5.5 | 18 April 2008 |
| Participant Consent Form | 5.5 | 18 April 2008 |
| Notice of Substantial Amendment (non-CTIMPs) | | 18 April 2008 |
| Covering Letter | | 18 April 2008 |

**Membership of the Committee**

The members of the Committee who were present at the meeting are listed on the attached sheet.

Nephrol Dial Transplant (2012) 0: 1–9
doi: 10.1093/ndt/gfs075

NDT
Nephrology Dialysis Transplantation

*Original Article*

# Hypoalbuminaemia, systemic albumin leak and endothelial dysfunction in peritoneal dialysis patients

Zanzhe Yu[1,2], Boon Kay Tan[1,2], Stephen Dainty[3], Derek L. Mattey[2,4] and Simon J. Davies[1,2]

[1]Department of Nephrology, University Hospital of North Staffordshire, Stoke on Trent, UK, [2]Institute of Science and Technology in Medicine, Keele University, Keele, UK, [3]Medical Physics, University Hospital of North Staffordshire, Stoke on Trent, UK and [4]Staffordshire Rheumatology Centre, Haywood Hospital, Stoke on Trent, UK

*Correspondence and offprint requests to:* Simon J. Davies; E-mail: simondavies1@compuserve.com

## Abstract

**Background.** Inflammation, hypoalbuminaemia and peritoneal protein clearance are important predictors of survival in patients treated with peritoneal dialysis (PD). We hypothesized that the common link is abnormal endothelial barrier function. To test this, we explored associations between hypoalbuminaemia, systemic albumin leak and soluble markers of systemic inflammation and endothelial injury.

**Methods.** This was a cross-sectional study of 41 prevalent PD patients. Endothelial barrier function was measured as transcapillary escape rate of $^{125}$I albumin [transcapillary escape rate of albumin ($TER_{alb}$)]. Seventeen plasma biomarkers including pro-inflammatory cytokines, endothelial biomarkers and metalloproteinases were measured. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) were used to explore the hypothesis.

**Results.** The mean $TER_{alb}$ was 13.7 ± 8.9 (%/h), higher than in non-uraemic subjects 8.22 ± 5.8 (%/h). Three patient clusters were defined from HCA according to their biomarker patterns. Cluster 1 was characterized by inflammation, hypoalbuminaemia, overhydration and intermediate $TER_{alb}$. Cluster 2 was non-inflamed, preserved muscle mass and more normal $TER_{alb}$. Cluster 3 had highest $TER_{alb}$, platelet activation, preserved plasma albumin and intermediate high-sensitivity C-reactive protein levels. Two principal components (PCs) were identified from the biomarker matrix, PC1, indicating platelet activation and PC2, pro-inflammatory. $TER_{alb}$ was positively related to PC1 but not PC2. Diabetes and ischaemic heart disease were associated with PC1 and PC2, respectively.

**Conclusions.** This exploratory analysis indicates that endothelial barrier function is decreased in PD patients and is associated with diabetic status and markers of platelet activation more than inflammation. In contrast, hypoalbuminaemia is associated more with inflammation and atherosclerotic disease indicating a more complex relationship between systemic endothelial barrier function, inflammation and hypoalbuminaemia which requires further validation.

**Keywords:** biomarker pattern; body composition; inflammation; platelet activation; transcapillary escape rate

## Introduction

Inflammation and hypoalbuminaemia are both important predictors of survival in patients treated with dialysis for advanced renal failure [1–3]. Endothelial dysfunction is a feature of uraemia [4], also present in complicated diabetes and cardiovascular co-morbidities that commonly occur in these patients. Indeed, it may represent the link between increased systemic and/or peritoneal protein leak, inflammation, hypoalbuminaemia and worse survival through its association with reduced capillary barrier function [5] (see Figure 1a). However, the picture is further complicated in peritoneal dialysis (PD) patients by the important daily losses of protein from the peritoneal cavity which likely explains why for a given degree of hypoalbuminaemia, survival in PD patients is superior to those on haemodialysis [1]. The observation that peritoneal protein clearance (the main determinant of absolute protein losses) predicts survival independent of effective peritoneal surface area [5–7] led us to test the hypothesis that the underlying common factor is impaired systemic endothelial barrier function.

To do this, we explored the relationship between the systemic transcapillary escape rate of albumin ($TER_{alb}$) as an indicator of endothelial barrier function, a panel of biomarkers of endothelial dysfunction and associated clinical phenotypes. We used the techniques of hierarchical clustering (HC) and principal component analysis (PCA) to generate patient groups from the patterns of biomarkers observed and then subsequently compared their clinical phenotypes so as to avoid any pre-conceived assumptions.

### Materials and methods

*Study design and patient population*

This was a cross-sectional study of prevalent PD patients in a single centre. Sequential patients were approached to participate and studied while undergoing their routine 6-monthly assessments of peritoneal membrane

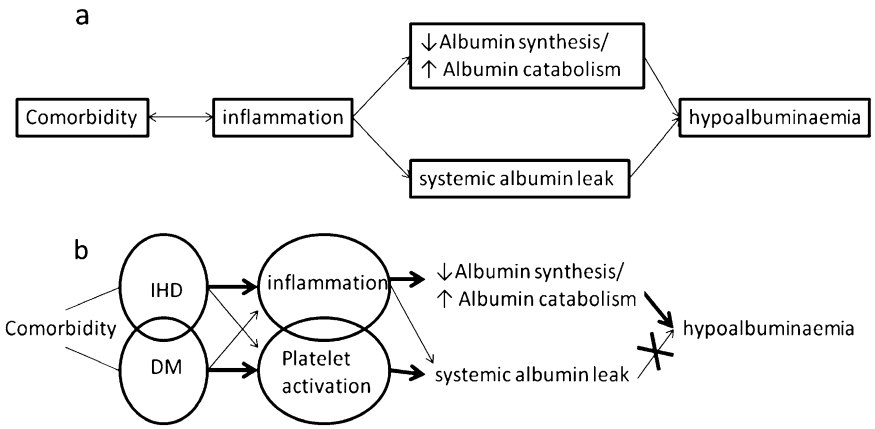



**Fig. 1.** (**a**) Simplified linear hypothesis linking co-morbidity to hypoalbuminaemia invoking reduced endothelial barrier function as the central unifying mechanism. (**b**) Modified relationship between co-morbidity, inflammation, systemic protein leak and hypoalbuminaemia in the light of current findings.

function and dialysis adequacy tests, provided they were not acutely ill or within 1 month of peritonitis. The study was peer reviewed and approved by the local ethics committee and all patients signed the consent form.

*$TER_{alb}$ and plasma volume*

After a bolus intravenous injection of 10 mL 0.185 MBq $^{125}$I-human serum albumin (HSA), blood samples were taken at 10, 20 and 30 min at a remote venous site. The isotopic concentration in each case was plotted against time on a semi-logarithmic scale and the best linear fit line was performed from these points. $TER_{alb}$, expressed as percentage loss per hour (%/h) was estimated from the disappearance rate of the $^{125}$I HSA from the gradient of this line. The plasma volume was calculated by extrapolating the line to time zero. The method was also applied for clinical purposes as the measurement of plasma volume in the normalization of red cell mass and diagnosis of primary polycythaemia. These patients who were mainly referred from haematologists during the time of this study served as non-uraemic contemporaneous 'in-house' controls.

*Biomarker measurement*

The plasma sample for biomarker analysis was collected just before $^{125}$I HSA injection on the study day and stored at −20°C. A panel of 17 biomarkers, which are involved in inflammation, endothelial function and tissue remodelling processes, [interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), IL-1β, IL-10, monocyte chemotactic protein-1, inter-cellular adhesion molecule 1, vascular cell adhesion protein 1 (VCAM-1), E-selectin, P-selectin, CD40 ligand (CD40L), vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-1, MMP-2, MMP-3, MMP-8, MMP-9], was measured on a Luminex suspension array system (Bio-Plex 200TM platform; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) using commercially available multi-analyte cytokine kits [MILLIPLEX MAP; Millipore (UK) Ltd, Walford, Hertfordshire, UK] or Fluorokine multi-analyte profiling kits for MMPs (R&D Systems, Abingdon, UK). Assays were carried out according to the manufacturer's instructions.

*Body composition, solute clearance, membrane function and blood biochemistry*

Estimated total body water (TBW) and extra-cellular water were evaluated by bioimpedance (BIA, multi-frequency Xitron Hydra device, Model 4200; Xitron Technologies, San Diego, CA). At the same time, absolute TBW was measured by deuterium ($D_2O$) dilution technique. The detailed methodologies have been described previously [8]. In brief, baseline and equilibrated blood samples were taken before and 2.5 h after an oral dose of 99.8% $D_2O$ (Cambridge Isotope Laboratories). The difference between the headspace HDO abundance of the two samples, measured by flowing-afterglow mass spectrometry, was used to determine $TBW_D$ after accounting for equilibration with dialysate and 4% D exchange with H in body proteins. The difference between BIA estimated ($TBW_{BIA}$) and D dilution measured TBW ($TBW_D$) reflects tissue overhydration [8, 9].

The dialysis dose and residual renal function were calculated as the weekly $Kt/V_{urea}$ from the 24-h urinary and dialysate clearance by direct measurement of urea in urine and dialysate. Peritoneal dialysate protein loss was measured from the collection of 24-h dialysate effluent. A validated equation was used for the calculation of protein clearance, (Pcl), = 24-h dialysate protein loss/(serum albumin/0.4783).

Solute transport was measured by standard 4-h peritoneal equilibrium test with 2.27% glucose concentration 2-L exchange. The dialysate:plasma ratio of creatinine at the completion of the 4-h dwell period (D/P creatinine) was used to estimate low-molecular-weight solute transport.

Plasma albumin was estimated using the bromocresol purple colorimetric method, peritoneal dialysate and urine protein by the biuret method. C-reactive protein (CRP) was measured using a latex enhanced immunoturbidimetric method.

*Statistical analysis*

One-way analysis of variance and unpaired *t*-test were used to examine differences in normally distributed continuous data among groups, chi-square for categorical data. Univariate correlations were examined by Pearson correlation coefficient. Variables not normally distributed were $\log_2$ transformed for further analysis. Significance was considered at P-values $<0.05$. Statistical analyses were performed using IBM SPSS Statistics version 19 except for the HC analysis (HCA).

*Hierarchical cluster analysis*

The biomarker levels were first converted to $\text{Log}_2$ and expressed relative to the mean value for normalization. These measurements were used to generate heat map (Figure 2) using Genesis software (version 1.7.2, Alexander Sturn; Institute for Genomics and Bioinformatics, Graz University of Technology). The Genesis programme uses a two-dimensional HC method, using the average linkage clustering agglomerative rule that enables groups of variables with similar expression levels to be clustered together as well as grouping together patient samples with similar expression patterns.

*Principal component analysis*

To decrease the dimensionality of the biomarker data set while retaining as much of the variance as possible, an exploratory PCA was employed. Principal components (PCs) were extracted using varimax rotation, with the factor selection based on an eigen value cut-off of 1.0. Because of limitations of the sample size, eight biomarkers only were processed in the final analysis. These biomarkers were selected from the first two PCs, which contributed the most variance of the whole matrix. Those biomarker variables not normally distributed after log transformation were converted to binary data.

## Results

A total of 41 prevalent patients (M/F 22/19, mean age 61 ± 17 years, median time on PD 20 [9–33] months, diabetics

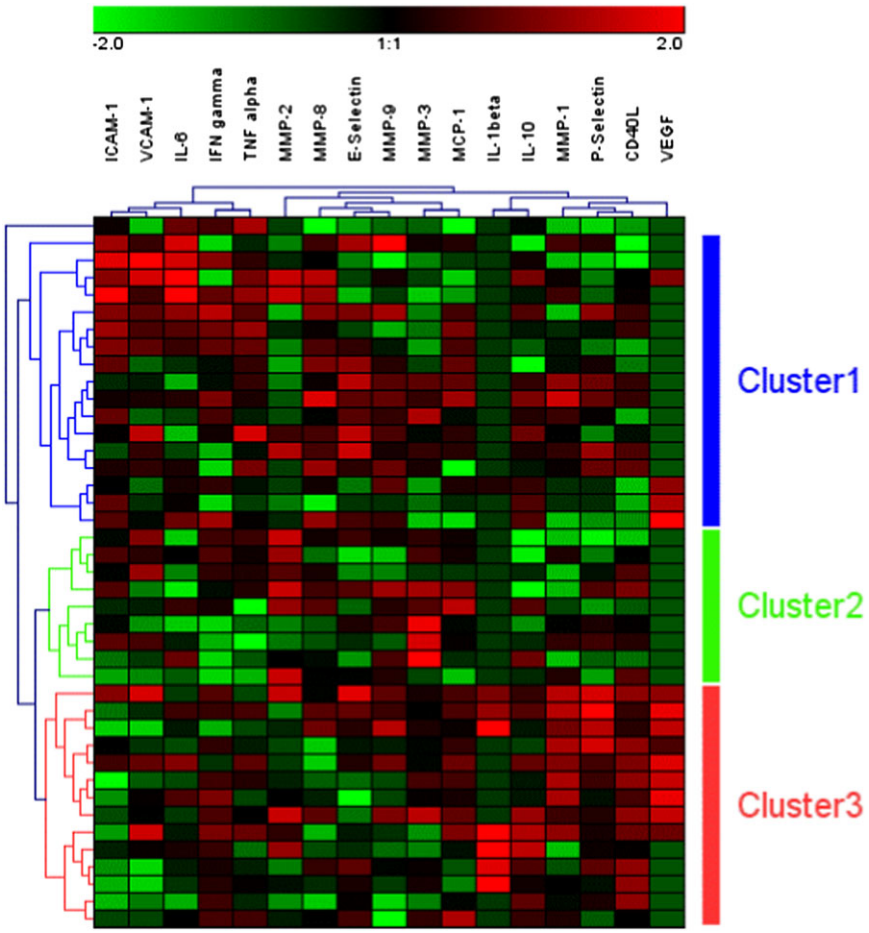



**Fig. 2.** Plasma concentration profiles of 17 biomarkers in 41 patients expressed as a HCA heat map. Plasma concentrations of biomarker close to higher and lower than the mean values are represented by black, red and green colours, respectively. Three patient clusters, Cluster 1 (blue bar), Cluster 2 (green bar), Cluster 3 (red bar) and one outlier are generated by HCA.

29%) were studied. The $TER_{alb}$ was higher in the PD patients 13.74 ± 8.8 (%/h) than the non-uraemic comparator group 8.22 ± 5.8 (%/h) ($n = 13$, $P < 0.05$).

Three clusters and one outlier were defined from the HCA (Figure 2). Patient characteristics, peritoneal membrane function and $TER_{alb}$ (Table 1) and absolute biomarker concentrations (Table 2) are shown by cluster. Cluster 1 was characterized by an inflammatory profile, associated with higher high-sensitivity CRP, lower albumin, intermediate $TER_{alb}$ and overhydration indicated by the big discrepancy between measured TBW ($TBW_D$) and estimated ($TBW_{BIA}$). Cluster 2 was non-inflamed according to the lowest CRP levels, had the lowest $TER_{alb}$ and the most normal body composition as evidenced by the highest body water by body weight but good agreement between D dilution and BIA measures, indicating well-preserved muscle mass but least overhydration. Cluster 3 was characterized by biomarkers consistent with platelet activation, had the highest $TER_{alb}$ but only moderate CRP levels, less inflammation and less overhydration than Cluster 1 patients.

The PCA was first carried out in the whole panel of the 17 biomarkers. Seven PCs were identified, explaining 74.9% of the total variance, Figure 3a. The eight biomarkers that composed the two strongest PCs, contributing the majority of the variance, were selected for the final PCA and are shown with their eigen values (>1) in Figure 3b. The first PC (PC1-platelet activation) was composed mainly of MMP-1, P-selectin, CD40L and VEGF. The second PC (PC2 pro-inflammation) featured an inverse relationship between MMP-3 and positive association with IFN-γ, TNF-α and VCAM-1. This component showed a positive relationship with CRP ($r = 0.31$, $P = 0.047$) and was negatively correlated with plasma albumin ($r = -0.50$, $P = 0.001$).

The relationship between the HCA and PCA is shown graphically in Figure 4a. It can be seen that despite differing dimensions that these two analytical techniques give patterns that are in agreement. Patients with different co-morbidities also showed differences in PCs such that patients with ischaemic heart disease (IHD) and diabetes mellitus (DM) have different, albeit overlapping, patterns of biomarkers (Figure 4b). Diabetes had higher PC1, while PC2 was higher in IHD (Table 3).

To further explore the relationship between clinical factors and biomarker profiles with our primary end point,

**Table 1.** Characteristics of the three patient clusters derived from HCA[a]

| | Cluster 1 ($n = 17$) | Cluster 2 ($n = 9$) | Cluster 3 ($n = 14$) | Single outlier |
|---|---|---|---|---|
| $TER_{alb}$ (%/h) | 13.88 ± 9.23 | 9.12 ± 5.29[b] | 17.29 ± 9.11 | 3 |
| Age (years) | 62.1 ± 17.2 | 61.4 ± 19.2 | 60.3 ± 15.7 | 60 |
| Gender (M, %) | 65 | 56 | 36 | M |
| BMI (kg/m$^2$) | 26.1 ± 5.7 | 25.8 ± 3.9 | 28.3 ± 5.1 | 25.1 |
| PD duration (months) | 22.9 ± 20.3 | 27.6 ± 21.0 | 26.8 ± 21.2 | 10.5 |
| Solute transport (4 h D/P creatinine ratio) | 0.81 ± 0.13 | 0.78 ± 0.12 | 0.74 ± 0.17 | 0.7 |
| Albumin (g/L) | 29.1 ± 4.9[c] | 31.3 ± 3.3 | 33.2 ± 4.1 | 33 |
| CRP (mg/L) | 14.3 (4.8–21)[d] | 1.5 (0.6–4) | 4.6 (0.8–9.5) | 16.8 |
| Peritoneal Pcl (mL/day) | 95.6 ± 48.3 | 67.3 ± 21.7 | 69.0 ± 24.7 | 64.04 |
| Peritoneal protein loss (g/24 h) | 6.3 ± 3.2 | 4.9 ± 1.6 | 5.0 ± 1.8 | 4.28 |
| Urine protein loss (g/24 h) | 0.58 ± 0.5 | 0.88 ± 0.85 | 0.74 ± 0.51 | 1.12 |
| Total protein loss (g/24 h) | 7.11 ± 3.00 | 6.06 ± 2.06 | 6.02 ± 2.07 | 5.4 |
| Urine volume (mL) | 709 ± 631 | 1128 ± 626 | 1059 ± 759 | 997 |
| Adjusted PV (mL/m$^2$) | 1439 ± 300 | 1441 ± 173 | 1446 ± 197 | 2158 |
| $TBW_D$/Weight | 0.51 ± 0.04 | 0.53 ± 0.07[b] | 0.47 ± 0.06 | 0.52 |
| $TBW_{BIA}$/Weight | 0.45 ± 0.05 | 0.53 ± 0.08[d,e] | 0.45 ± 0.06 | 0.56 |
| $TBW_D$/Weight − $TBW_{BIA}$/Weight | 0.049 ± 0.041 | 0.006 ± 0.025[f] | 0.023 ± 0.055 | −0.046 |
| Ever smoked (%) | 44 | 22 | 23 | Yes |
| IHD (%) | 41 | 22 | 29 | No |
| Diabetes (%) | 17 | 22 | 50 | No |

[a]Adjusted PV, plasma volume adjusted by body surface area; BMI, body mass index; Pcl, protein clearance corrected for dialysis regime (modality, dry day and overfill); $TBW_D$/Weight, TBW normalized to body weight as determined from deuterium (D) dilution; $TBW_{BIA}$/Weight, TBW normalized to body weight as estimated by BIA. Clusters 1, 2 and 3 were compared using one-way analysis of variance (ANOVA) and when significant the *post hoc* between group differences are shown.
[b]Cluster 2 versus Cluster 3, $P < 0.05$.
[c]Cluster 1 versus Cluster 3, $P < 0.05$.
[d]Cluster 1 versus Cluster 2, $P < 0.01$.
[e]Cluster 2 versus Cluster 3, $P < 0.01$.
[f]Cluster 1 versus Cluster 2, $P < 0.05$ (ANOVA).

**Table 2.** Mean/median biomarker concentrations of the three patient clusters derived from HCA[a]

| | Cluster 1 ($n = 17$) | Cluster 2 ($N = 9$) | Cluster 3 ($n = 14$) | Single outlier | P-value (ANOVA) |
|---|---|---|---|---|---|
| ICAM-1 (μg/mL) | 0.49 ± 0.09 | 0.4 ± 0.07 | 0.34 ± 0.08 | 0.41 | <0.01 |
| VCAM-1 (μg/mL) | 0.83 ± 0.18 | 0.74 ± 0.16 | 0.72 ± 0.2 | 0.52 | NS |
| E-selectin (ng/mL) | 68.21 ± 31.07 | 44.16 ± 18.08 | 56.23 ± 27.51 | 26.13 | NS |
| P-selectin (ng/mL) | 94.63 ± 31.68 | 79.94 ± 27.23 | 126.52 ± 46.95 | 48.53 | <0.05 |
| MMP-1 (pg/mL) | 33.4 (15–104.8) | 10.1 (1–60.8) | 345 (35.9–821.8) | 1 | <0.05 |
| MMP-2 (μg/mL) | 0.24 (0.17–0.28) | 0.76 (0.34–1) | 0.29 (0.23–0.44) | 0.24 | 0.081 |
| MMP-3 (ng/mL) | 11.5 ± 5 | 22.76 ± 12.76 | 13.52 ± 4.49 | 9.04 | <0.01 |
| MMP-8 (ng/mL) | 3.69 ± 1.66 | 2.26 ± 0.8 | 2.04 ± 1.15 | 0.33 | <0.01 |
| MMP-9 (ng/mL) | 67.66 ± 38.23 | 55.54 ± 26.02 | 53.13 ± 30.4 | 30.7 | NS |
| IFN gamma (pg/mL) | 1.4 (0–4.6) | 0.5 (0–1.4) | 1.7 (1.1–2.3) | 2.1 | NS |
| IL-1β (pg/mL) | 0.1 (0.1–0.1) | 0.1 (0.1–0.1) | 0.4 (0.1–4.1) | 0.1 | <0.01 |
| IL-6 (pg/mL) | 5.4 (3–11.4) | 2.2 (1–4.2) | 3.5 (2.8–5.1) | 8.7 | <0.05 |
| IL-10 (pg/mL) | 2.7 ± 2 | 1.4 ± 1.9 | 4.5 ± 4 | 1.4 | <0.05 |
| MCP-1 (pg/mL) | 357.8 ± 156.9 | 377.5 ± 175.2 | 417.1 ± 131.2 | 61.7 | NS |
| CD40L (ng/mL) | 3.1 ± 2.3 | 4.16 ± 2.58 | 7.85 ± 2.61 | 1.16 | <0.01 |
| TNF-α (pg/mL) | 12.1 ± 4.9 | 6.4 ± 4 | 8.9 ± 3 | 19.5 | <0.01 |
| VEGF (pg/mL) | 3 (3–3) | 3 (3–3) | 50.9 (3–135.7) | 3 | <0.01 |

[a]ANOVA, analysis of variance; ICAM-1, inter-cellular adhesion molecule 1; NS, non significant.

$TER_{alb}$, univariate regression analysis was undertaken. None of the clinical measures, including demographics, membrane function, PD duration, CRP or systemic dynamic factors (pulse pressure, mean blood pressure), showed significant correlation to $TER_{alb}$ whereas the biomarker derived PC1 score (platelet activation) showed a positive relationship ($r = 0.33$ $P = 0.03$) (Table 4). Multivariate analysis found this relationship to be independent of age, gender and PD duration.

## Discussion

This is the first study to measure systemic endothelial barrier function in patients with chronic kidney disease (CKD) Stage 5 and relate this to a wide panel of circulating biomarkers of inflammation and endothelial dysfunction. It confirms that endothelial barrier function is abnormally decreased and suggests that this is not solely related to inflammation but more so to platelet activation in this

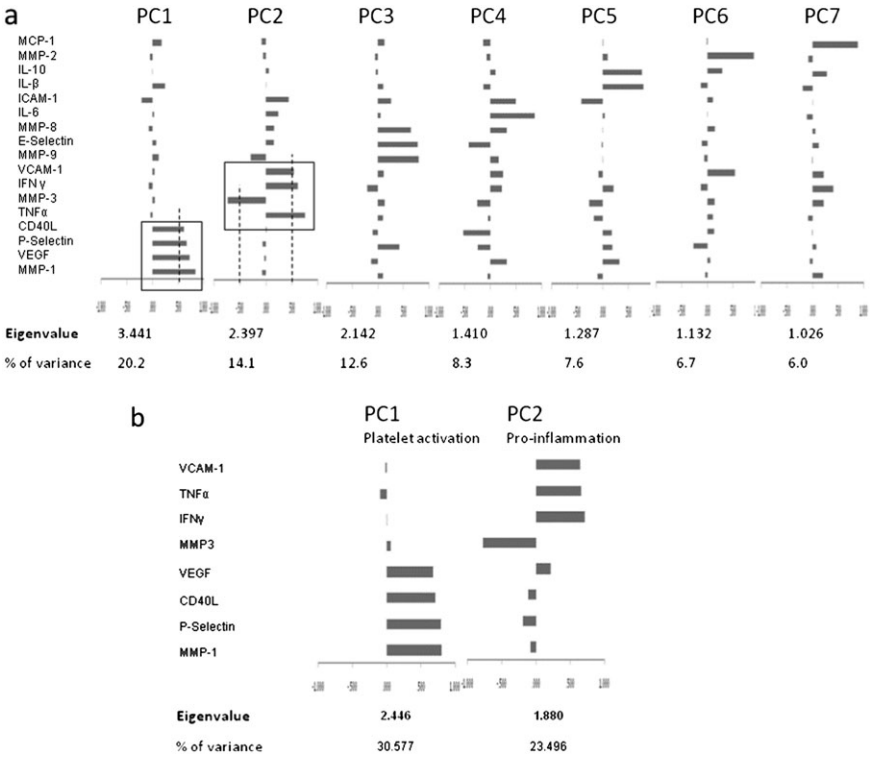



**Fig. 3.** (**a**) PCA from the whole panel of the 17 biomarkers eigen values and percentage contributing to the total sample variance of each of the seven PCs is presented. Individual composition feature is showed along with their rotated loading coefficients, a measure of the importance of each biomarker to the factor, displayed as a bar chart. (**b**) PCA from reduced number of biomarkers. The eight biomarkers that compose the two strongest PCs, contributing the majority of the total sample variance are selected for the final PCA. The composition of the 2 PCs derived from the final model is displayed along with their rotated loading coefficients expressed as a bar chart.

clinically stable cross-sectional cohort. Furthermore, patients with IHD and DM have different, albeit overlapping, patterns of biomarkers, although both exhibit endothelial dysfunction (Figure 4b).

The $TER_{alb}$ is a widely accepted measurement of endothelial barrier function, first described as early as 1973 [10, 11]. $TER_{alb}$ is increased in several conditions associated with endothelial dysfunction such as diabetes, hypertension and atherosclerosis [12–15] and also sepsis where it is clearly associated with marked inflammation. However, endothelial barrier function has been less well investigated in end-stage renal disease. This study found that mean $TER_{alb}$ is higher in CKD Stage 5 patients than in non-uraemic controls who likely had other pathologies as they were undergoing investigation for polycythaemia. A previous study of 29 non-diabetic CKD patients (glomerular filtration rate 11–44 mL/min/1.73 $m^2$) whose other markers of endothelial function (e.g. von Willebrand factor) were normal unless subjects were current smokers [16] showed no difference between CKD patients and normal controls. This suggests that severe renal failure is associated with more severe endothelial dysfunction and is in broad agreement with other rather small previously published studies in dialysis patients. $TER_{alb}$ was increased in 11 diabetics treated with PD (but not in non-diabetics) [17] and 9 predialysis haemodialysis patients [18]. This is supported by *in vivo* experiments in which human uraemic plasma increased frog mesenteric micro- vascular permeability to both water and protein, [19] subsequently confirmed in other studies. [20] One of the strengths of our study, which demonstrated a relatively high median $TER_{alb}$, was that we recruited sequential patients undergoing routine assessment of their therapy with <5% of patients declining investigation, so reducing selection bias.

Although a relationship between $TER_{alb}$ and blood pressure was seen in some early studies in hypertensive patients, we did not observe this to be the case in this cross-sectional study. The relationship may be more complex in this dialysis cohort with multiple co-morbidities and concomitant medication. A study comparing $TER_{alb}$ according to the angiotensin-converting enzyme (ACE) gene polymorphism in essential hypertension showed that ACE D/D homozygosis, which is associated with high risk of atherosclerotic vascular disease, is related to higher $TER_{alb}$ despite identical 24-h blood pressure readings [23]. Furthermore, endothelial function can be influenced by medications used in the patients with related effect on $TER_{alb}$. High-dose simvastatin reduces low-density lipoprotein cholesterol by 39% and is reported to normalize $TER_{alb}$ [24]. No clear relationship between medications and $TER_{alb}$ was observed in this study.

The measurement of a wide range of biomarkers combined with hierarchical cluster and PCA allowed us to investigate the overall pattern of their association with endothelial barrier dysfunction. Using this approach, we were able to identify three main patient clusters and there was good agreement between these and the two main

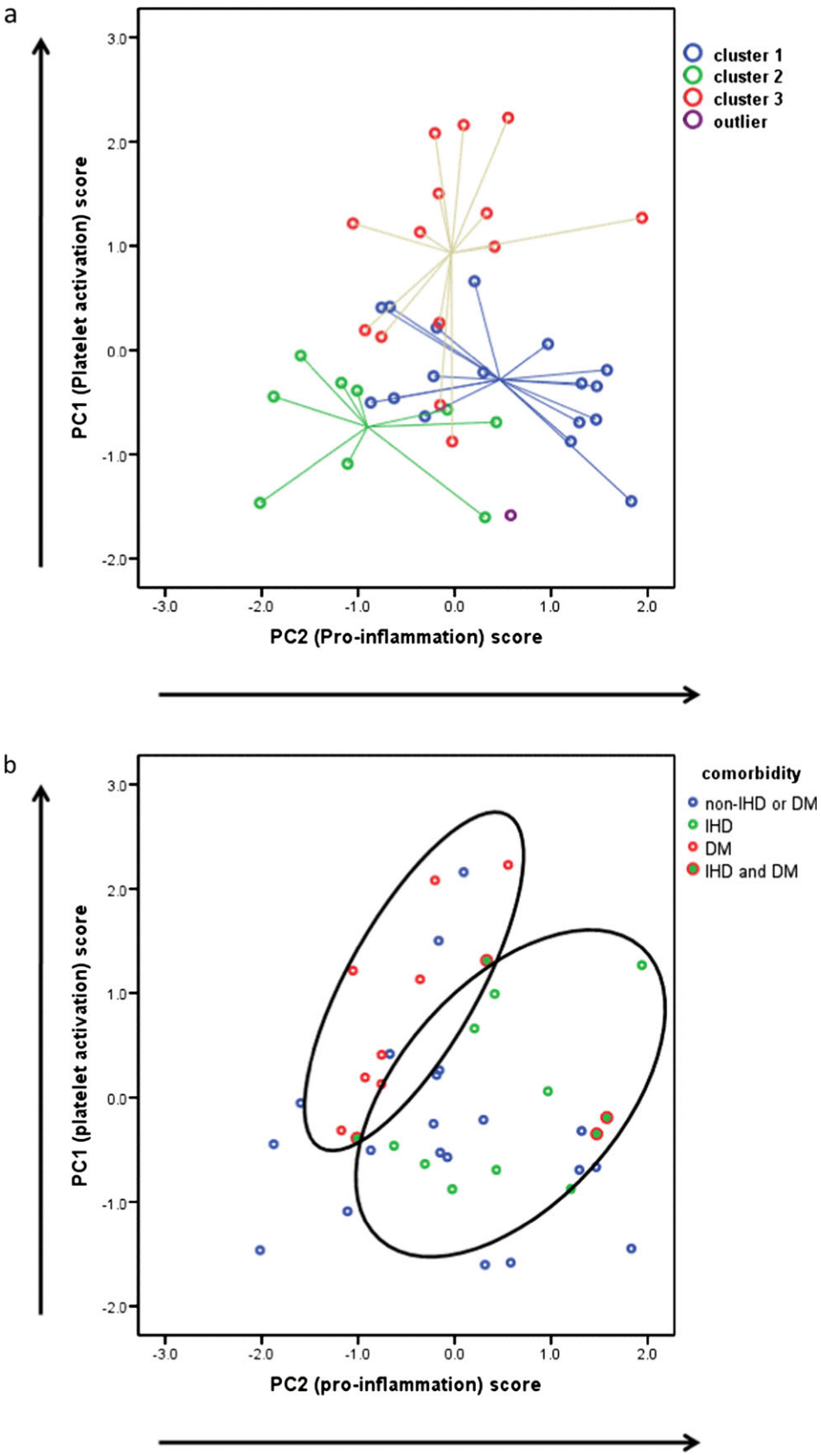



**Fig. 4.** (**a**) Combined graphical representation of the HCA (centroid plot) and PCA (dimensionless weighted scores); there is good agreement between the methods showing association between Cluster 3 and increased platelet activation, Cluster 1 with inflammation and Cluster 2 with the absence of markers of endothelial dysfunction. (**b**) Combined graphical representation of PC scores and co-morbidity; IHD and DM are clustered according to their different pattern of PC scores.

**Table 3.** PCs in different co-morbidity status[a]

| | IHD ($n = 13$) | Non-IHD ($n = 28$) | P-value[b] | DM ($n = 12$) | Non-DM ($n = 29$) | P-value[b] | PVD ($n = 12$) | Non-PVD ($n = 29$) | P-value[b] |
|---|---|---|---|---|---|---|---|---|---|
| PC1 | −0.01 ± 0.80 | 0.01 ± 1.09 | 0.951 | 0.62 ± 0.94 | −0.26 ± 0.92 | 0.009** | −0.01 ± 0.76 | 0.01 ± 1.10 | 0.96 |
| PC2 | 0.51 ± 0.89 | −0.23 ± 0.51 | 0.025* | −0.19 ± 0.97 | 0.08 ± 1.02 | 0.436 | −0.06 ± 0.85 | 0.02 ± 1.07 | 0.814 |

[a]PVD, Peripheral vascular disease.
[b]Unpaired *t*-test.
*P < 0.05.
**P < 0.01.

**Table 4.** Univariate correlation of $TER_{alb}$[a]

| | Correlation coefficient | P-value[b] |
|---|---|---|
| Age (years) | −0.21 | 0.19 |
| BMI (kg/m$^2$) | −0.09 | 0.57 |
| PD duration (months) | 0.26 | 0.11 |
| Pulse pressure (mmHg) | 0.10 | 0.54 |
| Albumin (g/L) | −0.09 | 0.56 |
| $Log_2$CRP(mg/L) | −0.04 | 0.81 |
| D/P creatinine | 0.12 | 0.46 |
| UF capacity (mL) | −0.03 | 0.88 |
| Peritoneal protein loss (g/24 h) | 0.23 | 0.16 |
| Peritoneal Pcl (mL/day) | 0.15 | 0.36 |
| Urine protein loss (g/24 h) | −0.16 | 0.34 |
| Total protein loss (g/24 h) | 0.20 | 0.23 |
| Urine volume (mL) | −0.22 | 0.17 |
| Gender | | 0.1 |
| Ever smoking | | 0.28 |
| IHD | | 0.39 |
| Diabetes | | 0.19 |
| PVD | | 0.71 |
| PC 1 | 0.33 | 0.03* |
| PC 2 | 0.07 | 0.69 |

[a]PVD, Peripheral vascular disease; UF, ultrafiltration.
[b]Pearson or Spearman correlation for continuous variables as appropriate according to the distribution and unpaired *t*-test for between group difference.
*P < 0.05.

components identified through PCA; additional PCs were identified that warrant further investigation in a larger validation cohort but were excluded from further analysis here to avoid Type 1 statistical error. The advantage to this approach is that while biomarkers are likely to correlate to each other as they all reflect endothelial function, it enables identification of possible multiple biomarker functions or different metabolic or signalling pathways involved in endothelial dysfunction. For example, CD40L is expressed in lymph cells, epithelial cells, fibroblasts, endothelial cells and platelets in response to pro-inflammatory cytokines, platelet activators and nitric oxide signalling. After combining with CD40, it subsequently up-regulates the pro-inflammatory and pro-atherogenic genes [25]. Although results from our PCA should be taken as exploratory, we find that PC1 (HC3 phenotype) is composed primarily of MMP-1, P-selectin, CD40L and VEGF, all of which are released in significant amounts from platelets and reflect platelet activation [26]. PC2, composed by IFN-γ, TNF-α and VCAM-1 and negatively contributed by MMP-3, is mainly involved in pro-inflammatory pathways and defined the inflamed, overhydrated and hypoalbuminaemic HC1 phenotype. HC2 was a relatively healthy phenotype with most normal body composition and least abnormal $TER_{alb}$. PC1 and PC2 could explain a significant proportion (34.3%) of the variance in the whole biomarker matrix.

One important finding of this study is the demonstration that the link between platelet activation and increased endothelial permeability may not be just through inflammation. The links between inflammations as a trigger for platelet activation in vascular lesions and between inflammation, atherosclerosis and endothelial dysfunction in uraemia are well established [27]. These pathways may, however, act either independently or sequentially; activated platelets can induce secretion of chemokines for monocyte recruitment [28] whereas single micro-vessel perfusion with TNF-α alone, without platelet activation, does not alter endothelial permeability [29]. In an aseptic animal injury model, systemic depletion of neutrophils with antibody failed to prevent the increase in vascular permeability, whereas anti-platelet pre-treatment reduced this by 25% [30]. These findings suggest that platelet activation may have a critical role in endothelial hyperpermeability that may not necessarily be through inflammation, especially in the low-grade inflammatory status seen in uraemia. Local release of VEGF by activated platelets, especially associated with HCA Cluster 3 and increased $TER_{alb}$ in this study, may explain this.

This study found that DM and IHD tend to cluster with different patterns of biomarkers associated with endothelial dysfunction and in keeping with other studies, we found that diabetics more commonly display an increase in platelet reactivity [31]. Worse cardiovascular outcomes in diabetics may reflect inadequate responses to anti-platelet therapy [32]. Another important finding of this study is that the systemic leak of albumin from the circulation to extravascular space is not correlated to hypoalbuminaemia and thus is unlikely to be causally related. It is well established that the single strongest predictor of plasma albumin concentration in PD patients is the daily peritoneal protein loss [33, 34], in turn strongly correlated to the rate of peritoneal small solute transport, an indicator of the effective vascular area in contact with dialysate. There is, however, residual variability in peritoneal protein losses which has now been shown in several studies to be an independent predictor of patient survival [6, 7]. This link between peritoneal protein leak and survival can at least in part be explained by an association with increasing age and cardiovascular co-morbidity, raising the possibility that some of the variability may reflect endothelial dysfunction.

We did not see any clear relationship between systemic protein leak and peritoneal protein losses in this study probably because the latter is dominated by effective peritoneal vascular area in contact with dialysate. It is also possible, given that this was a study of prevalent patients, that some had acquired membrane changes that could influence protein losses. The reason that plasma albumin is more strongly associated with peritoneal protein losses than with systemic leak is that the former represents net daily losses (typically 5–10 g/day) whereas the latter reflects the re-circulation between intra- and extravascular pools of up to 10 times this amount daily. Net plasma albumin concentration will also be determined by the balance of synthesis and catabolism, and the former in relatively fit PD patients has been shown to be increased above normal.[33] The patients with the lowest albumin in this study were those with inflammation compared to markers of platelet activation, in keeping with previous observations that hypoalbuminaemia in inflamed patients is associated with reduced synthesis.

This study has a number of limitations. Firstly, the number of patients would ideally be greater to enable a more extensive correlation of the PCA with clinical phenotype. Despite this, it remains the largest study of $TER_{alb}$ in dialysis patients to date and the numbers were limited by the ethical permission which was primarily obtained to measure plasma volume in an adequately powered study of fluid status and hypoalbuminaemia [8]. Secondly, the biomarker patterns identified, while good evidence that the relationship in more complex that originally envisaged can only be considered as hypothesis generating at this stage. Further validation in a larger patient cohort of selected biomarkers is planned. Thirdly, we did not have ethics permission to study normal subjects, so limiting our comparison to a non-uraemic control group under clinical investigation, which might be expected to have worse endothelial function than normal subjects [35]. As acknowledged above, the cross-sectional design means that there may be confounding of the data by factors known and unknown that change with time on treatment.

In conclusion, this study elaborates a more complex relationship between abnormal endothelial dysfunction and clinical phenotype in patients on PD, emphasizing the importance of platelet activation as well as inflammation (Figure 1b). In addition, it further clarifies our understanding of the mechanisms of hypoalbuminaemia, an important predictor of survival in patients on PD.

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