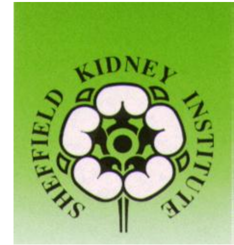

Characterisation of ECM protein processing mechanisms underlying simple peritoneal sclerosis and encapsulating peritoneal sclerosis

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Dedication

This thesis is dedicated

To

Mr Nichervan Barzani (Prime Minister of Kurdistan of Iraq), who supported me to undertake this PhD

My father, who encouraged me to complete my postgraduate studies (Master and PhD in UK), who sadly died during my first year of studying for a PhD

My mother for her continuous support

My brothers and sisters

My wife, who has stood by me continuously & created a supporting atmosphere

Thank you very much

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Publications out of this study

1) Abstracts- posters

1. Do changes in the Matrix Metalloproteinase (MMP) system underlie the development of Peritoneal Sclerosis (PS) or Encapsulating Peritoneal Sclerosis (EPS) during peritoneal dialysis (PD)? S Osta, M Lambie, N Topley, S Davies, A Summers, P Brenchley, L Huang, M Wilkie & TS Johnson. Sheffield University Medical School Research day, June 2011, **Sheffield**.
2. Do changes in the Matrix Metalloproteinase system underlie the development of Peritoneal Sclerosis or Encapsulating Peritoneal Sclerosis? S Osta, M Lambie, N Topley, S Davies, A Summers, P Brenchley, L Huang, M Wilkie & TS Johnson. Proceedings of The British Renal Society and Renal Association joint annual conference, **Brirmingham**, June 2011.
3. A pilot study of the impact of the residual peritoneal volume on the parameters from the peritoneal equilibrium test. D Abdellatif, Y Jackson, S Osta and M Wilkie. Proceedings of the 10th European Peritoneal Dialysis Meeting, **Birmingham**, October 2011.
4. Can changes in the Matrix metalloproteinase (MMP) system predict peritoneal membrane damage in peritoneal dialysis (PD)?. S Osta, M Wilkie & TS Johnson. Department of Infection & Immunity Research Day, **Sheffield**, January 2013.
5. Can Omentin (Intelectin-1), Dermatopontin, and collagen (alpha 1) I predict peritoneal membrane damage in peritoneal dialysis (PD)?. S Osta, M Wilkie & TS Johnson. BTS and RA Joint Congress, **Bournemouth**, March 2013.
6. Can changes in the Matrix metalloproteinase (MMP) system predict peritoneal membrane damage or EPS in peritoneal dialysis (PD)?. S Osta, N Topley, S Davies, M Lambie, P Brenchley, A Summers, M Wilkie & TS Johnson. BTS and RA Joint Congress, **Bournemouth**, March 2013.
7. Can omentin (Intelectin-1), Dermatopontin, and collagen (alpha1) I predict peritoneal membrane damage in peritoneal dialysis (PD)?. S Osta, M Wilkie & TS Johnson. World Congress of Nephrology, **Hong Kong**, June 2013.
8. Can changes in the Matrix metalloproteinase (MMP) system predict peritoneal membrane damage or Encapsulating Peritoneal Sclerosis (EPS) in peritoneal dialysis (PD)?. S Osta, N Topley, S Davies, M Lambie, P Brenchley, A Summers, M Wilkie & TS Johnson. World Congress of Nephrology, **Hong Kong**, June 2013.
9. Omentin (Intelectin-1), Dermatopontin, and collagen (alpha1) I relationships to risk factors of peritoneal membrane damage. Samir Osta Muhammad, Martin Wilkie, Timothy Johnson, Sheffield Kidney Institute, Nephrology, Sheffield, UK. ERA-EDTA 52nd Congress, London, May, 2015.
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2) Abstract - Oral presentations

1. Do changes in the Matrix Metalloproteinase (MMP) system underlie the development of Peritoneal Sclerosis (PS) or Encapsulating Peritoneal Sclerosis (EPS) during peritoneal dialysis (PD)? S Osta, D Abdellatif, M Lambie, N Topley, S Davies, A Summers, P Brenchley, L Huang, M Wilkie & TS Johnson. Proceedings of the 10th European Peritoneal Dialysis Meeting, **Birmingham**, October 2011.
2. Can changes in the Matrix metalloproteinase (MMP) system predict peritoneal membrane damage in peritoneal dialysis (PD)?. University of Sheffield Faculty of Medicine, oral Presentations, **Sheffield**, July 2012.

Prizes related to my PhD project

1. Two abstracts were presented by me during the World Congress of Nephrology, Hong Kong, June 2013. I also reported the scientific update from the congress to other nephrologists around the world via the Global Kidney Academy (GKA) website (Approximately 3,500 nephrologists worldwide are registered on GKA website). I got the GKA Ambassador Award for my poster presentations and reporting from the congress.
2. My abstract on the MMP system in peritoneal dialysis at ERA-EDTA 52nd Congress, London, May, 2015 was chosen as one of the **best abstracts presented by the young authors** and I received the prize with certification during the congress.

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Abstract

Introduction and hypothesis: Peritoneal dialysis (PD) is an important option for renal replacement therapy. Peritoneal sclerosis (PS) limits PD duration due to loss of ultrafiltration (UF) capacity, while about 3% of PD patients experience a condition termed encapsulating peritoneal sclerosis (EPS). In many fibrotic diseases reduced Extracellular matrix (ECM) breakdown due to lowered matrix metalloproteinase (MMP) activity occurs, often from over-expression of tissue inhibitors of MMP (TIMPs) that underlie fibrotic remodeling. Furthermore, recent application of 2D gel proteomics on peritoneal dialysis effluent (PDE) samples has identified several proteins that are elevated in patients with membrane damage. These observations have led to the hypothesis that: changes in proteins in PDE samples, in particular those associated with ECM breakdown have value as non-invasive biomarkers of PS and the switch to EPS. To test this hypothesis, PDE samples from 3 patient cohorts was analysed for ECM proteolytic activity. A range of ECM processing proteins and 3 proteins identified from previous proteomic studies of patients developing EPS (intellectin-1, dermatopontin and collagen α1 (I)) were analysed in PDE samples.

Methods: Three patient cohorts were studied: two were from Sheffield Kidney Institute (SKI) that consisted of 32 spot PDE samples (SKI-1) that included 1 EPS patient & 51 PDE & plasma samples collected during a peritoneal equilibrium test (PET) with multiple dwell times in patients who did not have EPS (SKI-2). The third cohort consisted of 209 samples from the Global Fluid Study (GFS) including sequential samples from 12 EPS & 42 matched controls patients. MMP activity was assessed using the ENZchek assay system. Plasmin activity was assessed by using cleavage of the V0882 substrate. TIMPs, MMPs, intelectin-1, dermatopontin, and collagen (α1) I were quantified by commercial ELISA in PDE and plasma samples. PDE cytology (macrophages, leukocytes, fibroblasts and mesothelial cells) was performed to determine if changes in any protein could be associated with changes in cell types. Clinical data were recovered from either the peritoneal dialysis database (PDDB) at Sheffield or the GFS archives. The analysis was performed using Microsoft Excel 2010 software, SPSS, and Graphpad prism (prism 5.01 for windows).

Results: Plasmin activity in PDE samples decreases with long duration of PD therapy. Minimal MMP activity was found in all PDE samples. In the SKI-1 cohort, MMP-1, -9, & -13 were almost undetectable with only MMP-2 & -3 being measurable with levels of ((mean±SD) 46±37 & 2.1±2.2 ng/mL respectively). In contrast TIMP-1 and TIMP-2 and to lesser extent TIMP-3 had significant levels in PDE samples from commencing PD (109±88, 17±12, and 0.28±0.33 ng/mL respectively). All TIMPs & MMP-2 were raised in the single patient who had a diagnosis of EPS. In samples from the GFS cohort, there was a rapid 6 fold increases in TIMP-1 within 100 days of the diagnosis of EPS, which when normalised to TIMP-2 levels was a good predictor of EPS. Calculation of the plasma to dialysate transfer rate by reference to that of circulating proteins with no peritoneal production and of known molecular weight (albumin, beta2microglobulin (B2M), transferrin, IgG, and creatinine) demonstrated that TIMPs & MMPs (especially TIMP-1 and MMP-2) have significant peritoneal production. Plasma levels for TIMP-1,-2, MMP-2,-3, and intelectin-1 (mean±SD) were 121±27, 85±16, 176±35, 11±5, and 374±136 ng/mL in healthy individuals respectively. Plasma levels in PD patients for TIMP-1,-2, MMP-2,-3, and intelectin-1 (mean±SD) were 297±78, 158±33, 309±112, 42±28, and 749 ±722 ng/mL respectively. None of the proteins identified by proteomics as predictors of EPS were able to be validated by ELISA. However TIMP-1,-2, MMP-2, intelectin-1, and collagen (α1) I in PDE samples had significant correlations with the loss of ultrafiltration and thus membrane damage. PDE cytology showed that peritoneal fibroblast and leukocyte numbers increase with time on PD, while peritoneal macrophage decreases with time on PD. There were no significant changes in mesothelial cells.

Conclusions: Negligible MMP activity in PDE samples results from high TIMP levels which could underlie the development of PS. The rapid increase in TIMP-1 within 100 days of EPS development offers value as a diagnostic tool or a late biomarker. Plasma levels of TIMP-1,2, MMP-2,3, and intelectin-1 are higher in patients on PD compare to healthy individuals. The increase in peritoneal fibroblasts may be a source of TIMP-1.

List of Abbreviations

A	Angstrom
ACEI	Angiotensin converting enzyme inhibitor
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AGE	Advanced glycation end products
APD	Automated peritoneal dialysis
APMA	Aminophenylmercuric acetate
ARB	Angiotensin receptor blocker
B2M	Beta 2 microglobulin
BGG	Bovine gamma globulin
BMI	Body mass index
BP	Blood pressure
B R S	British renal society
BSA	Bovine serum albumin
CA 125	Cancer antigen 125
CAPD	Continuous ambulatory peritoneal dialysis
CCPD	Continuous cyclic peritoneal dialysis
CG	Chlorhexidine gluconate
CKD	Chronic kidney disease
CMMC	Central Manchester and Manchester children's university hospital
COL1A1	Collagen type one alpha one
COL1A2	Collagen type one alpha two
CrCl	Creatinine Clearance
CT	Computerised tomography
D/D0 glucose	Ratio of dialysate glucose at 4 hours dwell time to dialysis glucose at 0 dwell time
D/P	Dialysate to plasma ratio
D/Pcr	Dialysate-to-plasma ratio of creatinine
Da	Dalton
DAB	Diaminobenzidine
DPT	Dermatopontin
ECM	Extracellular matrix
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transformation
EPS	Encapsulating peritoneal sclerosis
ESRF	End-stage renal failure
FGF	Fibroblast growth factor

Fl	Fluorescence
FSP-1	Fibroblast specific protein one
g	Gram
GAG	Glycosaminoglycan
GalNAc	N-acetylgalactosamine
GBM	Glomerular basement membrane
GDP	Glucose degradation product
GFS	Global fluid study
GlcNAc	N-acetylglucosamine
GN	Glomerulonephritis
H&E stain	Hematoxylin and eosin stain
HA	Hyaluronic acid
HBME-1	Hector battifora mesothelial epitope one
HD	Hemodialysis
HDL	High-density lipoprotein
HK-2	Human kidney proximal tubular epithelial cells
HPMC	Human peritoneal mesothelial cells
hr	Hour
HSP27	Heat shock protein 27
HSP47	Heat shock protein 47
IGAN	IgA nephropathy
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IPD	Intermittent peritoneal dialysis
IPP	Intra peritoneal pressure
IQR	Interquartile range
ISPD	International society for peritoneal dialysis
ITRAQ	Isobaric tags for relative and absolute quantification
Kg	Kilogram
Log	Logarithm
LOXL2	Lysyl oxidase-like 2
m	Metre
MAP	Mean atrial pressure
MC	Mesothelial cell
mg	Milligram
min	Minute
mL	Millilitre
MMP	Matrix metalloproteinase
MMT	Mesothelial to mesenchymal transition

MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MT-MMP	Membrane-type matrix metalloproteinase
MW	Molecular weight
NIPD	Nocturnal intermittent peritoneal dialysis
nm	Nanometre
NRK-49F	Normal rat kidney fibroblasts
OCPD	Optimized continuous peritoneal dialysis
OD	Optical density
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCKD	Polycystic kidney disease
PD	Peritoneal dialysis
PDDB	Peritoneal dialysis database
PDE	Peritoneal dialysis effluent
PDGF	Platelet-derived growth factor
PDOPPS	Peritoneal Dialysis Outcomes and Practice Patterns Study
Peritoneal Kt/v	K - peritoneal clearance of urea, t - dialysis time, and V - volume of distribution of urea
PET	Peritoneal equilibrium test
pg	Picogram
PIS	Participant information sheet
pNA	p-Nitroaniline
pNPP	p-nitrophenyl-phosphate
PP	Pulse pressure
PS	Simple peritoneal sclerosis
QA	Quenching agent
RCO	Reactive carbonyl compound
RRF	Residual renal function
RV	Residual volume
S	Serine
SD	Standard deviation
SE	Standard error
SKI-1 cohort	Sheffield kidney institute PD cohort 1
SKI-2 cohort	Sheffield kidney institute PD cohort 2
SLE	Systemic lupus erythematosus
SMA	Smooth muscle actin
SPSS	Statistical package for the social sciences
T	Threonine
TG	Triglyceride

TG2	Transglutaminase type 2
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TNP-470	An angiogenesis inhibitor
tPA	Tissue-type plasminogen activator
TPD	Tidal peritoneal dialysis
UF	Ultrafiltration
UFF	Ultrafiltration failure
UO	Urine output
uPA	Urokinase type plasminogen activator
Val-Leu-Lys	N-L-valine-L-leucine-L-lycine
VEGF	Vascular endothelial growth factor
µm	Micrometre
µg	Microgram
µL	Microlitre
2D	Two dimensional

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CHAPTER ONE

General introduction

1.1 Peritoneal dialysis (PD)

PD was originally described in the 1970s as an option for renal replacement therapy in patients with end-stage renal failure (ESRF) (Barratt 2009). Solute and fluid exchange occur between dialysate placed in the peritoneal cavity and peritoneal capillary blood across the peritoneal membrane. In order to perform a peritoneal dialysis exchange, a dialysis solution of approximately 2 litres is drained by the gravity into the peritoneal cavity via a surgically placed silastic catheter allowing dialysis. The fluid is left to dwell for a variable period (usually 4 – 6 hours) in the peritoneal cavity while dialysis occurs by means of diffusion and convection across the peritoneal membrane (Levy, Morgan et al. 2004). At the end of the dwell, the spent effluent is drained out using the catheter.

1.1.1 Types of peritoneal dialysis

There are two main types of peritoneal dialysis.

1.1.1.1 Continuous ambulatory peritoneal dialysis (CAPD)

In this form of dialysis the patient will be trained to do three to five exchanges/day. There are three phases during each exchange. The infusion (or fill) phase for 10 minutes, followed by the dwell phase (when the dialysis fluid remains inside the peritoneal cavity). The dwell time is dependent on the peritoneal dialysis technique (usually 6 to 8 hours). Finally the drain phase is where the dialysis fluid is removed under gravity (20-30 minutes). Each cycle of draining and refilling is named as an exchange. Dwell time is the time that the solution remains in the peritoneal cavity between exchanges.

The equipment required to perform CAPD consists of:

- dialysate fluid bag
- a drain bag for collecting waste products
- connecting tubes and clips. These are used for connecting both bags to the catheter
- a wheeled stand for hanging both bags.

1.1.1.2 Automated Peritoneal Dialysis (APD)

In this form of dialysis the patient uses an automatic cycling device. The aim is to perform multiple (6-7) exchanges during the night time. In APD, patients usually have one or two exchanges manually in the day time when they are off the machine (this is known as a “wet-day” i.e. dialysate in the *peritoneum* in the day time).

1.1.2 Different modes of peritoneal dialysis

- Nocturnal intermittent peritoneal dialysis (NIPD) is a high-volume dialysis for short-duration that takes place for 8-12 hours/day (Brophy, Sowinski et al. 1999). The exchanges are overnight only with no exchange during day time (dry peritoneum during the day).
- Continuous cyclic peritoneal dialysis (CCPD) in this mode of dialysis there are exchanges during night time with one exchange during day time. The day exchange will be at various times.
- Optimized continuous peritoneal dialysis (OCPD) is a combination of APD with extra exchanges (classically day time). OCPD is ideal if the patient needs maximal solute transfer e.g. in anuric patients
- Tidal Peritoneal Dialysis (TPD) is a manipulation of the prescription in which a significant percent of the dwell is left remaining in the peritoneal cavity, for example 30% to suit the individual patient needs. TPD is used if there are difficulties with catheter flow since it avoids drainage alarms and is useful to

prevent catheter tip pain that can occur when the peritoneal cavity is empty.

- Intermittent Peritoneal Dialysis (IPD) is an old modality of peritoneal dialysis and can be used to treat acute kidney injury in hospitalized patients. It can be used as a chronic treatment where the patient does a period of dialysis in part of the week then has a few days off.

1.1.3 Advantages of Peritoneal dialysis

There are several advantages of PD in comparison to the alternative dialysis modality of hemodialysis (HD). These include relative patient independence since PD is performed at home. It does not require anti-coagulation therapy, avoids vascular access and related complications. There is some suggestion that PD may be preferable for patients who have cardiovascular instability, although not all studies demonstrate better cardiac outcomes for patients on PD in comparison with HD (Sens, Schott-Pethelaz et al. 2011). Patients are free to move around while they are in the dwell phase. In most health care systems PD is less expensive than the alternative, HD. Several studies have indicated better preservation of residual renal function (RRF) for patients on PD than on HD (Tam 2009). RRF is of vital significance in patients with ESRF such as better control in blood pressure, and reduction in left ventricular hypertrophy and is an important indicator of patient survival. Sodium removal will be increased and fluid status will be improved (Marron, Remon et al. 2008). PD patients have less fluid restriction than hemodialysis patients.

1.1.4 Disadvantages of Peritoneal dialysis

Peritoneal dialysis also has disadvantages, these include the requirement to perform PD daily, while HD is usually performed three days a week. PD patients need enough space at home to store their dialysis supplies. The time taken to perform the dialysis exchanges can impact patient lifestyle. Nutritional complications can result from protein loss across the peritoneal membrane

(Blumenkrantz, Gahl et al. 1981).Reduced appetite, dyslipidaemia, and weight gain due to the absorption of glucose from the dialysate are also issues. Increased intra-abdominal pressure from dialysis fluid can result in hernias, fluid leaks, chronic back pain, rectal and/or vaginal prolapses and loss of appetite (Levy, Morgan et al. 2004).

Peritoneal Dialysis Outcomes and Practice Patterns Study (PDOPPS) was established for better understanding in causes of peritoneal dialysis technique failure. PDOPPS and ISPD (International Society for Peritoneal Dialysis) identified 7 primary causes for PD technique failure with a hope that this will help in standardizing international registry data reporting (Table 1.1) (Lambie M, Davies S et al. 2012, Perl, Davies et al. 2015). The most frequent and important complication of PD is infection. This can occur at several sites such as the exit-site for the peritoneal dialysis catheter (most common causative agents are Staph. aureus and Pseudomonas spp.), catheter tunnel infection, and peritonitis (this could be bacterial, fungal, tuberculous or water-borne atypical mycobacterial peritonitis). Peritoneal membrane change is another important complication of PD that may lead to decline in ultrafiltration (UF). Encapsulating Peritoneal Sclerosis (EPS) is a rare but potentially fatal complication for PD. The other potential PD complications include inflow pain and eosinophilic peritonitis (Levy, Morgan et al. 2004).

Primary cause	
Infection-related	Peritonitis
	Exit-site infection
Catheter-related problems	Catheter displacement
	Catheter blockage
Peritoneal leaks/hernia	
Problems with solute/ water clearance	Fluid/UF-related
	Solute-related
Risk or diagnosis of EPS	
Psychosocial /medical	
Other	For example, intra-abdominal pathology, and hemoperitoneum

Table 1.1 Standardizing Causes of Peritoneal Dialysis Technique Failure

The primary causes for technique failure modified from Perl, J et al 2015 (Perl, Davies et al. 2015).

1.2 Anatomy and physiology of the peritoneal membrane

The space between abdominal wall and the abdominal viscera is called the peritoneal cavity. The peritoneal membrane lines the peritoneal cavity. It consists of parietal and visceral peritoneal membranes. The parietal membrane lines the abdominal wall, whereas the visceral layer covers the intra-abdominal viscera. The peritoneal membrane is lined by mesothelial cells (MCs). Mesothelial cells are supported by submesothelial connective tissue containing lymphatics and peritoneal capillaries. The peritoneal vasculature has a central role in PD. The peritoneal capillary acts as a barrier to fluid and solute removal between dialysis fluid and the blood. The peritoneal membrane is semipermeable. PD uses this membrane to filter patient's blood. In addition, peritoneal lymphatics have a role in fluid removal from the peritoneal cavity (Barratt 2009).

The movement of solute and water movement between dialysis fluid and the patient's blood across the peritoneal membrane is generally descried using a three-pore theory. There are three different sizes of pores (Figure 1.1) (Saxena and West 2006), which are large (>200 angstrom (A)), small (40-60 A), and ultra-small pores (3-5 A). Large pores are located in inter-endothelial cell clefts and are relatively few in number. They allow macromolecules such as protein to move from the patient's blood to the dialysate. Small pores make up the majority of the overall pore area. They are located in the gaps between endothelial cells. They allow diffusion of small solutes between dialysate in the peritoneal space and blood including the absorption of glucose from dialysate and the removal of urea and creatinine from blood to the dialysate. Small pores also play a role in fluid removal by ultrafiltration. Finally, ultra-small pores are transcellular aquaporin water channels that allow passage of water only, but impermeable to solutes. They are responsible for 50% of water removal during a glucose based dialysate exchange (Barratt 2009).

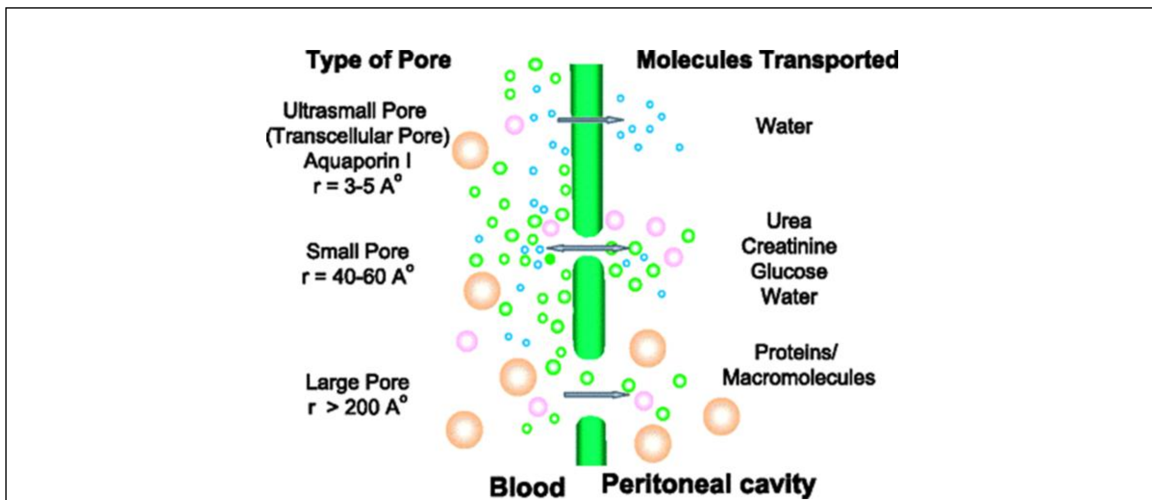


Figure 1.1 <u>The three-pore model of peritoneal transport</u>

Solute and water transport through the three pores (Ultra-small or transcellular pores, Small pores, large pores). It is reproduced by permission of the American Board of Family Medicine (Saxena and West 2006)

Aquaporin-1 (28 kDa) protein is a water-specific membrane channel which is impermeable to urea and glycerol (Zeidel, Nielsen et al. 1994, Agre 2004). It is found in the endothelial lining of the peritoneal capillaries and post capillary venules (Devuyst, Nielsen et al. 1998, Devuyst and Yool 2010). Aquaporin-1's role in peritoneal water permeability was first recognised in studies using Mercury chloride in a rat model (Carlsson, Nielsen et al. 1996). Aquaporin-1 knockout mice also demonstrated that osmotically driven water transport was significantly decreased across the peritoneal membrane compared to the wild-type littermates (Yang, Folkesson et al. 1999). Further studies using a standard peritoneal exchange test in mice (Ni, Cnops et al. 2005), revealed that aquaporin-1 knockout mice had an approximately 50% reduction in UF during peritoneal dialysis with a hypertonic dialysate as same as the three pore model prediction (Ni, Verbavatz et al. 2006).

1.3 Peritoneal membrane permeability

The un-physiological nature of the dialysate results in time dependent changes to the membrane such as the development of mesothelial intracellular oedema, oedema of the interstitium, destruction of organelles, collagen fiber deposition in the submesothelial region and a reduction in the number of microvilli (Levy, Morgan et al. 2004). Episodes of peritonitis may lead to destruction of the microvilli on the mesothelium and the formation of layers of fibrin on the peritoneal surface. After a single episode of peritonitis the membrane can recover to a large extent, however if there are repeated episodes, the changes are likely to become irreversible and the damage will alter membrane permeability (Levy, Morgan et al. 2004). Several studies suggest an impact of peritoneal membrane function on patient survival (Ates, Nergizoglu et al. 2001, Brown, Davies et al. 2003, Jansen, Termorshuizen et al. 2005). Therefore, it is important to identify the causative factors that damage the peritoneal membrane and to design strategies to preserve peritoneal membrane function (Davies, Phillips et al. 1998). There is increasing evidence that with time

on PD, there will be an increase in peritoneal solute transport rate (Struijk, Krediet et al. 1991, Selgas, Fernandez-Reyes et al. 1994, Struijk, Krediet et al. 1994, Davies, Bryan et al. 1996, Davies, Phillips et al. 1998). High solute transport increases the risk of mortality, which is independent of age, residual renal function and co-morbidity of the patient (Davies 2006). UF capacity and the small solute transport rate are linked and they are affecting the clinical outcome. However, patients with severe UF failure, have a combination of high solute transport and reduced osmotic conductance of the membrane – a measure of the effectiveness by which UF is induced by glucose (Heimburger, Waniewski et al. 1990, Davies 2004, Smit, Schouten et al. 2004, Parikova, Smit et al. 2005, Waniewski, Sobiecka et al. 2005). Loss of osmotic conductance is due to membrane and interstitial changes.

1.3.1 High peritoneal solute transport

High solute transport is defined as an increase in the ratio of creatinine in the dialysate to plasma after a dwell time of 4 hr (D/Pcr4) during PET (Chang, Park et al. 2010). PD duration is frequently limited by failure of the peritoneal membrane, which is associated with increase in solute transport, UF dysfunction with degenerative changes of the peritoneal membrane such as loss of mesothelial cells, sub-mesothelial ECM accumulation and vasculopathy (Williams, Craig et al. 2002, Fraser and Topley 2009).

Early studies of high peritoneal solute transport revealed it was associated with increased hospital admissions (Heaf 1995), increased technique failure (Wu, Huang et al. 1996) and increased peritoneal protein and creatinine clearance (Nolph, Moore et al. 1993, Heaf 1995). One of the largest studies published from the Australia and New Zealand dialysis and transplant (ANZDATA) registry has confirmed an increase in mortality and technique failure among those patients on PD having high transport rates (Rumpsfeld, McDonald et al. 2006).

Managing patients with high transport status requires two strategies. First, using appropriate dwell times with the aim of draining the peritoneal cavity at optimal UF volume and before fluid starts to be reabsorbed across the peritoneal membrane. Second, the use of icodextrin to prevent fluid reabsorption. These two points can be achieved by allowing shorter exchanges to occur overnight by APD and using icodextrin. Icodextrin provides slow sustained UF, avoiding glucose absorption and consequently fluid reabsorption (Davies 2006). Icodextrin in diabetic patients showed improvement in PD technical survive (Takatori, Akagi et al. 2011).

A case series was published in 2001 (Sheffield) following 6 PD patients with EPS. All of them had UF inadequacy, and five of them were treated with icodextrin for variable durations (Jenkins, Leng et al. 2001). The icodextrin role in these cases is not clear. However, icodextrin use is associated with an extension in technique survival (Wilkie, Plant et al. 1997), as well as use in PD patients with UF inadequacy and high transport status (Paniagua, Ventura et al. 2009) Both of these are defined EPS risk factors (Sampimon, Coester et al. 2011). In general, PD patients with EPS are on PD for long periods and nowadays it is likely that for this reason they had been treated with icodextrin (Wilkie 2011).

1.3.2 Ultrafiltration (UF)

Ultrafiltration is equal to the volume of fluid that is drained from the peritoneal cavity minus the volume of fluid that was infused to the peritoneal cavity. UF is obtained after a standard dwell time during PD. It is varies according to the glucose concentration of the dialysate. Ultrafiltration failure (UFF) diagnosis is made if the net UF was less than 100 mL after a dwell time of 4 hours with using a dialysate of 2.5% glucose, or less than 400 mL after a dwell time of 4 hours with using a dialysate of 4.25% glucose, in the absence of fluid leaks, catheter malfunction or extensive intraperitoneal adhesions (Twardowski,

Nolph et al. 1987, Davies, Brown et al. 1993, Krediet, Imholz et al. 1993, Ho-dac-Pannekeet, Atasever et al. 1997). The formula that describes the forces involved in ultrafiltration is as following:

Transcapillary UF = Co-efficient of UF x hydrostatic pressure + oncotic pressure + osmotic pressure

In general, the Co-efficient of UF defines as the ability of the dialyzer to transfer water across the membrane at a certain transmembrane pressure (Kher, Schnaper et al. 2006). In peritoneal dialysis, Co-efficient of UF is the amount of water across the peritoneal membrane in respond to the osmotic gradient by the dialysis solution, while other forces in the system are negligible.

There is individual variability in UF, and it can be positive or negative UF (negative UF, means retained fluid from dialysate to vascular compartment). It is mainly governed by the osmotic pressure gradient. Ultrafiltration failure is an important cause of long-term failure of PD. Ultrafiltration failure can be defined either clinically or by changes in peritoneal membrane function. Clinically, UF failure is the inability to maintain dry weight despite the requirement for excessive amounts of hypertonic glucose solutions and this would also requires a fluid restriction for the patient. UF failure can be predicted by the PET or any equivalent test, and is commonly associated with high solute transport (Davies, Brown et al. 1993, Krediet, Ho-Dac-Pannekeet et al. 1996, Ho-dac-Pannekeet, Atasever et al. 1997). The chance of getting ultrafiltration failure increases for those who have been on long term PD, and UF failure leading to technique failure (Heimburger, Waniewski et al. 1990).

It is important to overcome low ultrafiltration capacity of the peritoneal membrane by preventing long-term membrane damage. For example, by reducing the exposure of the membrane to the hypertonic glucose. The exact mechanism is unknown, but it could be due to glucose degradation product injury

in addition to hypertonic glucose itself (Wieslander 1996). There is evidence that using icodextrin and APD in anuric patients over two years preserves UF better (Davies, Brown et al. 2005). There is no long-term data to confirm that newer biocompatible solutions prevent long-term membrane damage.

There are 3 types of the Ultrafiltration failure (UFF). Type I membrane failure and this is accompanied by the rapid solute transport. Type II membrane failure and this is accompanied by the impaired solute transport. Finally, Type III membrane failure is associated with excessive lymphatic absorption.

Type I membrane failure is the commonest cause of UFF in comparison to the other 2 types. One of the proposed mechanisms is where aquaporin mediated water transport is impaired (Monquil et al.1995). UFF prevalence increases with longer time on PD. This is may be due to increase in glucose derived substances and reactive carbonyl compounds (RCOs) (Buemi et al. 2004). Type I UFF may also arise during peritonitis, but the UF losses during peritonitis is usually reversible. There is an increase in the effective peritoneal surface area in Type I UFF through increase in vascular permeability or vascular neoproliferation.

Type II membrane failure is comparatively rare and mainly seen in patients who are suffering from peritoneal sclerosis. Solute transport is frequently intact, but there is reduction in peritoneal surface area which is due to fibrosis or extensive adhesions. Type II UFF can cause reduction in the overall solute and fluid removal. Finally, Type III membrane failure is associated with increase in dialysate resorption from the peritoneal cavity which is because increase in lymphatic flow. Mechanical problems such as catheter dysfunction and leaks are among the differential diagnosis of normal membrane function and low UF.

1.4 Adequacy of Peritoneal dialysis

The dose of peritoneal dialysis is calculated by two methods: weekly creatinine clearance and weekly urea clearance or Kt/V urea (NKF-DOQI 1997) The updated Renal Association Clinical Practice Guidelines has suggested that a combined peritoneal and urinary Kt/V urea of >1.7/week or a creatinine clearance of >50L/week/1.73m2 should be regarded as minimal treatment doses. The treatment dose should be raised in patients experiencing uraemic symptoms. Methods of calculations and units for Kt/V urea and creatinine clearance are provided in appendix 1.

1.5 Simple peritoneal sclerosis

There are several changes of the peritoneum that happen during the course of PD treatment. These changes are mesothelial layer denudation, progressive fibrosis of peritoneal vasculature as well as media hyalinization due to collagen IV deposition (vasculopathy). In addition to the development of new vessels (neoangiogenesis), there is an increase in thickness of the submesothelial compact zone due to interstitial fibrosis which consists of different types of collagen such as collagen IV as well as myofibroblasts. Furthermore, accumulation of advanced glycation end products (AGEs) in the mesothelial and submesothelial layers as well as vascular wall after a long time on PD. AGE deposition is linked with the peritoneal fibrosis in previous studies (Nakayama, Kawaguchi et al. 1997, Honda, Nitta et al. 1999). Peritoneal morphological changes may impact its function such as increased peritoneal permeability and decrease in UF (Korte, Sampimon et al. 2011)

Simple peritoneal sclerosis develops with time on peritoneal dialysis. It is common and characterised by increase in peritoneal membrane thickness. Uraemic patients already have an increase in peritoneal membrane thickness prior to dialysis (Williams, Craig et al. 2003). There are changes in morphology

of the parietal peritoneal membranes. In normal subjects, the median thickness is 50μm for the submesothelial compact collagenous zone. In PD patients, the median thickness is 270μm. There is an increase in compact zone thickness with the time of PD therapy from 180μm in first 2 years on PD to 700μm for patients on PD for more than 8 years (Williams, Craig et al. 2002). There is an absence of encapsulation in simple peritoneal sclerosis (Garosi 2009)

1.5.1 Pathology and pathogenesis of simple peritoneal sclerosis

Simple peritoneal sclerosis is associated with a decrease in arteriolar and venular lumen/vessel diameter ratio (Augustine, Brown et al. 2009). Triggering factors for PS include dialysate (which contains glucose and glucose degradation products), bacteria, peritoneal catheters, plastic particles and plasticizers (Ronco 2005). At a cellular level, dialysis-related peritoneal sclerosis is associated with numerous changes in the immunobiology of the peritoneum such as leukocyte attraction, production of inflammatory mediators such as interleukins and chemotactic factors, as well as macrophage activation. Interleukin-1 (IL-1) subsequently activates endothelial cells increasing vascular permeability and causing vasodilation. IL-1 also instigates fibroblast proliferation. Lymphocytes show irregular chemical mediator production and changes in surface antigens. All these biochemical changes influence fibroblast and stimulate fibrosis (Ronco 2005). In animal models, after 2 months of peritoneal dialysis in rabbits, it is possible to observe the typical submesothelial and mesothelial modification of simple peritoneal sclerosis, but it is not possible to recognize the factors activating the transformation from PS to EPS in many cases (Ronco 2005).

1.6 Encapsulating Peritoneal Sclerosis (EPS)

EPS is a rare but serious complication of PD. The mortality rate of EPS is 60-93% (Spence, Gillespie et al. 2013). EPS was described in 1980 for the first time (Gandhi, Humayun et al. 1980). In EPS, fibrous tissues encapsulate the

viscera leading to decreased intestinal motility, intestinal malfunctions and sometimes ascites. A prospective study performed in Japan showed an increase in the incidence and mortality rates with time on PD. The incidence rates were 0% at 3 years, 5.8% at 10 years, and 17.2% over 15 years on PD (Kawanishi and Moriishi 2005).

1.6.1 Pathology and pathogenesis of EPS

The initiating event in EPS is unknown; however a 'two-hit' hypothesis has been suggested. First hit is long term exposure to dialysate fluid which will lead to changes in peritoneal/mesothelial physiology. The second hit could be peritonitis, surgical procedures, genetic tendency or discontinuation of PD. In EPS there is denudation of the mesothelium, capillary angiogenesis, fibrosis of the interstitium and vascular sclerosis There is deposition of fibrin, mainly disturbing the visceral membrane (Honda and Oda 2005). The Pathobiology of EPS is unclear. One theory connects plasma exudation and deposition of fibrin with the formation of adhesions and fibrosis (Figure 1.2). Inflammation of the peritoneal membrane will lead to fibrinous exudation, which may lead to adherence of membrane surfaces. This adhesion either be re-absorbed or will be invaded by fibroblasts which lead to a permanent adhesion. Mesothelial cells lose normal physiological responses, including the production of fibrinolytic agents which are pre-disposed to fibrinous adhesion formation (Augustine, Brown et al. 2009). Plasmin plays a vital role in fibrin degeneration, ECM breakdown, and activation of both metalloproteinases and uPlasminogen activator (Holmdahl 1997). Increased expression of transforming growth factor-β1 (TGF-β1) is linked to adhesion formation, probably via increasing production of plasminogen activator inhibitor type 1 (Holmdahl, Kotseos et al. 2001). The molecular mechanisms involved in EPS are complex and include subclinical bowel ischemia and growth factor dysregulation.

Apart from fibrosis, there is also vasculopathy and progressive angiogenesis i.e. increase in capillary number. This leads to UF failure because of increased solute transport through the peritoneal membrane (Aguilera, Yanez-Mo et al. 2005). Angiogenesis inhibition with TNP-470 reduced the number of vessels and inhibited thickening of the submesothelial zone in a mouse model (Yoshio, Miyazaki et al. 2004). Angiostatin (angiogenesis inhibitor) reduced the number of vessels and improved peritoneal function, but it did not change the thickness of the peritoneal membrane in a rat model (Margetts, Gyorffy et al. 2002). The difference in these results may be due to a difference in mechanism of the action for TNP-470 and angiostatin (Miyazaki and Yuzawa 2005)

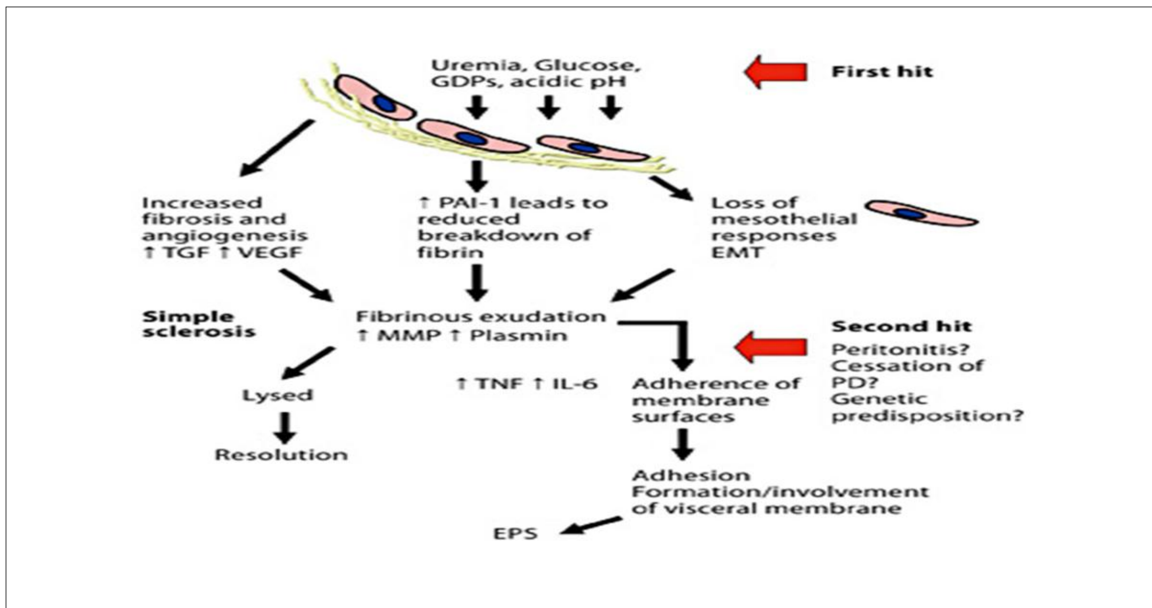


Figure 1.2 Two-hit hypothesis for EPS

The diagram shows a possible pathogenetic mechanism for EPS development. Abbreviations: EMT = Epithelial mesenchymal transformation; GDP = glucose degradation product; IL-6 = interleukin-6; MMP = matrix metalloproteinase; PAI-1 = plasminogen activator inhibitor type 1; TGF = transforming growth factor; VEGF = vascular endothelial growth factor; TNF = Tumor necrosis factor. It is reproduced by permission of the Karger Publishers. Copyright © 2009, Karger Publishers (Augustine, Brown et al. 2009).

1.6.2 Histology of PS and EPS

Histologically EPS tissue consists mainly of organized fibrin which is most likely derived from plasma exudation that comes from the peritoneal microvasculature (Honda and Oda 2005). The fibroblasts in the peritoneum appear swollen with an increased level of cellularity. They are accompanied by the appearance of various activation and proliferation markers. Fibrin deposition and alteration of fibroblast phenotype are proposed as significant findings for early detection of EPS. In addition, EPS can be predicted by the presence of persistent inflammatory changes (Honda and Oda 2005). Vasculopathy and neoangiogenesis may be of more significance in early EPS cases. Most of these histological findings can be found in patients on long-term peritoneal dialysis even without EPS (Sherif, Yoshida et al. 2008). The capsular membrane in EPS is derived from exudative fibrin. In addition, perivascular bleeding is frequently observed (Figure 1.3 A). Enlarged fibroblasts are distributed throughout the fibrous tissue (Figure 1.3 B). Peritoneal fibroblasts produce large amounts of ECM proteins and their accumulation will drive fibrosis (Figure 1.3 C). Capillary angiogenesis and mononuclear cell infiltration are present (Figure 1.3 D). Mesothelial denudation and interstitial fibrosis are due to PD-induced chronic tissue damage after a long history of PD (Figure 1.3 E). Specific microvasculopathy can be seen (Figure 1.3 F) (Honda and Oda 2005).

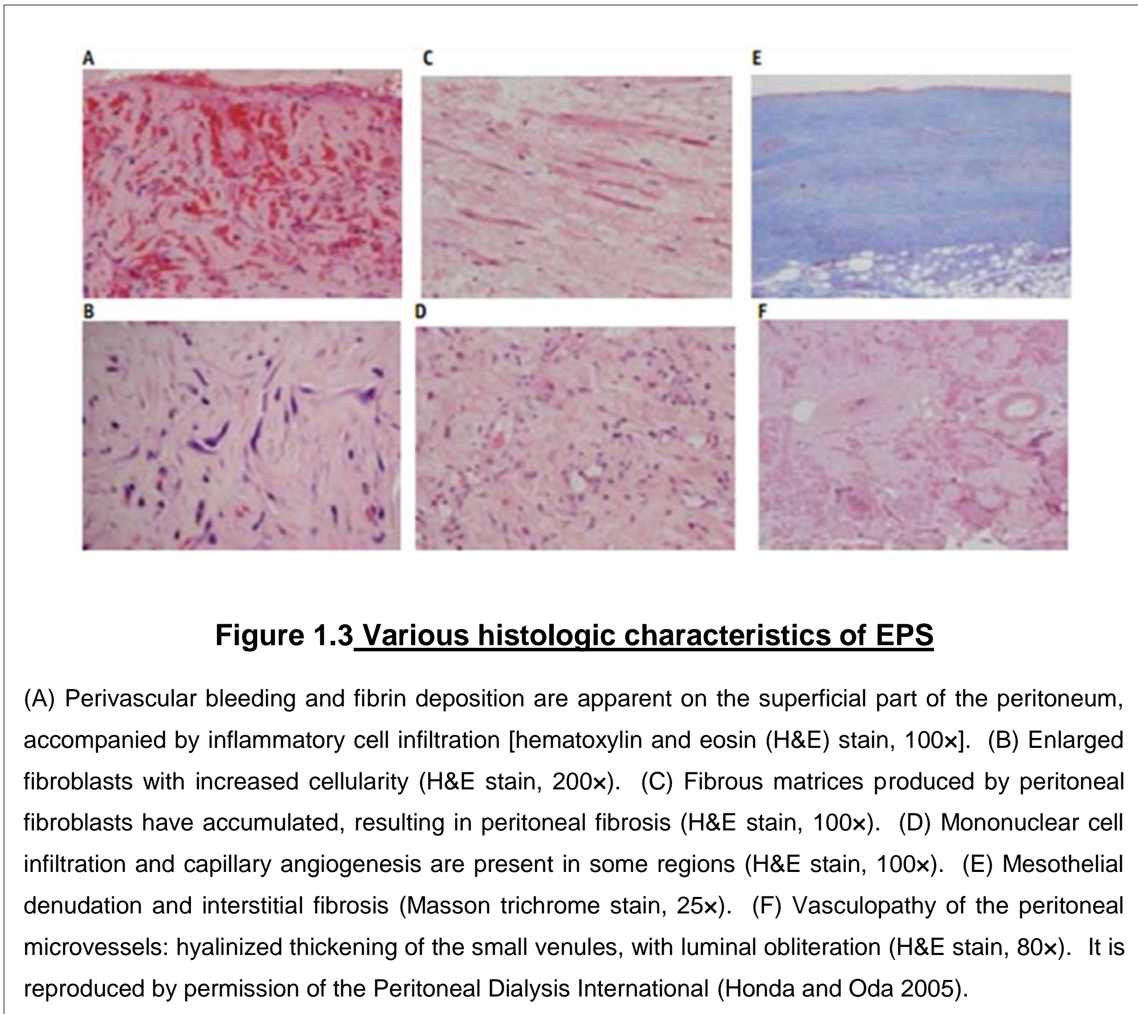


Figure 1.3 Various histologic characteristics of EPS

(A) Perivascular bleeding and fibrin deposition are apparent on the superficial part of the peritoneum, accompanied by inflammatory cell infiltration [hematoxylin and eosin (H&E) stain, 100×]. (B) Enlarged fibroblasts with increased cellularity (H&E stain, 200×). (C) Fibrous matrices produced by peritoneal fibroblasts have accumulated, resulting in peritoneal fibrosis (H&E stain, 100×). (D) Mononuclear cell infiltration and capillary angiogenesis are present in some regions (H&E stain, 100×). (E) Mesothelial denudation and interstitial fibrosis (Masson trichrome stain, 25×). (F) Vasculopathy of the peritoneal microvessels: hyalinized thickening of the small venules, with luminal obliteration (H&E stain, 80×). It is reproduced by permission of the Peritoneal Dialysis International (Honda and Oda 2005).

1.6.3 Comparison between simple PS and EPS

It is not even clear whether EPS is an evolution of a simple sclerosis or is a different disorder. The positive fibrin stain on the membrane surface and a thick degenerative compact zone layer are main pathological findings in patients with EPS. Angiogenesis, vasculopathy, fibrosis, degenerative changes of the compact zone layer, and new membrane formation are common pathological findings in both simple sclerosis and EPS (Figure 1.4) (Sherif, Yoshida et al. 2008).

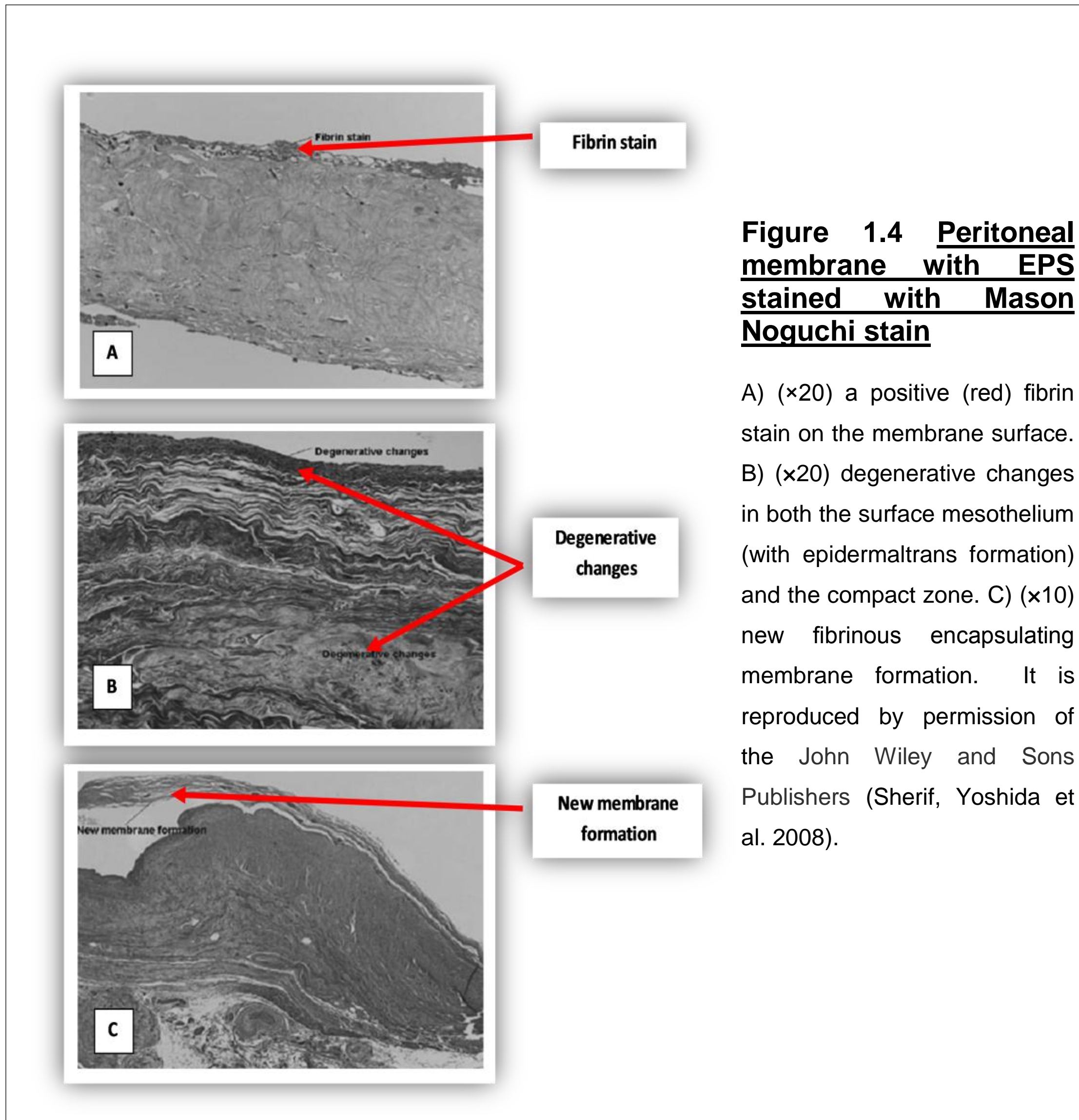


Figure 1.4 Peritoneal membrane with EPS stained with Mason Noguchi stain

A) (×20) a positive (red) fibrin stain on the membrane surface. B) (×20) degenerative changes in both the surface mesothelium (with epidermaltrans formation) and the compact zone. C) (×10) new fibrinous encapsulating membrane formation. It is reproduced by permission of the John Wiley and Sons Publishers (Sherif, Yoshida et al. 2008).

1.6.4 Risk factors

There are several factors behind our poor understanding of the risk factors for EPS development. These include the sporadic nature of EPS, difficulty in diagnosis during the early stage and the requirement for better animal models. Clinical associations of EPS have been identified. These include acetate buffer (Slingeneyer 1987), exposure to glucose in dialysate (Hendriks, Ho-dac-Pannekeet et al. 1997), low UF (Yamamoto, Otsuka et al. 2005), and absence of residual renal function (at least in children) (Hoshii, Honda et al. 2000).

Discontinuing PD sometimes acts as a trigger for EPS and this may explain the increase rate of developing EPS after renal transplantation (Fieren, Betjes et al. 2007). There is a possibility of genetic predisposition for developing EPS (Davies, Phillips et al. 1998).

Several studies show that progression to EPS correlates with high membrane permeability, long-term PD and loss of UF (Nomoto, Kawaguchi et al. 1996, Rigby and Hawley 1998, Yamamoto, Nakayama et al. 2002, Kawanishi, Kawaguchi et al. 2004). However, EPS has also been reported in patients with adequate UF and low membrane transport status (Balasubramaniam, Brown et al. 2009). There is an association between EPS and icodextrin (Wilkie 2011). The patient, who starts PD at a younger age, is at greater risk of developing EPS (Korte, Sampimon et al. 2011).

Patients at high risk for EPS are those patients who have been on peritoneal dialysis for a long time (more than 8 years on PD), with high peritoneal transport and a history of repeated episodes of peritonitis (Korte, Yo et al. 2007), although a history of peritonitis is not essential for developing EPS (Nomoto, Kawaguchi et al. 1996). This is not surprising because patients with a greater frequency of peritonitis tend to have shorter technique survival (Balasubramaniam, Brown et al. 2009). Those PD patients, who do not get peritonitis, continue longer on PD and they are more likely to get EPS due to a “survivor” bias.

The literature reports on the significance of peritonitis in the EPS development remain unclear. Peritonitis episodes caused by the fungal peritonitis, Haemophilus Influenza, Pseudomonas spp, and Staphylococcus Aureus have been linked to the development of EPS (Chew, Clarkson et al. 1997, Afthentopoulos, Passadakis et al. 1998, Rigby and Hawley 1998, Nakamura, Okada et al. 1999). A prospective study in Japan showed that quarter of the EPS cases were associated by bacterial peritonitis, whereas a

single centre controlled study displayed no relationship (Hendriks, Ho-dac-Pannekeet et al. 1997, Kawanishi, Kawaguchi et al. 2004). Both the Dutch multi-centre study and the Scottish registry could not prove the association between EPS and peritonitis incidence (Betjes, Fieren et al. 2009, Brown, Simpson et al. 2009). However, the frequency of peritonitis episodes caused by fungal peritonitis, Pseudomonas spp and Staphylococcus Aureus were higher in the EPS compared to control groups. This may indicate that it is not the incidence, but the source of the peritonitis episode that is important for the EPS development (Korte, Sampimon et al. 2011, Korte, Sampimon et al. 2011).

1.6.5 Clinical presentation

The onset of EPS is often insidious. The presenting features vary from one patient to another, but typically features of inflammation, abdominal pain and weight loss are seen. In advanced cases, the patient may suffers from malfunction of the gastrointestinal tract and death (Augustine, Brown et al. 2009).

1.6.6 Diagnosis

There are three aspects to EPS diagnosis; clinical presentation, radiological imaging and the pathology. The development of these diagnostic criteria was undertaken by the International Society of Peritoneal Dialysis (ISPD). EPS is a clinical syndrome with the presence of intestinal obstruction (persistent or recurrent). The clinical syndrome is with or without the presence of inflammation. There are peritoneal thickening, sclerosis, calcifications and encapsulation, which are confirmed by radiological findings or macroscopic inspection (Kawaguchi, Kawanishi et al. 2000, Korte, Fieren et al. 2011).

Diagnosis of EPS is made after establishing both functional and structural abnormalities. Initially any symptom that suggests intestinal obstruction, ranging in severity from mild symptoms such as anorexia to severe symptoms such as marked weight loss would raise concern. Diagnosis is confirmed by the

demonstration of peritoneal membrane thickening by imaging techniques or at laparotomy. The increasing thickness is a pre requisite to encapsulation and bowel cocooning. Gastrointestinal symptoms may include loss of appetite, abdominal pain and fullness, disturbance in bowel motions, early satiety, nausea, vomiting, weight loss (Nakamoto 2005). There may be signs of inflammation (fever, raised CRP, anaemia, ascites and blood in dialysate), abdominal masses and pain (features of adhesions in the peritoneum and/or cocooning), signs of intestinal obstruction either acute or subacute, failure of peritoneal ultrafiltration capacity and a rapid rise in small solute peritoneal transport.

EPS is not only associated with PD, but it is also associated with other conditions such as systemic autoimmune disease, gastrointestinal tract diseases, malignancies involving the peritoneum or intra-abdominal organs, exposure of the peritoneum to talc or particulate material, peritoneal lavage by intraperitoneal disinfectant, and administration of β-blockers (Kawanishi and Moriishi 2005). In animal models, infusing a variety of intraperitoneal sclerosing agents can induce peritoneal sclerosis as well as encapsulation (Hoff 2005). Adenovirus can be used to introduce pro-fibrotic agents to the peritoneum of the animal models (Hoff and Margetts 2006). The differential diagnosis for EPS includes tuberculous peritonitis, small bowel lymphoma especially after transplantation, carcinomatosis and peritoneal mesothelioma (Augustine, Brown et al. 2009).

1.6.7 Imaging techniques

1.6.7.1 Computed tomography scan (CT scan)

The CT scan is useful for diagnosis, but not screening. Abdominal CT could be normal in up to 50% of EPS patients up to two years before diagnosis (Tarzi, Lim et al. 2008). There are peritoneal abnormalities (thickening and enhancement being most common) in approximately 7% of patients, who are on long-term PD, but without developing EPS (Stafford-Johnson, Wilson et al. 1998). Despite all of the above, CT scanning of the abdomen is the investigation

of choice for those patients with established EPS (Stafford-Johnson, Wilson et al. 1998, George, Al-Zwae et al. 2007). The progression of EPS can be monitored by abdominal CT.

The CT scan findings show peritoneal enhancement following the administration of intravenous contrast media (Figure 1.5) and it is usually accompanied with peritoneal thickening. The differential diagnoses of peritoneal enhancement and thickening are peritoneal carcinomatosis, pseudomyxoma peritoneii, tuberculosis in addition to EPS. There could also be peritoneal calcification and bowel tethering. Bowel tethering is due to fibrosis in the mesenteric root as well as previsceral membrane formation. Bowel tethering and peritoneal calcification are more specific radiological features than others. Other CT features are bowel wall thickening and dilatation with or without obstruction (Stafford-Johnson, Wilson et al. 1998, George, Al-Zwae et al. 2007, Tarzi, Lim et al. 2008).

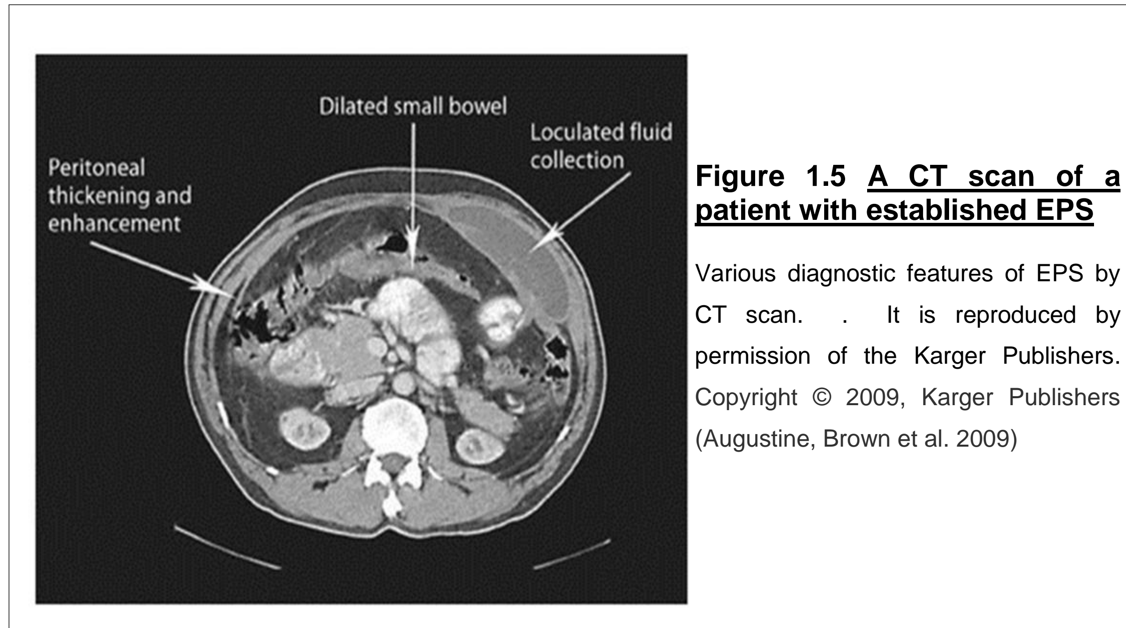


Figure 1.5 <u>A CT scan of a patient with established EPS</u>

Various diagnostic features of EPS by CT scan. . It is reproduced by permission of the Karger Publishers. Copyright © 2009, Karger Publishers (Augustine, Brown et al. 2009)

1.6.7.2 Other imaging techniques

Ultrasound findings include increase of intestinal peristalsis, tethering of the bowel, visualisation of a previsceral membrane and ascites (Krestin, Kacl et al. 1995). The main feature in barium studies is delayed transit with possibility of sudden change in calibre in the joining point of normal and encapsulated bowel segment and disconnection of rigid bowel loops with disordered peristalsis (Holland 1990, Krestin, Kacl et al. 1995). MRI is not particularly useful because of poor assessment of peritoneal calcification. Furthermore, there is a risk of nephrogenic systemic fibrosis in dialysis patients, if gadolinium used as a contrast in MRI.

1.6.8 Management

There are some evidence-based management regimens for EPS. There are case reports and clinical series rather than randomized controlled trials. Reported treatments include the use of steroids in the early 'inflammatory' phase (Kawanishi, Kawaguchi et al. 2004), immunosuppressants (Junor and McMillan 1993, Rigby and Hawley 1998, Rajani, Smyth et al. 2002, Lafrance, Letourneau et al. 2008), tamoxifen (an estrogen receptor antagonist) (Allaria, Giangrande et al. 1999, Summers, Clancy et al. 2005, Eltoum, Wright et al. 2006, Moustafellos, Hadjianastassiou et al. 2006), nutritional support, and surgery including enterolysis/adhesiolysis to remove the fibrotic material (Kawaguchi, Saito et al. 2005). In established EPS, surgery is possibly the only definitive line of treatment at the current time (Kawanishi, Moriishi et al. 2006). The surgery is difficult and requires to be conducted by a surgeon with expertise in EPS (Yamamoto, Otsuka et al. 2005). EPS requires multidisciplinary input including a nephrologist, dietitian, gastroenterologist and surgeons. Management includes medication, nutritional support, control of the symptoms control and surgery (Spence, Gillespie et al. 2013)

1.6.9 Prevention

It is necessary to understand the aetiology of EPS as this will lead to better ways to reduce the risk of developing EPS for patients on PD (Mactier 2000). Preserved residual renal function, low glucose exposure, low small solute transport status, and little or no peritoneal infection may give a lower individual risk. It is important to minimise glucose exposure and peritonitis rates. According to Japanese data, the incidence rate of EPS is markedly increased after 8 years on PD. Pre-emptive discontinuation of PD is recommended at this stage (Kawaguchi, Saito et al. 2005). The problem is that discontinuation of PD by itself is a risk factor for some patients developing EPS (Fieren, Betjes et al. 2007). Therefore the decision to discontinue PD is an individual decision and is based on a balance of risks. For example a younger PD patient with lower co-morbidity who starts PD during young age is at more risk to develop EPS and this influences the clinical decision.

1.7 Factors associated with peritoneal fibrosis

The human bodies' responses to acute and chronic wounding are very different. In chronic wound, there are continuous insults to organs leading to what most people believe is an aberrant unresolved wound response with perpetual inflammation in contrast to acute wounding which fully resolves. The unresolved nature of chronic wounding leads to a total remodeling of the organ in an attempt to protect it from the continuous insult. This remodeling in itself is ultimately harmful as the formation of scar tissue on such a gross level affects architecture and thus organ function.

Peritoneal fibrosis has many similarities to fibrosis in other organs. There are major pathological changes in the peritoneal fibrosis such as. First, there are chronic inflammatory responses to the peritoneal dialysate fluid. Second, there are a proliferation and activation of fibroblasts which have multiple sources that

produce a large amount of interstitial ECM proteins. Third, there are changes in cytokine and growth factor signaling in response to the above. Fourth, there are metabolic changes to the glucose and ROS that alter cellular signaling and biology. Fifth, there are loss of MCs and a change in their biology. Finally, there are changes in ECM processing and deposition to stabilize the peritoneal membrane. The following changes are described in more detail below (sections 1.7.1 – 1.7.7 and 1.8). There are also major fibrosis contributors such as inflammation, profibrotic signaling, aberrant ECM processing (low clearance with elevated ECM deposition), fibroblast proliferation, ROS generation, and cellular death.

1.7.1 Epithelial to mesenchymal transition (EMT)

EMT is a potential mechanism whereby the epithelial cell de-differentiates into a mesenchymal cell via a complex process. The event begins by the dissociation of intercellular junctions and loss of microvilli followed by loss of apical-basal polarity. Finally, there is basement membrane degradation and invasion with up-regulation of MMPs (Aroeira, Aguilera et al. 2007). Myofibroblastic cells may arise from conversion of mesothelial cells (MCs) through EMT during PD (Margetts, Bonniaud et al. 2005). Transdifferentiated MC may play a crucial role in the beginning of fibrosis and subsequent decline of peritoneal function (Aroeira, Aguilera et al. 2007). More details on EMT are illustrated in (section 6.1.4).

1.7.2 Myofibroblasts in peritoneal fibrosis

After peritoneal injury, myofibroblasts can also accumulate in the peritoneum as a consequence of activation and proliferation of resident fibroblasts in the peritoneum, infiltration and proliferation of circulating and resident fibrocytes, transformation of local pericytes, mesenchymal transition and dedifferentiation of peritoneal MCs, mesenchymal transition and dedifferentiation

of endothelial cells, and differentiation of local mesenchymal stem cells (Abraham, Eckes et al. 2007, Liu, Dong et al. 2015, Witowski, Kawka et al. 2015).

Myofibroblasts represent a subset of the fibroblast population that are highly activated, producing large quantities of ECM proteins, are highly proliferative and invasive. They are believed to be involved in various scarring and fibrotic diseases, where they play an important role in the fibrotic process after the initial of the injury. In acute and resolving wounding, Myofibroblasts will undergo apoptosis after the tissue integrity has been adequately restored. (Darby, Skalli et al. 1990, Desmouliere, Redard et al. 1995). However, myofibroblasts fail to undergo apoptosis in many scarring and fibrotic conditions thus providing a much higher percentage of the cell population and this contributes significantly to the fibrosis (Aarabi, Bhatt et al. 2007, van der Veer, Bloemen et al. 2009). Myofibroblasts apoptosis is controlled by a decline in the local growth factors which sustain and drive differentiation in the myofibroblasts. In particular, endothelin-1 and TGF-β1 local concentrations play an important role in the myofibroblast survival by activation of the protein kinase B (AKT) (Kulasekaran, Scavone et al. 2009).

Peritoneal fibrosis is much less well characterised than fibrosis in many other organs, but it is likely that many of the processes described above do play key roles in peritoneal fibrosis. Recent in vitro studies have suggested that MCs to be an important source of myofibroblasts via epithelial to mesenchymal transition (EMT), but there are no in vivo studies to support this currently. Duffield and colleagues used primary cultures of MCs and submesothelial fibroblasts that were expressing alpha smooth muscle actin under a TGFbeta-1 stimulus. In these studies alpha smooth muscle actin was only expressed in the submesothelial fibroblasts after peritoneal fibrosis induction in mice (Chen, Chang et al. 2014). In renal fibrosis, blockade of platelet-derived growth factor (PDGF) receptor signaling decreases the numbers of myofibroblast and

decreases renal fibrosis (LeBleu and Kalluri 2011). In peritoneal fibrosis, PDGF receptor expressed by submesothelial fibroblasts was antagonised pharmacologically and this led to a reduced the peritoneal fibrosis (Chen, Chang et al. 2014).

Numerous studies have suggested that MCs are the main source of myofibroblasts through the EMT process (Yanez-Mo, Lara-Pezzi et al. 2003, Yang, Chen et al. 2003, Margetts, Bonniaud et al. 2005, Patel, West-Mays et al. 2010, Bajo, Pérez-Lozano et al. 2011). These are in vitro experiments only which is limiting in translatability, but they do suggest that MCs can be stimulated to express alpha smooth muscle actin and are thus taking on a fibroblastic phenotype with associated matrix protein production under a TGFbeta-1 driver (Wong, Phillips et al. 2003, Yanez-Mo, Lara-Pezzi et al. 2003, Yang, Chen et al. 2003, Margetts, Bonniaud et al. 2005, Bajo, Pérez-Lozano et al. 2011) .

1.7.3 Heat shock protein 47 (HSP47)

HSP47 presence has been implicated in different fibrotic disorders including fibrotic peritoneal tissue obtained from patients on PD (Shioshita, Miyazaki et al. 2000), peritoneal fibrosis in rats induced by chlorhexidine gluconate (CG) (Mishima, Miyazaki et al. 2003), kidney scarring (Razzaque, Ahsan et al. 2000), liver fibrosis (Masuda, Fukumoto et al. 1994), and lung fibrosis (Iwashita, Kadota et al. 2000).

The degree of HSP47 expression shows a significant relationship with collagen types I and III accumulation in humans and with peritoneal fibrosis induced by CG (Miyazaki and Yuzawa 2005). Changes induced by CG in the expression of HSP47, α smooth-muscle actin and collagen types I and III are abrogated by HSP47 anti-sense oligonucleotides. In addition, the number of infiltrating vessels and macrophages are reduced after suppression of HSP47 (Nishino, Miyazaki et al. 2003).

1.7.4 Angiotensin II

Angiotensin II has a variety of biological activities, including cell proliferation, collagen synthesis, promotion of macrophage infiltration, and induction of many cytokines and growth factors (Luft 2002, Suzuki, Ruiz-Ortega et al. 2003). Angiotensin II may also play a role in peritoneal fibrosis. The possible benefit of using angiotensin converting enzyme inhibitors (ACEI) and angiotensin II type 1 receptor blockers for the prevention and/or therapy of peritoneal fibrosis remain to be determined.

In an experimental animal model, peritoneal fibrosis was induced by injection of CG, then angiotensin II modulated by benazepril (ACEI) and candesartan (angiotensin II type 1 receptor blocker) (Abe, Miyazaki et al. 2002, Miyazaki and Yuzawa 2005). The effect of Angiotensin II inhibition reduced the number of CD31+ vessels, the thickness of the submesothelial tissue, and expression of Transforming Growth Factor-β (TGFβ) and vascular endothelial growth factor (VEGF). In another experiment the inhibition of ACE ameliorated peritoneal fibrosis in mice (Sawada, Ishii et al. 2002).

In humans, little is known about the effects of ACEI and ARB in PD patients. There are some studies on effects of these drugs on peritoneal membrane transport. These studies are divided into short-term and long-term. One of the short-term studies showed a decline in peritoneal protein loss in CAPD patients treated with captopril (ACEI) (Coronel, Hortal et al. 1989), and irbesartan (ARB) (Coronel, Berni et al. 2004). In contrast, another study showed higher β2-microglobulin and peritoneal clearances of creatinine in enalapril (ACEI) treated PD patients in comparison to clonidine and nifedipine treatment (Favazza, Motanaro et al. 1992). Other short-term studies were unable to show any effect of losartan or enalapril on peritoneal membrane transport in CAPD (Ripley, Gehr et al. 1994, Rojas-Campos, Cortes-Sanabria et al. 2005). More studies with long

follow-up are needed to resolve this difference of results between different research groups (Kolesnyk, Struijk et al. 2010).

1.7.5 Growth factors and cytokines

Several key fibrogenic cytokines have been identified in tissue scarring. Most notable amongst them is TGFβ1, which has been found in numerous types of fibrosis (Cutroneo 2007). TGFβ1 has been shown to have a direct effect on peritoneal fibrosis (Margetts, Kolb et al. 2001). TGFβ1 is unique in that it cannot only induce numerous ECM components such as collagen I and III, but also decrease the transcription of several MMPs (Yan and Boyd 2007) and elevate the expression of several TIMPs (Cotton, Herrick et al. 1998) as well as both PAI1 and 2 (Willis and Borok 2007, Samarakoon, Higgins et al. 2008). Further, TGFβ1 can induce EMT and cause fibroblast proliferation (Willis and Borok 2007).

Peritoneal thickening is induced by gene transfer of TGFβ, and this thickening is resolved naturally. Decorin is a natural inhibitor of TGFβ, that can prevent TGFβ-induced peritoneal thickening (Honda, Nitta et al. 2003). There is some evidence to suggest that fibroblast growth factor (FGF) and TGFβ1 play a role in both PS & EPS (Mlambo, Hylander et al. 1999, Honda, Nitta et al. 2003). During PD there is production of VEGF, which plays an essential role in peritoneal neoangiogenesis and decline in PD function (Aguilera, Yanez-Mo et al. 2005). Other signaling molecules have also been ascribed roles in tissue scarring including platelet-derived growth factor (PDGF) (Floege, Eitner et al. 2008), insulin-like growth factor 1 (IGF-1) (Krein and Winston 2002) and tumor necrosis factor-alpha (TNF-α) (Tilg 2001). Dialysate which contains glucose and glucose degradation products may trigger VEGF and TGF-β1 production by mesothelial cells. TGF-β1, which is a strong pro-fibrotic factor, will affect mesothelial cells by inducing EMT (Aguilera, Yanez-Mo et al. 2005).

Growth factors have a major effect on the protein levels of ECM, but the same growth factor may causes difference responses from a range of cell types. A growth factor may lead to increased deposition of ECM in some cell types, however the same growth factor may also lead to decreased deposition of ECM in other cell types (Ito, Aten et al. 1998). Cell signaling is triggered by various growth factors and cytokines such as TNF-α, TGF-β, interferons, and interleukins. Cell signaling causes activation of promoters for MMPs (Yan and Boyd 2007). TGFβ can work with other growth factors to produce a synergistic effect such as TNFα. TGFβ alone is able to induce production of collagen to around 25% of its maximum. TNFα alone can also induce the production of collagen to around 25% of its maximum. TGFβ and TNFα together maximally induce production of collagen to around 100%, although their levels were less than maximum level of TGFβ and TNFα independently (Border 1994).

1.7.6 The role of inflammation in peritoneal fibrosis

The role of inflammation in the development of peritoneal fibrosis remains unclear. Histologically, advanced peritoneal fibrosis demonstrated little infiltration by macrophages. Elevation in levels of interleukin-1β (IL-1β) and interleukin-6 (IL-6) are seen in the ascites of EPS patients and also in an animal experimental model of EPS (Imai, Nakamoto et al. 2003, Masunaga, Muto et al. 2003). Expression of adhesion molecules was observed in the peritoneum of rats with EPS in conjunction with an increase of IL-6 in the dialysate (Imai, Nakamoto et al. 2003). Gene transfer of TNFα and IL-1β into rat peritoneum caused an increased peritoneal inflammatory cell infiltration, particularly by neutrophils (Margetts, Kolb et al. 2002). However, peritoneal membrane thickening induced by TNFα eventually resolved and the membrane returned to normal. In contrast, peritoneal membrane thickening associated with IL-1β did not return to normal. Net ultrafiltration declined in both TNFα and IL-1β induced rats, however UF returned to normal levels in the TNFα group, but remained low in the IL-1β induced rats (Miyazaki and Yuzawa 2005).

1.7.7 The roles of proteolytic enzyme systems in fibrosis:

Excessive extra cellular matrix accumulation can lead to fibrotic diseases in most organs such as the kidney (Johnson, Haylor et al. 2002) and is this likely to also occur in peritoneal fibrosis. Shutdown of proteolytic enzymes or over expression of their inhibitors will lead to ECM accumulation. Different studies have shown changes in the MMP and plasmin systems in PD. These may be useful as markers of increased solute transport, peritoneal injury or progression to EPS. Matrix metalloproteinases, cysteine/aspartic proteases and the serine proteases (e.g. plasmin) are the main proteolytic enzyme systems. An understanding of ECM metabolism is an important building block in exploring the role of these agents in fibrosis.

1.8 The Extracellular Matrix

1.8.1 Structure and Function of the ECM

The ECM is a multi-component protein material found outside the cell. It has several functions such as structural support, extracellular feedback, formation of basement membrane, storage of growth factors such as TGFβ1, cell adhesion and a role in the wound healing response (Levey, Eckardt et al. 2005). Components of ECM are synthesized intracellularly then passed extracellularly for assembly. The balance between matrix degradation and synthesis is crucial to preserve the function of matrix and the balance of tissue homeostasis (Figure 1.6).

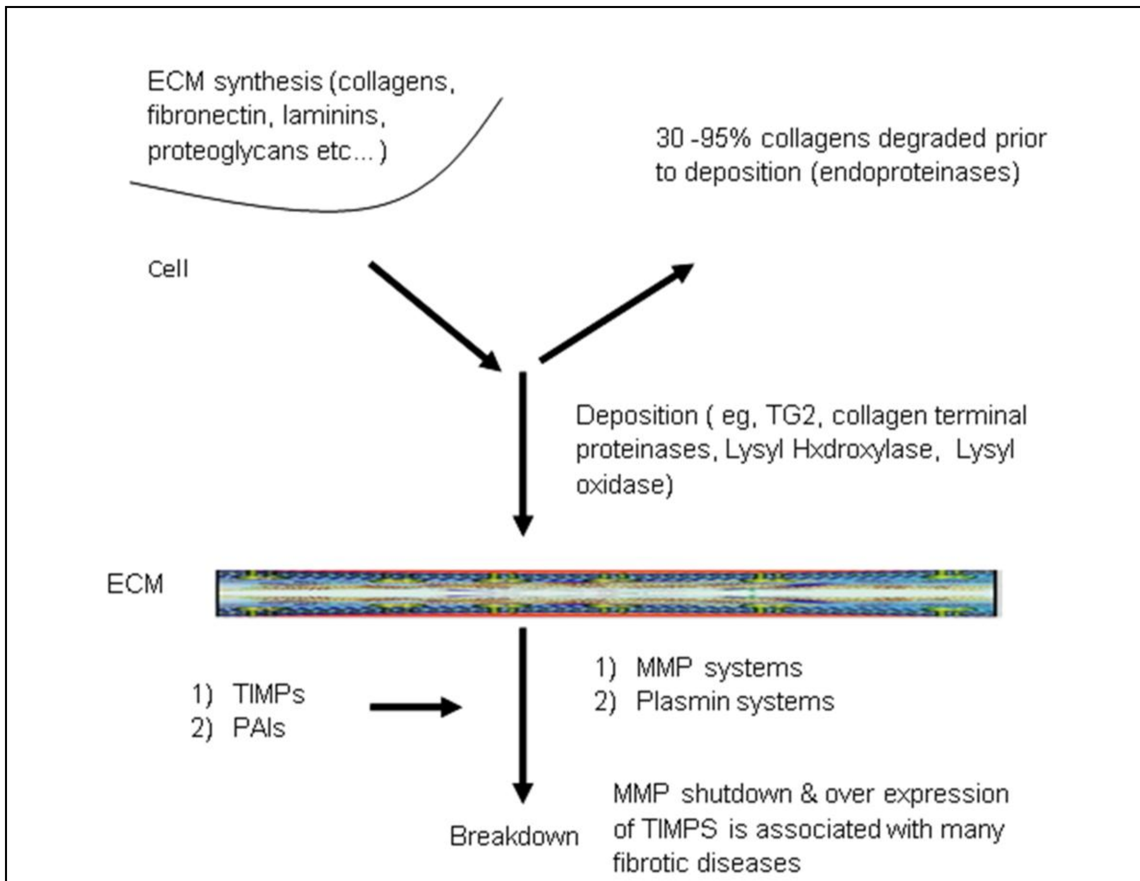


Figure 1.6 ECM synthesis and deposition

Components of ECM are synthesized intra-cellularly then passed extracellularly for deposition. Some of the collagen is degraded prior to deposition. Once deposited 2 main systems degrade the ECM. These are the MMP and Plasmin system. Each has its own regulatory system. MMP activity is inhibited by TIMPs, while plasminogen activator inhibitors (PAIs) prevent the activation of plasminogen. Note TG2 is abbreviation for (Transglutaminase type 2).

1.8.2 Components of ECM

The ECM may take different forms in various organisms and tissues, but it has a tendency to be composed of similar components of macromolecules (Figure 1.7) (Gerald 2013). The ECM consists of structural proteins such as collagens which are complemented by more specialized proteins such as elastin which can convey flexible properties. Fibronectin is involved in adhesion and proteoglycans are the key in determining charge.

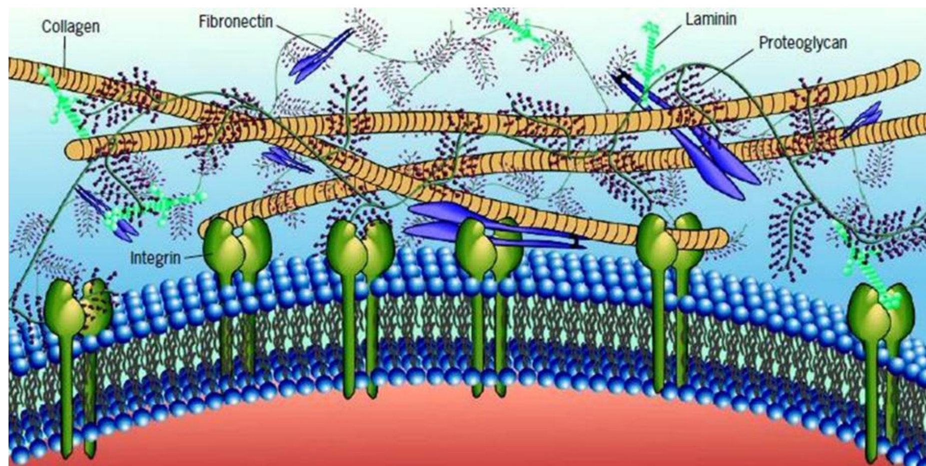


Figure 1.7 An overview of the extracellular matrix macromolecular organisation

The proteins shown are collagen, laminin and fibronectin. They contain sites for binding with each other, in addition to the binding sites for the integrins. Integrins are receptors which are located at the cell surface and are involved in cell adhesion, out-in cell communication and growth factor recruitment/activation. Large protein–polysaccharide complexes can form through the ECM which are typically formed of proteoglycans such as perlecan and syndecans. . It is reproduced by permission of the John Wiley and Sons Publishers (Gerald 2013).

1.8.2.1 Collagens

Collagen molecules have a helical structure consisting of three polypeptide chains coiled around each other in a triple helix (Alberts, Johnson et al. 2002). Fibrils are formed from staggered arrays of collagen molecules that are arranged to form collagen fibers (Figure 1.8) (Buehler 2006). Collagens in general are the most abundant proteins in mammals, especially collagen I (Di Lullo, Sweeney et al. 2002). Collagens vary in α chain composition of amino acids. So far 29 types of collagens have been described. Collagen I, II, III and IV form over 90% of body collagen. Localization of different types of collagen varies: for example, type I collagen is located in skin, tendon, bone, etc., type II collagen is located in cartilage, vitreous humor, type III collagen is located in skin, muscle, and type IV collagen located in basal lamina; eye lens, and basement membranes, including the glomerular, tubular basement and peritoneal membranes (Williams, Craig et

al. 2003). Type III collagen is frequently associated with type I collagen and they are known as interstitial collagens. Type IV collagen is composed of three α chains interrupted at several sites via non-collagenous sequences allowing collagen IV to self-associate with other components of ECM. This provides highly specialized characteristics of the basement membrane (Costigan, Chambers et al. 1995). Interstitial collagens are mainly synthesized by fibroblasts, with basement membrane collagens produced by epithelial and endothelial cells.

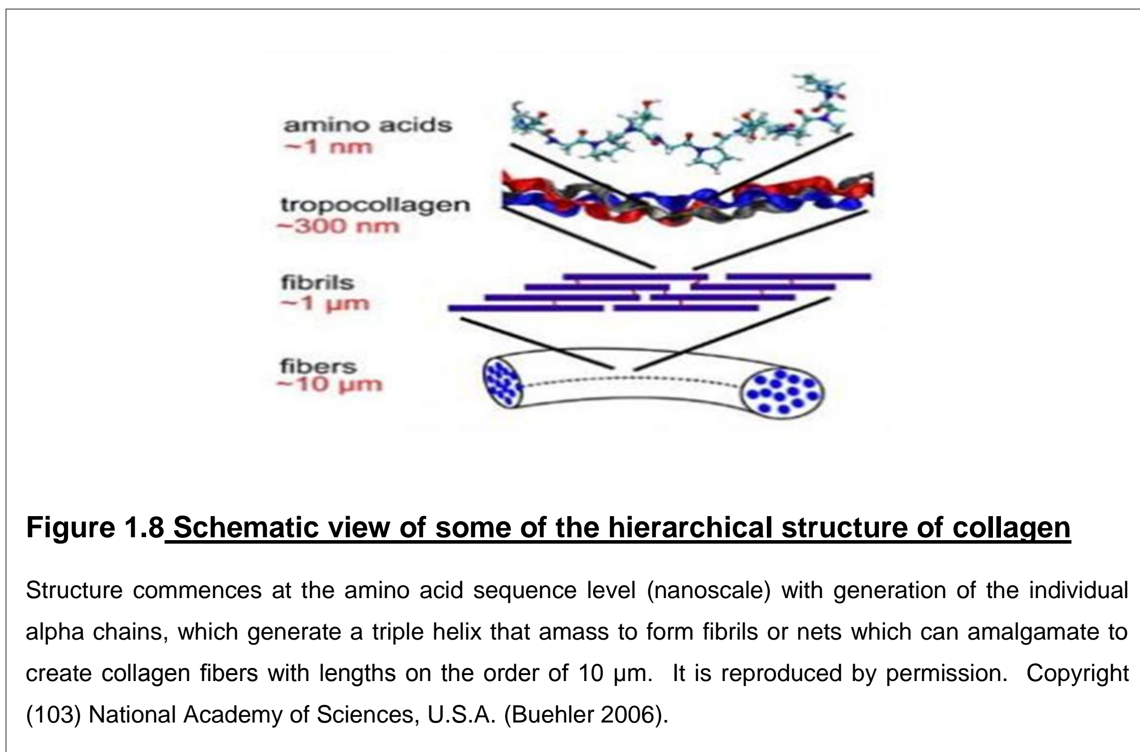


Figure 1.8 <u>Schematic view of some of the hierarchical structure of collagen</u>

Structure commences at the amino acid sequence level (nanoscale) with generation of the individual alpha chains, which generate a triple helix that amass to form fibrils or nets which can amalgamate to create collagen fibers with lengths on the order of 10 μm. It is reproduced by permission. Copyright (103) National Academy of Sciences, U.S.A. (Buehler 2006).

1.8.2.2 Elastin

Elastin is a structural protein in the ECM with elastic properties. It plays a role in stretch and recoil for example in alveoli of lungs, arteries, and skin. In elastin, the arrangement of fibrous proteins is somewhat amorphous (i.e. relaxed cross-linked coils) arrangement, while the arrangement in collagen is dense fibrous bundles (Alberts, Johnson et al. 2002).

1.8.2.3 Proteoglycans

Proteoglycans are formed by linkage between core proteins and glycosaminoglycans (GAGs). GAGs are side chains/sugars on proteoglycans. The structure of proteoglycans is a brush-like structure due to the extension of GAGs perpendicularly from the core. A specific tetrasaccharide is involved in the linkage between GAGs and the protein core. This tetrasacharide is composed of a xylose residue, two galactose residues, and glucuronic acid (Figure 1.9). The O-glycosidic bond links a tetrasaccharide and a serine (S) residue in the protein. Some forms of keratan sulfates link to the protein core through an N-asparaginyl bond. Multiple GAGs attach to the protein cores of proteoglycans because these cores are rich in both serine (S) and threonine (T) residues.

Glycosaminoglycans (Figure 1.9) are long unbranched polysaccharides and they include repeating disaccharide units. Glycosaminoglycans are the most abundant heteropolysaccharides in the body. The disaccharide units include either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) (modified sugars), and uronic acids such as iduronate or glucuronate. GAG molecules are highly negatively charged. They are located primarily in the extracellular matrix or on the surface of cells. These molecules confer high viscosity and low compressibility. In addition, they provide rigidity and which gives structural integrity to cells as well as forming passageways between cells to allow cell migration (Alberts, Johnson et al. 2002). In membranes such as glomerular basement membrane (GBM), proteoglycans provide a negative charge through their glycosaminoglycans side chains (Fischer, Mougenot et al. 2000).

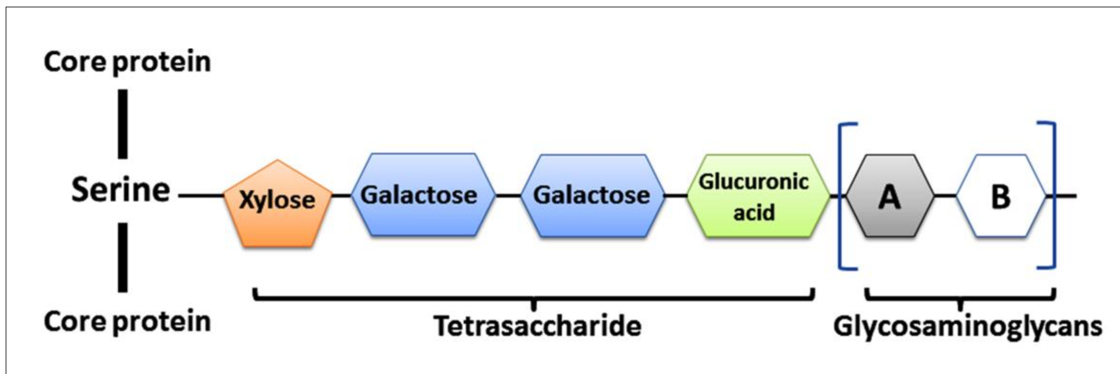


Figure 1.9 **Structure of proteoglycans**

This diagram demonstrates the linkage between glycosaminoglycan and protein core through tetrasaccharide link.

1.8.2.4 Laminin

Laminins are glycoproteins. Laminin is an integral part of almost every tissue and it is vital for their maintenance and survival. Structurally, laminin consists of three polypeptide chains (alpha, beta, and gamma chain subunits). In basement membranes laminins provide an integral part of the supporting structure of the membranes by binding to other macromolecules in the ECM. Laminins have unique and shared interactions with cells, mediated by dystroglycan, integrins and other receptors. Cell interactions give laminins the ability to contribute to cell differentiation, cell movement, cell shape, maintenance of tissue phenotypes and promotion of tissue survival (Colognato and Yurchenco 2000).

1.8.2.5 Fibronectin

A high molecular weight glycoprotein, fibronectin is also involved in interaction with different macromolecules of the ECM. It is involved in basement membrane assembly by acting as a scaffold protein to assist matrix deposition (Czirok, Zamir et al. 2006). Fibronectin contains at least 6 firmly folded domains. Each of these domains has a high affinity for different substrates such as

collagen, heparin sulfate, fibrin, and cell surface receptors (Magnusson and Mosher 1998). Fibronectin connects cells with collagen fibers, allowing movement of the cells through the ECM. It binds cell surface integrins and collagen, causing a reorganization of the cytoskeleton and facilitating cell movement. In addition, fibronectin assists at the site of tissue injury during wound healing by binding to platelets. This leads to blood clotting and facilitating movement of the cells to the affected area (Lewin 2007).

1.8.3 ECM accumulation

The ECM is in a continual state of turnover and under normal circumstances there exists a homeostatic balance between the rate of synthesis and deposition of ECM and its breakdown and clearance. If an imbalance occurs, this can lead to either ECM accumulation (such as may occur in kidney fibrosis) (Lenz, Elliot et al. 2000) or ECM loss. ECM accumulation can be due to increase in ECM synthesis due to qualitative and quantitative changes, as well as increased deposition and stabilization or decreased breakdown.

1.8.3.1 Increased synthesis and changes of ECM components

Increase in synthesis of ECM components such as collagens, elastin, and fibronectin to a greater extent than degradation will lead to ECM accumulation. The formation of scar tissue is also associated with a change in ECM composition. Collagen production increases with a relative increase in collagen types I and III as a percentage of the total collagen load (Johnson, Fisher et al. 2007). The regulation of ECM processing is controlled at many levels from response to metabolic, oxidative or physical stress to tissue tension. Often changes in the cell type can be the key to changes in ECM characteristics. In many cases, growth factors and cytokines are mediators of either production of ECM related molecules or changes in cell types.

1.8.3.2 Stabilisation of ECM

Stabilisation of the ECM to proteolytic decay favours ECM accumulation. Collagen fibrils can be stabilised by enzymes such as tissue transglutaminase (TG2) and lysyl oxidase. Up-regulation of these enzymes in disease will lead to ECM accumulation, with both lysyl oxidase and TG2 being implicated (Kagan 1994, Kagan 2000, Fisher, Jones et al. 2009). In addition TG2 released into the ECM during the wound response can accelerate the deposition of ECM components by short circuiting the standard deposition pathways (Telci and Griffin 2006). TG2's ability to both stabilize and accelerate ECM deposition is normally part of an efficient wound response, however when the wounding becomes chronic then the non-resolving elevated levels of TG2 are central to fibrotic disease.

1.8.3.3 Deposition and Degradation of the ECM

1.8.3.3.1 Deposition of the ECM

Matrix deposition increases, if syntheses of the ECM components go up or if the mechanisms essential for the deposition for these components are up regulated (Bennett and Schultz 1993). The collagens are synthesised de novo. The collagen and pro-peptides are making up the ECM. They are mainly controlled by cytokines and growth factors such as TGF β1. The other inducer for de novo synthesis is induced by feedback from the ECM (Bennett and Schultz 1993).

1.8.3.3.2 Degradation of the ECM

ECM breakdown is by three main proteolytic enzyme systems. These systems are matrix metalloproteinases, cysteine/aspartic proteases and the serine proteases (e.g. plasmin). They are the main proteolytic enzyme systems responsible for the degradation of extracellular matrix (Figure 1.6). There are other molecules involved in the ECM breakdown. For instance, phagocytes

control the deposition of the ECM components by removing apoptotic myofibroblasts (Douglass, Wallace et al. 2008). Moreover, macrophages slow the progression of fibrosis by removing extra collagen from damaged tissues or scared tissue (Atabai, Jame et al. 2009). The ADAMTSs play role in the breakdown of the ECM and this is explained in more detail in (section 1.8.4.4).

1.8.3.4 Impairment of ECM degradation

Net accumulation of ECM will increase if there is impairment of degradation. Excessive ECM accumulation can occur, for example as a result of shutdown of ECM proteolytic enzymes. This can be due to lower synthesis, failure to be converted to an active form or the presence of their natural inhibitors (Johnson, Haylor et al. 2002).

1.8.4 Proteolytic enzyme systems and their inhibitors

1.8.4.1 Matrix metalloproteinases (MMPs)

MMPs are a closely related family of zinc-dependent endoproteinases (Van Lint and Libert 2007). To date, at least 25 different vertebrate MMPs (23 in humans) have been identified. They are either anchored to the plasma membrane or secreted from the cell. MMPs can degrade ECM components involved in many essential processes such as cell proliferation, differentiation, migration and even cell death, in addition to cell–cell interactions (Elkington, O'Kane et al. 2005). MMPs also have many non-matrix substrates. For example, chemokines, growth factors, and receptors. This shows that MMPs have role in a wide range of physiological and pathological processes (McCawley and Matrisian 2001). Dysregulated inflammatory conditions are often associated with increased MMP activities (Parks, Wilson et al. 2004).

MMPs consist of three domains: pro-peptide, catalytic and the haemopexin-like C-terminal (Figure 1.10) (Lafleur, Handsley et al. 2003). The last two

domains are connected by a flexible hinge region. The significance of MMP types varies between organ types. For example in the kidney, MMPs 1, 2, 3, 8, 9, and 13 have significant expression (Lenz, Elliot et al. 2000). MMPs play a significant role in several biological processes, such as normal tissue remodeling, embryogenesis, angiogenesis, and wound healing. They also have been implicated in diseases such as arthritis, atheroma, tissue ulceration and cancer. MMPs can be classified into six groups by their domain organization, substrate specificity and sequence similarity (Figure 1.10) (Lafleur, Handsley et al. 2003). These groups are described below.

1.8.4.1.1 Interstitial Collagenases

These enzymes have the ability to preferentially breakdown interstitial collagens I, II, and III by cleavage of these collagens at a specific cleavage points towards(three fourths from the N-terminus) (Visse and Nagase 2003). In addition, interstitial collagenases can digest other ECM molecules such as collagens VII and X (Collier, Bruns et al. 1991). Interstitial collagenases have the ability to degrade triple-helical fibrillar collagens in to distinctive ¾ and ¼ fragments. The majority of degraded collagen fragments come from the major components of cartilage and bone. Interstitial collagenases include MMP-1, MMP-8, and MMP-13. MMP 14 (an MT-MMP) and MMP2 (a gelatinase) have some similarities to interstitial collagenases (Aimes and Quigley 1995, Ohuchi, Imai et al. 1997). The distinctive ¾ and ¼ fragments are unstable at normal body temperature and thus can be readily cleared. Aberrant collagen degradation may cause atherosclerosis, arthritis, cancer and fibrosis (Woessner 1998, Brinckerhoff and Matrisian 2002).

MMP1 needs a catalytic domain and a hemopexin domain to cleave the triple helix (Clark and Cawston 1992). In rodents, MMP-1 is absent and its role is taken by MMP-13. MMP8 is a neutrophil collagenase and is present in the connective tissue in most mammals. It can degrade interstitial collagenases (collagens I, II and III) (Hasty, Pourmotabbed et al. 1990) and process

interleukin-8 (Tester et al., 2007). MMP13 is cleaves type II collagen better than I or III. It has been ascribed a role in breast carcinoma as well as osteoarthritis (Freije, Diez-Itza et al. 1994). The collagenases have other functions such as processing insulin-like growth factor binding proteins and protease-activated receptor-1 (Overall, McQuibban et al. 2002, Boire, Covic et al. 2005).

1.8.4.1.2 Gelatinases

This group includes gelatinase A (MMP-2) and gelatinase B (MMP-9). Gelatinases have a fibronectin type II insert within their catalytic region. This insertion makes MMP-2 and MMP-9 different from other MMPS conveying membrane adhesive properties to gelatinases assisting attachment to basement membranes to facilitate degradation (Weckroth, Vaheri et al. 1996). Gelatinases can degrade collagen types I, IV, VII, X, IX, fibronectin, elastin, laminin, aggrecan (Overall, McQuibban et al. 2002), and pro-TNF (Gearing, Beckett et al. 1995).

1.8.4.1.3 Stromelysins

This group includes Stromelysin 1 (MMP-3) and stromelysin 2 (MMP-10). The stromelysins degrade many components of ECM proteins, but they are unable to degrade collagen I. MMP3 has the ability to degrade several collagens (collagen II, III, IV, IX and X), fibronectin, proteoglycans, elastin, and laminin. MMP-3 has the ability to initiate MMPs 1, 7 and 9 activation (Ye, Eriksson et al. 1996) and suggests why MMP-3 is important in tissue remodeling. MMP-3 can also process pro-interleukin-1 beta and E-cadherin (Overall, McQuibban et al. 2002). MMP10 is important in the degradation of proteoglycans and fibronectin (Muller, Quantin et al. 1988). The proteolytic efficiency of MMP-3 is higher than that of MMP-10. MMP-11 is known as stromelysin 3; however it is now not grouped with "Stromelysins" for reasons of substrate specificity and the sequence divergence from those of MMP-3.

1.8.4.1.4 Matrilysins

This group includes matrilysin 1 (MMP-7) and matrilysin 2 (MMP-26) (Uria and Lopez-Otin 2000). The lack of a hemopexin domain is a characteristic feature of this group. MMP-7 and MMP-26 digest a number of ECM components, such as gelatin and fibronectin (Salmela, Karjalainen-Lindsberg et al. 2001). MMP-7 also processes cell surface molecules such as pro–tumor necrosis factor (TNF)-α, pro–α-defensin, Fas-ligand, and E-cadherin. Matrilysins are also known as the minimal-domain MMPs because the C-terminal hemopexin-like domain is present in all MMPs except matrilysins (Bode 1995, Stamenkovic 2003).

1.8.4.1.5 Membrane-Type MMPs (MT-MMPs)

This group is subdivided to: type I transmembrane proteins that include (MMP-14, MMP-15, MMP-16, and MMP-24), and glycosylphosphatidylinositol (GPI) anchored proteins that include (MMP-17 and MMP-25). All MT-MMPs except MT4-MMP (MMP-24) are capable of activating MMP-2. They can digest a number of ECM molecules such as gelatin, laminin, and fibronectin (Jones, Sane et al. 2003). In addition MT1-MMP (MMP-14) digests type I, II, and III collagens (Ohuchi, Imai et al. 1997).

1.8.4.1.6 Other MMPs

Some MMPs are not classified in any groups mentioned above. These include MMP-12, MMP-19, MMP-20, MMP-22, MMP-23, and MMP-28.

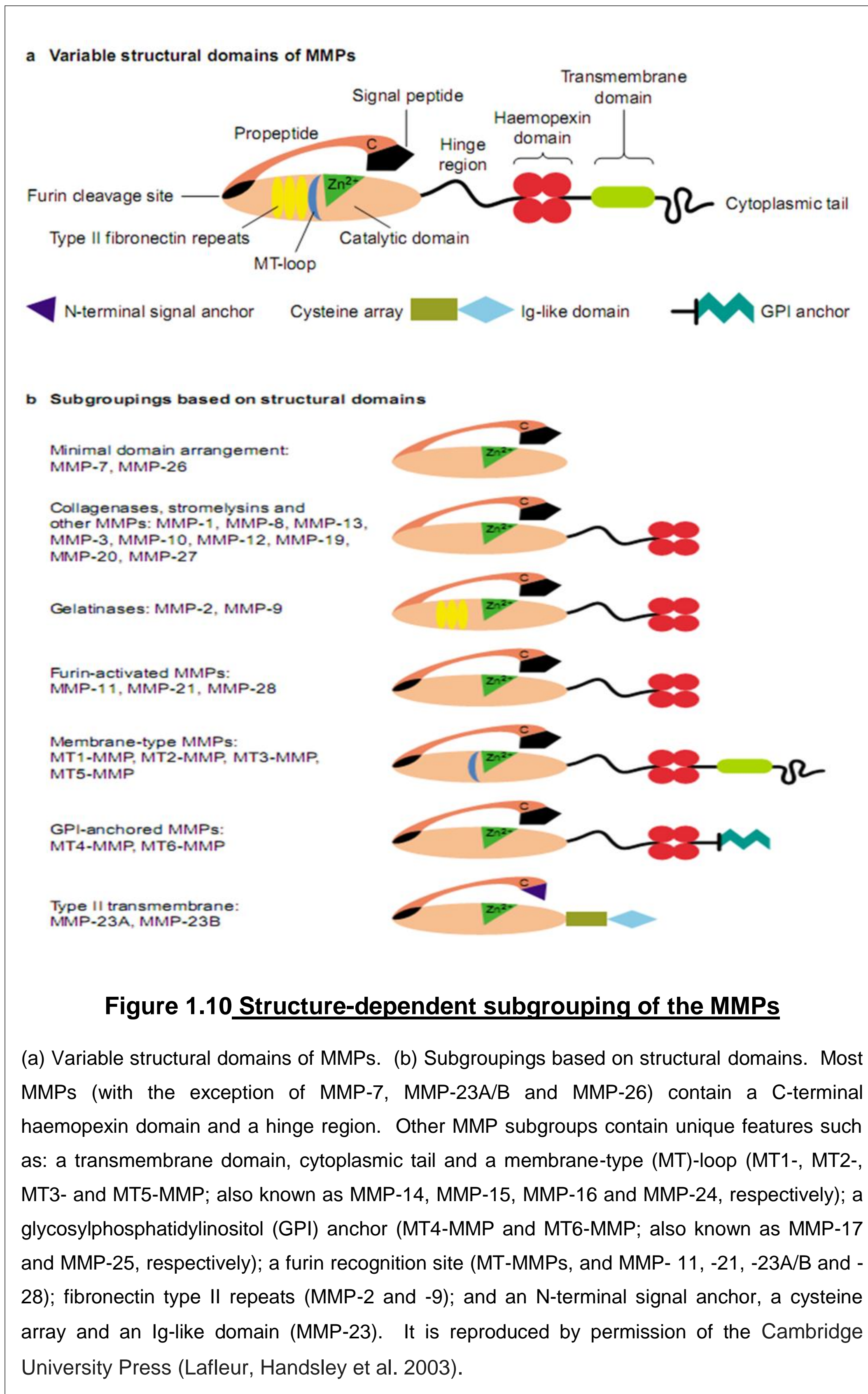


Figure 1.10 Structure-dependent subgrouping of the MMPs

(a) Variable structural domains of MMPs. (b) Subgroupings based on structural domains. Most MMPs (with the exception of MMP-7, MMP-23A/B and MMP-26) contain a C-terminal haemopexin domain and a hinge region. Other MMP subgroups contain unique features such as: a transmembrane domain, cytoplasmic tail and a membrane-type (MT)-loop (MT1-, MT2-, MT3- and MT5-MMP; also known as MMP-14, MMP-15, MMP-16 and MMP-24, respectively); a glycosylphosphatidylinositol (GPI) anchor (MT4-MMP and MT6-MMP; also known as MMP-17 and MMP-25, respectively); a furin recognition site (MT-MMPs, and MMP- 11, -21, -23A/B and -28); fibronectin type II repeats (MMP-2 and -9); and an N-terminal signal anchor, a cysteine array and an Ig-like domain (MMP-23). It is reproduced by permission of the Cambridge University Press (Lafleur, Handsley et al. 2003).

1.8.4.2 MMP Regulation

MMPs are not only regarded as proteinases of matrix catalysis, but also regarded as extracellular processing enzymes which are involved in a range of different processes for example cell-cell signaling (Vu and Werb 2000, Page-McCaw, Ewald et al. 2007). MMPs are regulated at four points. These points are gene expression, compartmentalization, activation and inactivation of enzyme (Ra and Parks 2007). In addition to that, they are controlled via substrate availability and substrate affinity. In general, MMP activity is higher in organs that are rich in basement membrane.

1.8.4.2.1 The Cysteine Switch.

MMPs are synthesized in a large latent form (pro-MMPs) which requires activation. Cysteine residue 73 (Cys73) is present in the propeptide which acts as a stabilizer of the proenzyme (Springman, Angleton et al. 1990, Van Wart and Birkedal-Hansen 1990) via interaction with a Zn^{2+} in the catalytic domain (Figure 1.11) (Snoek-van Beurden and Von den Hoff 2005). Activation of MMPs involves breakdown of this Cys73- Zn^{2+} bond. This mechanism is called “the cysteine switch” (Springman, Angleton et al. 1990, Van Wart and Birkedal-Hansen 1990). Here a water molecule must bind to the Zn^{2+} ion causing dissociation of the Zn^{2+} - cysteine bond. This leads to partial activation of the enzyme (Springman, Angleton et al. 1990). The pro-domain of the MMP is then detached via autolytic cleavage or via other proteases (Springman, Angleton et al. 1990, Van Wart and Birkedal-Hansen 1990). This cleavage leads to a reduction in molecular mass and a fully active enzyme. MMPs are generally activated via other proteinases *in vivo,* while *in vitro*, MMPs are also activated via chemical and physical agents such as low pH, aminophenylmercuric acetate (APMA) and heat treatment (Springman, Angleton et al. 1990, Nagase and Woessner 1999, Visse and Nagase 2003).

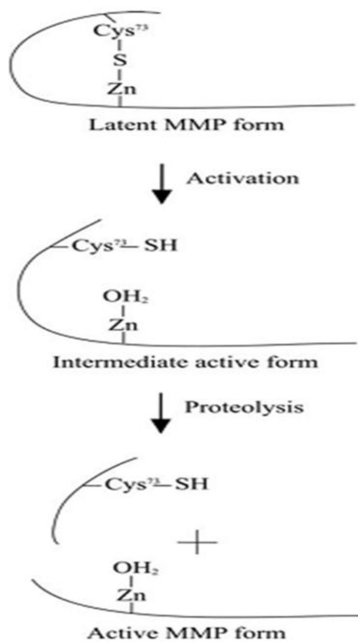


Figure 1.11 The activation of pro-MMPs

The activation of latent MMPs involves a disruption of the Cys73-Zn^{2+} bond that results in an intermediate active form. The fully active MMP form is formed in vivo through proteolysis. MMP, matrix metalloproteinase. It is reproduced by permission of the BioTechniques (Snoek-van Beurden and Von den Hoff 2005)

1.8.4.2.2 Allosteric Activation

Cysteine-zinc forms undergo allosteric disruption, which leads to a transitional active state that permits pro-domain autolytic cleavage (Ra and Parks 2007).

1.8.4.2.3 Furin Activation

MMPs are mostly activated by proteolysis and 1/3 of the MMPs have an RRKR or RXKR motif sequence and they are located in between the pro and catalytic domains. Furins (a subtilisin-like serine protease) or pro-protein convertases target these motifs. Furin locates in the trans-Golgi network (Thomas 2002). Furin cleavage of MMPs leading to activation occurs inside cells, with secretion of active enzyme (Pei and Weiss 1995, Illman, Keski-Oja et al. 2003).

1.8.4.2.4 Activation of Pro-MMPs by MMPs

Several MMPs have the ability to activate other MMPs by cleavage of the pro-domain of MMP zymogens. The final proteolytic step may be mediated by active MMPs to yield the fully active enzyme (Nagase 1997).

Cleavage initially takes place courtesy of a serine protease or an MMP; however it is not clear if the activation is a result of direct proteolysis or of downstream mechanisms. Cleavage of pro-MMP2 by MMP14 is an example of direct activation of Pro-MMPs by MMPs (Strongin, Collier et al. 1995, Caterina, Yamada et al. 2000). The activation occurs at the cell surface in a process in which pro-MMP2 requires MMP14 and TIMP2 with a stoichiometric ratio of 1:1:1. A specific amount of TIMP2 boosts proMMP2 activation, on the other hand additional amounts of TIMP2 block the activation (Strongin, Collier et al. 1995, Worley, Thompkins et al. 2003).

1.8.4.2.5 Activation by Plasmin

The MMP and fibrinolytic (plasminogen/plasmin) systems work to breakdown most ECM components. Some ECM components, such as fibronectin and laminin, are better degraded directly by plasmin (Lijnen 2001). MMPs also degrade other components like elastin and collagen. However, several pro-MMPs are activated by the plasmin system (Galis and Khatri 2002) such as MMPs-1, 3, 7, 9, 10 and 13 (Amalinei, Caruntu et al. 2007). In cell culture, pro-MMP2 and 9 are activated by plasmin without requiring other proteases (Monea, Lehti et al. 2002). Of note, other groups showed that plasmin does not activate pro-MMP9 (Okada, Gonoji et al. 1992).

1.8.4.2.6 Activation by other Serine Proteases

Mast cell chymases is another group of serine proteases that may play a role in activating MMPs (Fang, Wolters et al. 1999). Activation of pro-MMPs 2 and 9 is decreased in mice deficient in mast cell chymase-4 (Tchougounova,

Lundequist et al. 2005). MMP-1 is activated by many serine proteases such as trypsin, cathespin G, neutrophil elastase, chymase, and tryptase (Saunders et al., 2005).

1.8.4.2.7 Oxidative Control

Oxidants are generated by leukocytes. Oxidative control can inactivate or activate MMPs. Activation occurs by oxidation of the pro-domain thiol leading to and autolytic cleavage (Ra and Parks 2007). Reactive oxygen species (ROS) activate a number of pro-MMPs. Pro-MMPs 1, 7 and 9 are activated by hypochlorous acid (HOCl) (Fu, Kassim et al. 2001) and pro-MMPs 1, 2 and 9 are activated by peroxynitrate (Okamoto, Akaike et al. 1997). High concentrations of HOCl also inactivate the enzyme (Fu, Kao et al. 2004). HOCl may plays a role in MMPs activity regulations in an inflammatory setting (Ra and Parks 2007).

1.8.4.2.8 Compartmentalisation

In diseases such as chronic inflammation or cancer, all MMPs are expressed, however selectivity is conferred by compartmentalization and enzyme affinity (Ra and Parks 2007). Some substrates are degraded by some enzymes better than others. For example, type 4 collagen is degraded better by MMPs 2 and 9 in comparison to the other MMPs (Mackay, Hartzler et al. 1990). MMPs are selective catalysts in tissue, where they regulated by enzyme concentration, substrate concentration, and affinity.

The location as well as means by which an MMP will be released into the pericellular environment is important in the proteolysis specificity. This is called ‘compartmentalisation’. For instance, the $\alpha_2\beta_1$ Integrin is binding to the MMP-1 (Dumin, Dickeson et al. 2001), $\alpha_v\beta_3$ Integrin to the MMP-2 (Brooks, Stromblad et al. 1996), proteoglycans to the MMP-7 (Yu and Woessner 2000), CD44 to the MMP9 (Yu and Stamenkovic 2000). This may play a role as an enzyme

activation mediator and the recruitment of the substrate which leads to increase in the proteolysis (Ra and Parks 2007).

1.8.4.3 Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs are specific inhibitors for MMPs. The family of TIMPs consists of four members;TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Ronco and Chatziantoniou 2008). The common feature in the TIMP family is that all have 12 cysteine residues that form 6 disulphide bonds. Three of these bonds are in the N-terminal domain, while the other three are in C-terminal domain. The responsibility of N-terminal domain is to form tight but non-covalent binding with the active form of MMPs in a 1:1 stoichiometry.

TIMPs 1, 2, and 4 are secreted and act in the extracellular space, whereas TIMP3 is bound to the ECM. TIMPs roles are inhibiting MMP activity, stimulating proliferation of several cell types (Gomez, Alonso et al. 1997, Brew, Dinakarpandian et al. 2000, Clark, Swingler et al. 2008) and stimulating production of progesterone by steroidogenic cells (Boujrad, Ogwuegbu et al. 1995, Nothnick 2003). Regulation of proteolytic activity is made by a balance between active MMPs and TIMPs. Inhibition of MMPs by excess TIMPs is believed to underlie tissue scarring & fibrosis (Johnson, Haylor et al. 2002). TIMPs also have important effects in cell proliferation, anti-apoptotic processes, and anti-angiogenesis. Many of these are independent from the MMP activity (Brew and Nagase 2010).

Most of ADAM and ADAMTS families are inhibited by TIMP-3 (Murphy 2008). TIMP-3 is different from the rest of TIMPs by it is property of tightly binding to the ECM (Pavloff, Staskus et al. 1992). The rest of TIMPs have a limited role in inhibiting ADAMs and ADAMTS. ADAM10 and 12 are inhibited by TIMPs 1 and 2 respectively (Amour, Knight et al. 2000, Jacobsen, Visse et al. 2008). ADAMs17 and 28 are inhibited by N-TIMP4, along with TIMP-3 (Mochizuki, Shimoda et al. 2004, Lee, Rapti et al. 2005).

TIMPs have an N-terminal domain of ≈ 125 amino acids and a C-terminal domain of≈ 65 amino acids. Most of the biological functions of these proteins are attributable to the N-terminal domain, though the C-terminal domain mediates interactions with the hemopexin domains of MMP 2, 9 and with the catalytic domains of some MMPs (Brew, Dinakarpandian et al. 2000). The N-terminal domain folds like a separate unit and is able to inhibit MMP activity (Murphy, Houbrechts et al. 1991). TIMPs and their inhibitory effects are shown in (Table 1.2) (Ahmed 2009). The four types of TIMPs are described below.

1.8.4.3.1 Tissue inhibitor of metalloproteinase-1 (TIMP-1)

TIMP-1 (28 KD) was discovered in the 1970's and was at first considered to be a collagenase inhibitor (Bauer, Stricklin et al. 1975). Its name was changed to 'Tissue inhibitor of metalloProteinases' because it was found that it is inhibiting interstitial collagenases, proteoglycanase as well as gelatinases (Cawston, Galloway et al. 1981). It is a 184 amino acid residue glycosylated protein, however glycosylation is not essential for activity (Schultz, Silberman et al. 1988). It is produced by inflammatory cells and all mesenchymal tissues (Ahmed 2009).

1.8.4.3.2 Tissue inhibitors of metalloproteinase-2 (TIMP-2)

The molecular weight for TIMP-2 is 21 KD. Thirty eight percent of TIMP-2's amino acid sequence is similar to that of TIMP-1. In addition to the role of TIMPs in inhibiting metalloproteinase activity, TIMP-1 and TIMP-2 have other significant biological functions. TIMP-1 and TIMP-2 have cell growth–promoting activities (Hayakawa, Yamashita et al. 1992, Hayakawa, Yamashita et al. 1994) and erythroid-potentiating activity (Stetler-Stevenson, Bersch et al. 1992)

1.8.4.3.3 Tissue inhibitors of metalloproteinase-3 (TIMP-3)

TIMP-3 (24 KD) differs from the other members of the TIMP family by its potential inhibitory properties. TIMP-3 inhibits A Disintegrin and Metalloproteinase-17 (ADAM-17 (TACE)) (Amour, Slocombe et al. 1998), ADAM-

10 (Amour, Knight et al. 2000) , ADAM-12 (Loechel, Fox et al. 2000), and the aggrecanases (ADAMTS-4 and ADAMTS-5) (Kashiwagi, Tortorella et al. 2001). It has been found by kinetic studies that TIMP-3 is a better inhibitor for aggrecanases and ADAM-17 than for MMPs. Another unique characteristic of TIMP-3 is that it attaches tightly to sulfated glycosaminoglycans (Yu, Yu et al. 2000).

1.8.4.3.4 Tissue inhibitors of metalloproteinase-4 (TIMP-4)

TIMP-4 (26KD) is the most recent TIMP to be discovered. TIMP-4 is structurally similar to TIMP-2 and TIMP-3 (Leco, Apte et al. 1997). TIMP-4 binds with proMMP-2 in a similar way to that of TIMP-2 (Bigg, Shi et al. 1997).

TIMPS	Inhibitory effect
TIMP-1	All the latent proMMPs and active MMPs. Does not inhibit MMP-14, MMP-16, and MMP-24.Poor inhibitor for MMP-19 and a number of MT-MMPs.
TIMP-2	All the latent proMMPs and active MMPs
TIMP-3	All the latent proMMPs and active MMPs. Inhibits A Disintegrin and a Metalloproteinase (ADAMs), as well as the matrix-associated ADAM with a a thrombospondin like motif (ADAM-TS)
TIMP-4	MMP-1, MMP-2, MMP-3, MMP-7, MMP-9

Table 1.2 Different types of TIMPs with their inhibitory effects

Inhibitory profile for TIMP-1, TIMP-2, TIMP-3, and TIMP-4. From Ahmed, A.K.H. 2009 (Ahmed 2009).

1.8.4.4 ADAMTS

The A Disintegrin And Metalloproteinase with Thrombo Spondin motifs (ADAMTS) is a unique family of ECM proteases. ADAMTS are present in invertebrates and mammals. There is similarity a between A Disintegrin And Metalloproteinase (ADAM) and ADAMTS is their involvement in proteolytic processing of cell surface molecules (Wolfsberg, Straight et al. 1995). The difference is that the ADAM family lacks the binding motif for the catalytic zinc to

several proteins. That is why ADAM has lack in any catalytic activity. The ADAMTS interact with some components of ECM for example, procollagen (Jones and Riley 2005). Different ADAMTS degrade different ECM components. The known actions of ADAMTS are illustrated in (Table 1.3), however some actions of ADAMTS are unknown (Porter, Clark et al. 2005).

ADAMTS	Degradation
1, 4, 5, 8, 9 and 15 (known as proteoglycanases)	proteoglycans aggrecan, brevican, and versican (Apte 2009).
2 and 3	pro-collagens I, II and III N-propeptides.
13	von Willebrand factor (Zheng, Chung et al. 2001)
15	aggrecan (Sugimoto, Takahashi et al. 1999).

Table 1.3 Different ADAMTS targets within the ECM

ADAMTS have a specific but wide range of proteolytic targets.

1.8.4.5 The Plasmin system

The plasmin system has an important role in the coagulation and fibrinolysis cascades in the blood. The precursor plasminogen is activated to plasmin by two main activators named tissue-type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA). The inhibitors of plasmin are α2-macroglobulin and α2-antiplasmin. The tPA and uPA activities are regulated by plasmin activator inhibitors 1 and 2 (PAI-1 and PAI-2).

1.8.4.5.1 Plasminogen

The serine protease plasmin has a pro-enzyme precursor called plasminogen. Human plasminogen is a single chain, 90kDa, multidomain glycoprotein that is mainly produced in the liver (Forsgren, Raden et al. 1987). There are two forms of the plasminogen called Lys-Plasminogen and Glu-Plasminogen (Violand and Castellino 1976). The two main activators tPA and

uPA (physiological activators) cleave plasminogen to plasmin for activation (Dano, Andreasen et al. 1985).

1.8.4.5.2 Function of plasmin

Plasmin has several functions. The main function is the cleavage at specific sites of insoluble fibrin polymers to produce soluble fragments (Walker and Nesheim 1999). Plasmin also plays an important role as a proteolytic factor in many physiological processes including degradation of the ECM, tissue repair, angiogenesis, wound healing embryogensis as well as pathogen and tumour cell invasion (Strickland, Reich et al. 1976, Gross, Moscatelli et al. 1983, Ossowski and Reich 1983, Dano, Andreasen et al. 1985, Schafer, Maier et al. 1994).

Plasmin degrades ECM proteins such as collagens, laminins, and fibronectin (Bonnefoy and Legrand 2000, Nakagami, Abe et al. 2000, Netzel-Arnett, Mitola et al. 2002). Plasmin also degrades blood proteins such as von Willebrand factor, factor FVa, and thrombospondin (Hamilton, Fretto et al. 1985, Bonnefoy and Legrand 2000, Zeibdawi and Pryzdial 2001). The other function of plasmin is activation of signalling pathways via protease activated receptors. Plasmin also plays a role in nicotine reward regulation (Nagai and Yamada 2008).

1.8.4.5.3 Activators of plasminogen

The main physiological activators of plasminogen are tPA and uPA. They are multi-domain serine proteases. They catalyse the same reaction i.e. plasminogen is activated to plasmin through Arg^{561}-Val^{562} peptide bond. The difference is in their biological roles. tPA has a role in fibrin polymer dissolution by plasmin as part of vascular haemostasis, whereas uPA has role in the pericellular plasmin activity generation, for example, in degradation of the ECM (Schaller and Gerber 2011).

1.8.4.5.4 Plasmin Inhibitors

1) α-2 Antiplasmin

α-2 antiplasmin (67kDa) is a single-chain plasma glycoprotein. It is the main physiological plasmin inhibitor and is synthesised predominantly in the liver (Saito, Goodnough et al. 1982, Sumi, Ichikawa et al. 1989). It is a member of the serpin family.

2) Plasmin Activator Inhibitor 1 (PAI-1)

PAI-1 (50kDa) is a single chain plasma glycoprotein from the serpin family of proteases. It is produced by various cells such as liver, endothelial, and smooth muscle cells (Alessi, Peiretti et al. 1997). It is the main inhibitor for both activators of plasminogen. Other serine proteases, such as trypsin and thrombin, are inhibited by PAI, but at very low rates, (Hekman and Loskutoff 1988, Lawrence, Strandberg et al. 1989, Keijer, Linders et al. 1991).

The excessive accumulation of collagen in the ECM is determined by an increase in the collagen synthesis rate and/or decrease in the collagen degradation rate by proteolytic activities. The uPA/tPA and plasmin play important roles in the ECM proteolytic degradation that is required to maintain tissue ECM homeostasis. The activities of plasmin-dependent MMPs, tPA, uPA, and thus plasmin depend partly on the activity of PAI-1. In normal physiological conditions, the proteolytic activities of plasmin-dependent MMPs, tPA, uPA, and plasmin are controlled by PAI-1 which leads to maintains the tissue homeostasis. In contrast to the above (in the pathologic conditions), there are excessive collagen accumulation as well as other ECM proteins in the wound area due to excessive PAI-1. This will lead to scarring preservation (Ghosh and Vaughan 2012).

PAI-1 and tPA are involved in fibrogenesis of several organs and they are produced by MCs. PAI1 and t-PA were produced by HPMC in an in vitro study in response to glucose based peritoneal dialysis solutions (Katsutani, Ito et al. 2007). Peritoneal fibrosis in a mouse model showed that tPA exacerbates peritoneal fibrosis, peritoneal inflammation and neoangiogenesis (Kurata, Maruyama et al. 2009). It is not clear whether plasminogen is playing a role in the EPS development or not, but it had been shown that MCs express PAI-1 and PAI-2 under the effect of TGF-β (Rougier, Guia et al. 1998, Holmdahl, Kotseos et al. 2001). This inappropriate balance of fibrin deposition against breakdown will lead to increase in the probability of fibrous adhesion formation which may play a role in the EPS development (Moinuddin, Summers et al. 2014).

3) Plasminogen Activator Inhibitor 2 (PAI-2)

PAI-2 is a serpin protease produced by keratinocytes, monocytes, macrophages, and epithelial cells (Webb, Collins et al. 1987, Wohlwend, Belin et al. 1987). PAI-2 is a single chain and is poorly secreted (von Heijne, Liljestrom et al. 1991) which is why PAI-2 accumulates intracellularly as a non-glycosylated protein with molecular size of a 47kDa (Genton, Kruithof et al. 1987). PAI-2 has the ability to be secreted, but in small quantities as a plasma glycoprotein with size of a 60kDa (Belin, Wohlwend et al. 1989). It inhibits both activators of plasminogen (Ny and Mikus 1997), can inhibit apoptosis, change gene expression, and affect cell proliferation and differentiation (Schaller and Gerber 2011).

4) Neuroserpin

Another serpin protease is neuroserpin which is expressed mainly in the brain (Hastings, Coleman et al. 1997, Schrimpf, Bleiker et al. 1997). It is a single chain, 55kDa glycoprotein (Osterwalder, Contartese et al. 1996, Hastings, Coleman et al. 1997, Yazaki, Liepnieks et al. 2001) and is an inhibitor for both

activators of Plasminogen and plasmin (Hastings, Coleman et al. 1997, Ricagno, Caccia et al. 2009).

5) α2-Macroglobulin

α2 macroglobulin is a large glycoprotein, and is found in both vertebrates and invertebrates as well as reptile eggs (Buresova, Hajdusek et al. 2009, Raymond, Su et al. 2009, Ma, Wang et al. 2010). It is the main anti-proteinase in the plasma of vertebrates. It is the only inhibitor that has ability to inhibit almost all proteinases (Rehman, Ahsan et al. 2013). It inhibits cysteine-, aspartic-, serine-, metalloproteinases, fibrinolysis and coagulation (De Boer, Creasey et al. 1993). The mechanism of inhibition of fibrinolysis and coagulation is by inhibiting plasmin and inhibiting thrombin respectively.

1.9 Simple peritoneal sclerosis and encapsulating peritoneal sclerosis biomarkers and diagnostic tools

In summary, peritoneal dialysis is a successful renal replacement therapy that can be used alone or in conjunction with haemodialysis in patients with end-stage renal failure. PD has particular benefits including: it can be performing at home with increase mobility of the patient and less dietary and fluid restriction. However the long term use of peritoneal dialysis is restricted by sclerosis of the peritoneum and an increasing risk of encapsulating peritoneal sclerosis (EPS) where fibrotic tissue encapsulates the bowel, leading to obstruction and potentially death. The availability of dialysis fluid from PD patients gives us a unique opportunity to get materials from the site of disease both for routine screening and investigation of the disease pathobiology. By characterising changes in ECM related molecules it may be possible to develop noninvasive biomarkers that may predict EPS onset or how advanced PS is reached.

There is disagreement as to whether EPS represents the severe end of a continuum of peritoneal sclerosis (PS) or they are entirely two different pathological entities. Irrespective, it would seem sensible to assume that systems involved in normal extracellular cellular matrix (ECM) homeostatic balance that are altered in fibrotic disorders in other organs may well be implicated in PS and EPS. This would include the down regulation of enzyme systems involved in ECM breakdown such as matrix metalloproteinases (MMPs) and plasmin, or over expression of the natural inhibitors of these enzymes such as tissue inhibitors of matrix metalloproteinases (TIMPs) and plasmin activator inhibitors (PAIs), as well as excess production of ECM components. EPS is associated with severe morbidity and mortality and this complication cause strong negative view of the PD value as a long-term therapy. Moreover, peritoneal cellular study and it is link to ECM is important area to be studied. Last but not least, proteomics analysis of PDE samples to find the proteins that behave differently between EPS and control groups are also of high value.

1.9.1 Hypothesis

This thesis proposes the hypothesis that there are proteins present in the peritoneal dialysis effluent that will inform on the rate of simple peritoneal sclerosis and whether the patient is likely to subsequently develop encapsulating peritoneal sclerosis. These proteins are likely to be those involved in tissue remodeling or fibrosis. This overarching hypothesis is subdivided into 3 mini hypotheses that will be addressed individually

1. ECM proteolysis: Aberrations in systems involved in ECM regulation are already known to play role in numerous fibrotic diseases with perturbations in the MMP and TIMP leading to lower ECM clearance particularly prevalent. It is therefore likely that they may also underlie the development of PS and the switch to EPS. Therefore this thesis will test the hypothesis that changes in the MMP system in the peritoneum underlie PS &/or the switch to EPS and may have values as non-invasive prognostic markers or as diagnostic tools.

2. Peritoneal cellular changes with long duration of PD therapy: It is recognised that ECM processing is very dependent on the cells types present and there is some evidence that the cell population in the peritoneum changes during PD. This thesis will also test the hypothesis that changes in the peritoneal cell population will change with time on PD and in patients with EPS and that these will influence ECM processing

3. The proteomics proteins: Proteomic interrogation of PDE will reveal protein profiles that will link individual protein changes in PDE samples to the development of PS or EPS and these proteins can developed as biomarkers or diagnostic tools

1.9.2 Aims

To test the above hypotheses, Three cohorts of PDE samples; 2 from the Sheffield kidney institute and the global fluid study were used. The aims were divided to:

1.9.2.1 ECM proteolytic

1. Measure total MMP and plasmin activity in PDE samples from PD patients to establish any abnormal levels of ECM proteolysis and their relations with PS and EPS.
2. Determine the contributions of individual proteolytic enzymes and their natural inhibitors by ELISA
3. Determine the value of any identified enzyme or inhibitor in predicting EPS development
4. The rate of transfer of proteins of known molecular weight that are not produced in the peritoneum will be calculated. If TIMP and MMP levels in PDE samples have a potentially higher transfer rate this will indicate local production is occurring.

1.9.2.2 Peritoneal cellular changes with long duration of PD therapy

1. Cells will be isolated from overnight bags.
2. Cells will be stained for different peritoneal cell markers.
3. These will be quantified by cell counting and then comparing each of the peritoneal cells in 3 groups based on time on PD (<1 year, 1-3 years, and >3 years).

1.9.2.3 The proteomics proteins

The aim is to determine if identified proteins by proteomics are biomarkers of, or have diagnostic value in PS and EPS.

CHAPTER TWO

Materials and methods

2.1 Patient Cohorts

Three cohorts of PD patients were consented into the studies presented in this thesis. The three patient groups were termed:-

a) Sheffield Kidney Institute PD cohort 1 (SKI-1),
b) Sheffield Kidney Institute PD cohort 2 (SKI-2),
c) Global Fluid Study (GFS).

For the two Sheffield cohorts (a&b), clinical data was obtained from both the patient record system (Proton) and the peritoneal dialysis database (PDDB) at the Sheffield Kidney Institute (SKI), Northern General Hospital, Sheffield, UK. PD patients in the SKI-1 cohort were subdivided into groups, based on their duration on PD. Both Sheffield PD cohorts were included in the UK EPS Registry and DNA Bank. For the GFS cohort, patient samples were obtained from 4 centers from the UK wing of the Global Fluid Study. The Global Fluid Study consists of approximately 2,000 peritoneal dialysis patients, consented from a variety of centers worldwide. The GFS was designed to collect samples at 3-6 monthly intervals for the duration of renal replacement therapy by peritoneal dialysis. The GFS represents the largest PD patient group in the world available for clinical studies. Samples of peritoneal dialysis effluent (PDE) were collected in a different manner for each patient cohort:-

a) SKI-1 - single PDE samples were collected when patients consented for the EPS registry at a convenient time for the patient.
b) SKI-2 - single PDE samples were collected during the patient's visit to hospital for a peritoneal equilibrium test (PET),
c) GFS - PDE samples were collected sequentially but at irregular intervals.

2.1.1 Sheffield Kidney Institute PD Cohort 1 (SKI-1)

2.1.1.1 Ethics

The UK EPS and DNA Bank Study received national ethics approval on 12th July 2006 (REC reference number: 06/Q1407/94). Local ethics approval for Sheffield Hospitals Trustees was received on 6th December 2006. National ethics was extended in July 2011. PDE samples were collected at the Sheffield Kidney Institute, Northern General Hospital, Sheffield, UK. The appropriate consent forms (version 4), study protocol (version 4), and participant information sheet (PIS) (version 5) are attached in Appendix 2.

Informed, written consent was obtained for all those PD patients who agreed to participate in the study. The PIS sheet was given to all participants in advance of the study. Withdrawal from the study was permitted at any time, without requiring the patient to give any reason or explanation. The decision by any PD patient to withdraw from this study had no effect on the standard of care received within the renal unit.

2.1.1.2 Patients

Thirty-one patients on PD at the Sheffield Kidney Institute were consented into this cohort. PDE samples were collected from December 2007 to January 2011. The inclusion criteria included any patient on PD aged 18 years or over. The major exclusion criteria were a diagnosis of peritonitis in the two month period prior to sample collection and patients who were too ill to consent. Samples were collected from a range of patients; from those just starting on PD to patients who had been on PD for a period of up to 6 years. The study was cross sectional.

2.1.1.3 Sampling and clinical data

Single PDE sample was collected from each patient. PDE samples (20mL) were aliquoted into smaller volumes (10 x 1mL, 40 x 250 µL), and stored at -80°C. Aliquoted samples were used once for each assay to avoid the possibility of protein degradation by freeze/thaw cycles. Dwell times were not recorded for this cohort and unlike the SKI-2 cohort, samples were not collected during a peritoneal equilibrium test (PET). D/P creatinine (D/Pcr), 24hr urine output and UF data were also unavailable on the day of the samples were taken. Clinical data were therefore obtained on a date, as close as possible to the date of sample collection. The other data collected were age, gender, ethnicity, duration on PD, renal and peritoneal Kt/V, type of PD mode on the day of PDE sample, and number of peritonitis episodes. Samples collected when line flushing occurred were not differentiated from samples without and thus some differences could occur in sample dilution. Samples collected with line flushing were inevitably more dilute, leading to artificially low measurements of the concentration of different proteins in the PDE samples as determined by ELISA. The line flush samples would have the effect of diluting the samples by about 10% (a 200 mL flush in a 2 litre bag of dialysate), but it could be less or more than 10%.

2.1.1.4 Sample Grouping

Only 1 of the 31 samples collected in the SKI-1 cohort was taken from a PD patient after a diagnosis of EPS. PD patients contributing the other 30 samples of PDE were grouped according to their duration on PD. Patients providing samples within 1 month of starting PD formed a separate group because inflammation and other changes were expected during the first month on PD. An alternative view for separating this group is because it represents a more normal biology of Peritoneum.

2.1.2 Sheffield Kidney Institute PD Cohort 2 (SKI-2)

2.1.2.1 Ethics

The ethics and the study design were similar to the first Sheffield PD cohort, with a slight change in protocol (i.e. version 6 of protocol was followed). PDE samples were collected at the Sheffield Kidney Institute, Northern General Hospital, Sheffield, UK. The appropriate consent forms (version 6), study protocol (version 6), and participant information sheet (PIS) (version 6) are attached in the Appendix 3. Protocol amendment to version 6 was received on 23 March 2011 (STH ref: STH14477). PD patients consented into SKI-1 and SKI-2 were subsequently included within the UK EPS Registry and DNA Bank sponsored by Central Manchester and Manchester Children's University Hospital (CMMC). Version 6 of the ethical approval allowed collecting an increased volume of PDE sample (a whole PD bag). It also allowed repeat sample collection, cell recovery and plasma sample collection to measure molecules that might give information about the causes of EPS.

2.1.2.2 Patients

Fifty-one patients on PD at the Sheffield Kidney Institute were consented to the specified ethics and utilised for this cohort. Plasma and PDE samples were collected from April 2011 to July 2012. The inclusion criteria and the exclusion criteria were same as SKI-1. PDE samples were collected from patients ranging from those just started PD to patients who had been on PD for more than 10 years. This was a cross sectional study. There were no EPS patients in this cohort, but detailed clinical data was collected to help in identifying the peritoneal membrane status.

2.1.2.3 Samples and clinical data

Serial PDE samples were collected from each patient. PDE samples were collected from 4 different dwell times (0, 2hr, 4hr, overnight). Large volumes of

samples were aliquoted and stored at -80°C. All samples were collected during a PET (Figure 2.1). A plasma sample was also collected at the 2 hour dwell time. All PDE at 2hr dwell time were samples collected without a line flush. Clinical data collected included, age, gender, ethnicity (race), data on 24hr urine output, duration on PD, last year glucose exposure, UF, D/P creatinine at 2hr and 4hr, D/D0 glucose at 2hr and 4hr, renal and peritoneal Kt/v, number of peritonitis episodes, eGFR, drain time, peritoneal and renal creatinine clearance, residual volume (RV) and type of PD mode on the day of PDE sample. The glucose calculation was used to estimate the last year glucose exposure before the date of the PDE sample.

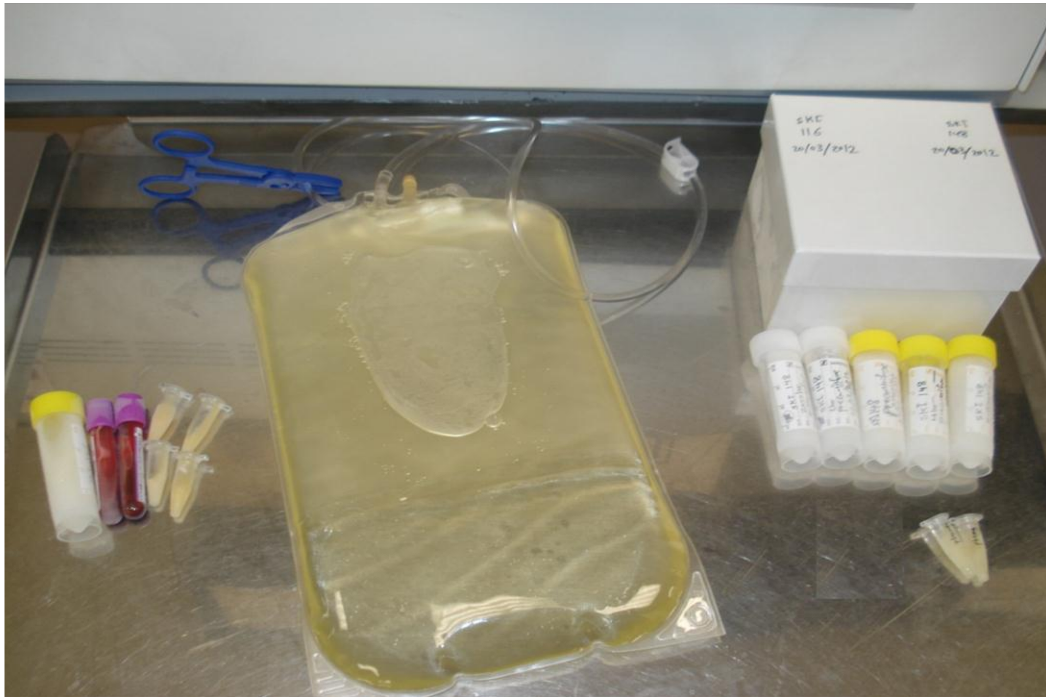


Figure 2.1 <u>Sheffield Kidney Institute Cohort 2 PD and plasma sample collection</u>

Far left, Aliquots of complete peritoneal dialysis effluent (PDE), blood and plasma samples collected to send to UK EPS registry in Manchester. Middle: overnight peritoneal dialysis bag before centrifuge. Far right, PDE samples (complete and cell free PDE samples) at dwell times of zero, 2hr, 4hr, and ON with 2hr plasma samples for Sheffield. Complete samples were collected before centrifuge, while cell free samples were collected after centrifuge.

2.1.3 The Global Fluid Study PD Cohort (GFS)

The Global Fluid Study (GFS) is an international biorepository of PDE and plasma samples. GFS was approved and supported by the British Renal Society (BRS) and the International Society for Peritoneal Dialysis (ISPD).

2.1.3.1 Ethics

The ethics approval for longitudinal evaluation of peritoneal membrane function, inflammation, and structural integrity in PD was given by the Multi-Centre Research Ethics Committee for Wales (Research protocol: MREC 02/9/14) on 16 April 2002. PDE and plasma samples were collected at multi-centres. PDE without plasma samples were utilized for this study. Informed consent was taken from all patients locally. Participants were free to withdraw from the study at any time. The ethical approval is attached in the Appendix 4.

2.1.3.2 Patients

Samples from fifty-four patients on PD were sent to Sheffield Kidney Institute for analysis. PDE and plasma were collected from June 2002 to August 2008. Inclusion criteria and exclusion criteria were same as SKI-1 and SKI-2. This was a longitudinal cohort

2.1.3.3 Samples and clinical data

For the purposes of this study in Sheffield, 201 samples from the UK arm of the GFS were made available which comprised of sequential samples from 54 patients collected at 4 centres. Approximately 2 to 3 mL of peritoneal dialysis effluent (PDE) from each patient was supplied to Sheffield, 400 ul of material was removed for this study & PDE aliquoted into smaller volumes (20 µL). PDE samples were stored at -80°C freezer. Samples were used once for every experiment to avoid freeze/defrosting cycle. Samples consisted of four hour dwell times with a few additional overnight samples available.

All clinical data pertaining to the patients was exported from the GFS database onto an Excel spread-sheet by the GFS team in Cardiff and Stoke. The clinical data included; age at the date of PDE sample, gender, data on duration on PD, 24hr urine output, UF, D/P creatinine at 4hr, renal and peritoneal Kt/v, renal and peritoneal CrCl, number of peritonitis episodes, peritoneal and renal creatinine clearance, reason for stopping PD, systolic and diastolic blood pressure (BP), Pulse pressure (PP), Mean atrial pressure (MAP), weight, height, BMI, type of PD mode on the day of PDE sample, daily UF, UF capacity, daily H20 removal, Glucose exposure rate, 24hr peritoneal protein loss, protein clearance, plasma level of Albumin, Cholesterol, TG's, and HDL.

2.1.3.4 Sample Grouping

GFS samples were classified into two groups. The first group, designated as the control group, included 42 patients who did not subsequently develop EPS. The second group contained 12 patients that ultimately developed EPS (EPS diagnosis according to ISPD criteria). The EPS & control groups are matched for time on PD, number of samples, gender, age, diabetes, and centre.

2.2 Peritoneal equilibrium test (PET)

This is a semi-quantitative test to assess the permeability of the peritoneal membrane (Pannekeet, Imholz et al. 1995, Teitelbaum and Burkart 2003) and to monitor peritoneal transport (Ram 1994). It was initially described by Twardowski et al (1989) to evaluate peritoneal function by assessing the movement of solute. The status of the peritoneal membrane can be grouped into 4 categories; high transporter, high average transporter, low transporter or low average transporter using the PET score (Twardowski, Nolph et al. 1987) (Appendix 5). Population data suggests progression to high solute status correlates with the time spent on PD. This change in transport status may be due to a change in the structure of the peritoneal membrane resulting in

ultrafiltration and/or technical failure (Davies, Phillips et al. 1998). Rather than using categories the D/Pcr, it is often displayed as a continuous variable.

Ultrafiltration failure may result in increased mortality and morbidity, fluid overload, cardiovascular disease and death. Minimizing glucose exposure by using an appropriate dwell length is an important protection against UF failure. For example, PD patients using short dwell times, icodextrin at day time, and utilizing amino acids at night. There is evidence that using icodextrin and APD in anuric patients over two years better preserves UF (Davies, Brown et al. 2005). The PET is used to determine the best mode of PD (Table 2.1), calculate predicted clearance for a given PD regime, measure residual volumes (Appendix 6) and measure UF (Twardowski 1987, Ho-dac-Pannekeet, Atasever et al. 1997).

BASELINE PET PROGNOSTIC VALUE			
Solute Transport	Predicted Response to CAPD		Preferred Dialysis
	UF	Dialysis	
High	Poor	Adequate	CCPD dry day or CAPD dry night
High Average	Poor-Medium	Adequate	Standard CAPD or APD
Low Average	Good	Adequate	Standard CAPD
Low Average	Good	Inadequate	High Dose PD or APD
Low	Very good	Inadequate	High Dose PD or Haemodialysis

Table 2.1 Prescription schedules

Best PD mode for different transport status of peritoneal membrane according to PET results (Twardowski 1989).

2.3 Sample collection

In the SKI-2 cohort, PDE samples were collected at four time points for each patient during the visit for a PET (9am – 2pm). A sample from the prior overnight dwell was collected, followed by, 0hr, 2hr and 4hr samples from the PET. Blood samples from patients were also taken at the 2hr dwell time. During

PET, a 2L of 2.27% Dianeal bag (Baxter Castlebar, Ireland) was warmed to body temperature. The overnight dwell (8-12 hours) was drained over 20 minutes. The patient must be in a vertical position (sitting or standing). Drained volume was measured and recorded. 120 mL of overnight sample (60 mL before centrifuge, and 60 mL after centrifuge) was collected, aliquoted, and stored at -80°C (Figure 2.2). For each sample a unique SKI study identification number was generated. Samples were also sent for PET i.e. for clinical chemistry laboratory for analysis for urea, creatinine and glucose.

Fresh solution was infused in a rate of 200 mL/minute (i.e. total infusion time = 10 minutes). The patient lies in a supine position and rolls from side to side after 400, 800, 1200, 1600, and 2000 mL have been infused to ensure solution mixing. The 0hr dwell sample was collected at completion of infusion. . Subsequent dialysate samples were collected at 2 and 4 hours dwell times. Samples 0, 2 and 4 hr dwell samples were sent for analysis for urea, cr and glucose (PET). Samples were also taken for UK EPS DNA study. They were aliquoted and stored at -80°C. Drain volume was weighed and recorded. Sample volumes were added to give the total drain volume. In addition to the above, 8 slides per patient were prepared for cell staining by cytospining cells recovered from generation of a cell free PDE (Figure 2.3). At 2 hour dwell time, **venous blood samples** were also drawn. For PET, blood samples were sent for analysis of urea, creatinine, and glucose, while for UK EPS DNA study, plasma samples were aliquoted and stored in -80°C (Figure 2.4). PET measures the D/Pcr and D/D_0 glucose. PDE samples, blood samples, slide preparation, and clinical data collection was performed by me, but the PET was performed by nurses with some help from Dr Dina Abdellatif specially in measuring hydrostatic intra peritoneal pressure (IPP) (cm).

Hydrostatic IPP measurement started by connecting the disconnect system at the extension tubing. The patient was placed by lying in supine position in a strictly horizontal plane. Then, zero level of the scale was adjusted on the medial

axillary line. A blue clamp stopped the infusion. The drainage bag was hung up on the stand. The extension catheter was opened and the drainage line was filled with the dialysis fluid. After that, waiting started until the level was stabilized. The patient was advised to breathe normally and hydrostatic IPP was recorded during inspiration and expiration. After readings, the drainage bag was lowered and drainage occurs as usual. The drainage volume was recorded too.

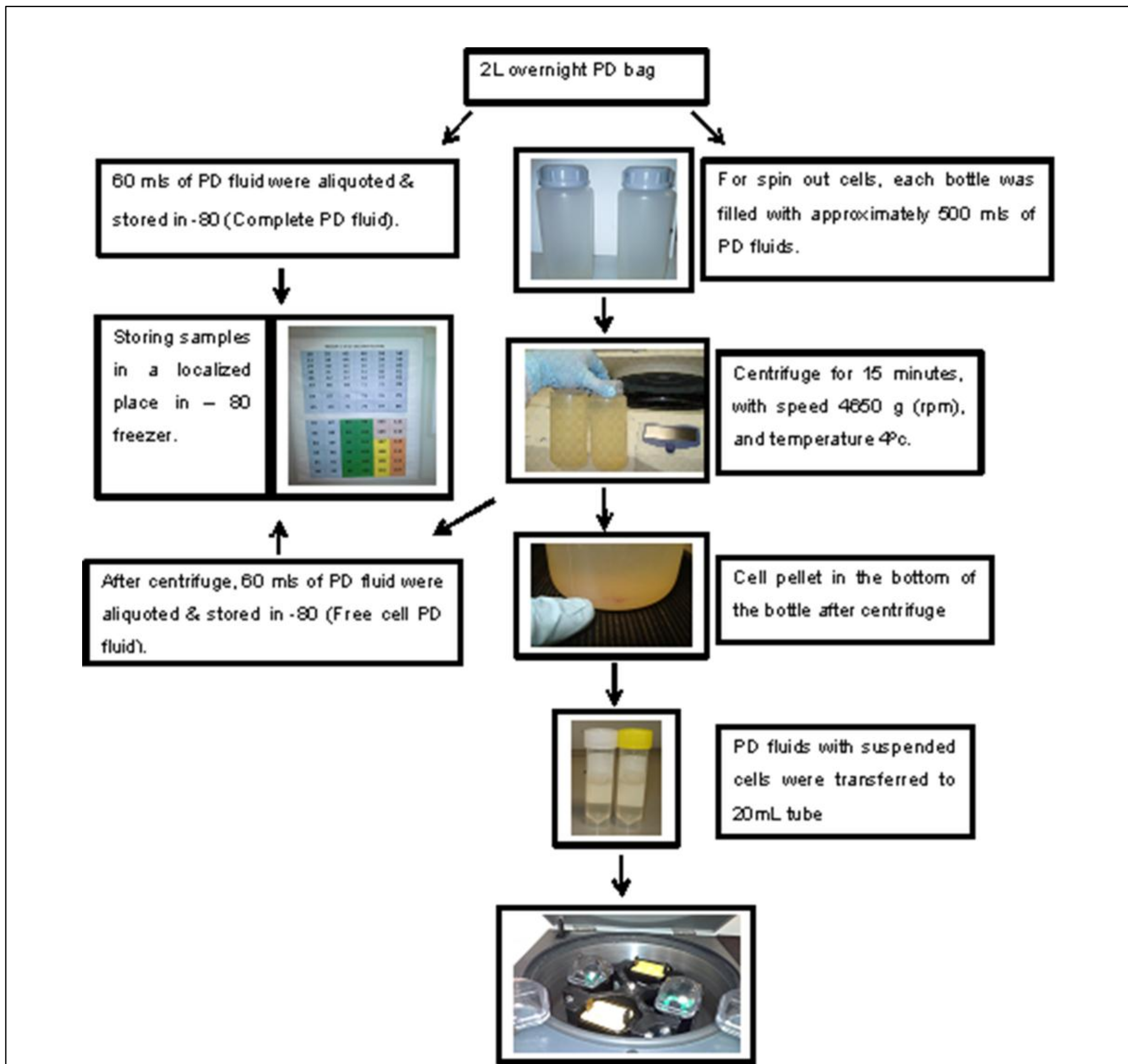


Figure 2.2 <u>Processing of the PDE samples in SKI-2</u>

Peritoneal dialysis effluent (PDE) samples were processed before and after centrifuge in Sheffield Kidney Institute PD Cohort 2 (SKI-2). In addition to that, cells were obtained from spun PDE for cytospin slide preparation.

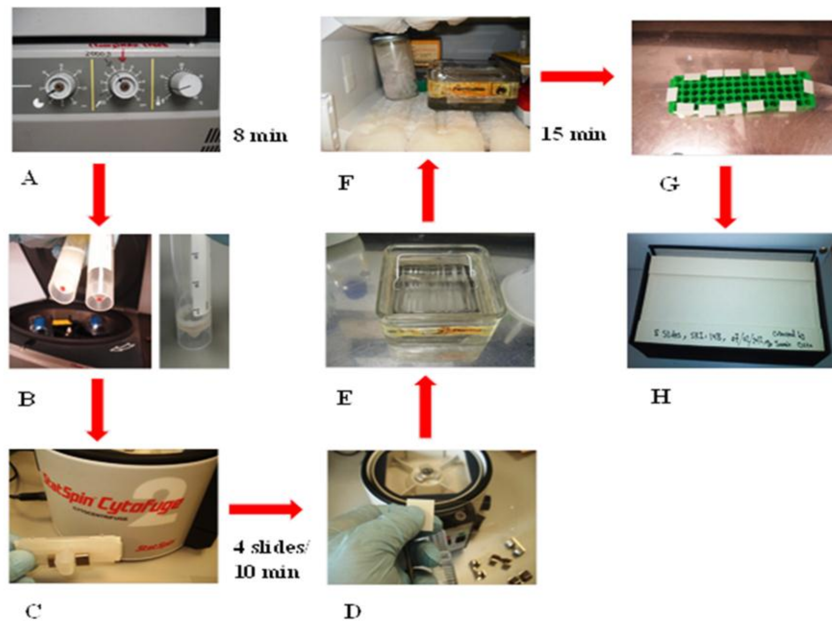


Figure 2.3 Processing PDE samples for cytospin slide preparation & cell staining in the SKI-2 cohort

(A) PDE samples were centrifuged with using a swing out rotor for 8 minutes, at 2000 g and 4ºC. (B) The pellet was suspended in 2ml of PDE and 300 µL loaded onto a cytospin. (C) Slides were spun for 10 minutes. (D) Four slides were obtained in each cycle. (E & F) Cells were fixed in acetone for 10 minutes at -20°C. (G) Slides were then left to dry at room temperature for 15 minutes. (H) Slides were labeled with a pencil & stored at -20°C prior to staining.

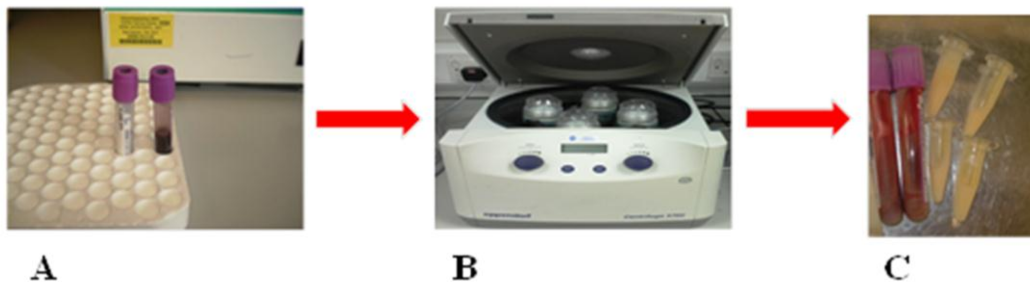


Figure 2.4 Processing blood samples in the SKI-2 cohort

(A) Heparin containing vacutainers were used for collecting blood sample to prevent blood clotting. (B) The tubes were balanced with another tube filled with water & centrifuged using an Eppendorf 5702 centrifuge for 10 minutes at 1100 g. (C) Plasma was separated into eppendorf microfuge tubes & stored at– 80 ^{o}C.

2.4 Healthy volunteers

Data on plasma samples from healthy volunteers was retrieved from studies performed under ethics application12/YH/0297, a parallel study looking at the natural history of diabetic and non-diabetic chronic kidney disease. Data from plasma samples from 18 consented healthy individuals was used in this study along with demographic data including age (at the date of plasma sample collection), gender and ethnicity. Plasma samples were aliquoted into smaller 20 µl volumes, were stored at -80°C and samples thawed just once for analysis to avoid protein degradation by freeze/defrosting cycle.

2.5 Peritoneal dialysis database (PDDB)

The patient record system (Proton) and the peritoneal dialysis database (PDDB) were used for collecting clinical data for the two Sheffield cohorts. In particular PDDB is easily used to facilitate quick access to the information on patients on PD (Figure 2.5). PDDB (developers are Prof Nick Topley, Prof Simon Davies, Dr Kit Huckvale and Dr James Chess) has a unique timeline feature that gives an easy, clear, direct view of the patients' renal replacement history. PDDB has the ability to identify patients who have consented into the UK EPS Study and those who have not. It contains a collection of information on PD patients in one place. It calculates Kt/V to ensure that all sites across the UK will be using same, standard calculation.

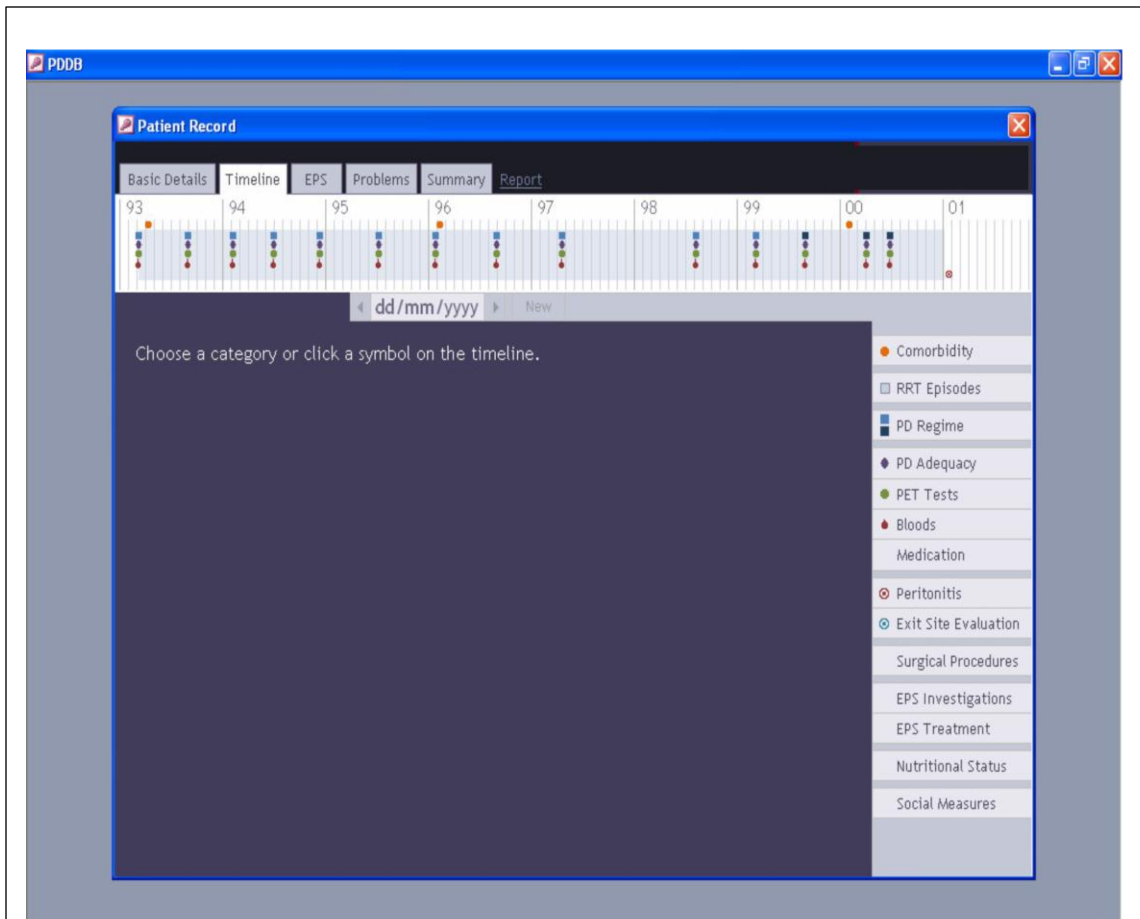


Figure 2.5 <u>Screen print of PDDB</u>

This screen print shows the timeline feature at the top of the entering area. Patient's name and hospital number is obscured for confidentiality reason. It is reproduced by permission of the UK EPS Registry & DNA Bank, 2008.

2.6 Calculations

2.6.1 Glucose exposure in last year

Glucose exposures for all patients in the SKI-2 cohort were calculated as illustrated in (Table 2.2). This example shows that this patient had different peritoneal regimes in the last year before donating sample for UK EPS DNA study. Therefore glucose exposure was calculated in each period. After that the sum of all glucose exposures in the last year was calculated.

Mode	[Glucose] (g/L)	Volume (L)	From	To	Glucose exposure (g)
APD	13.6	5.75	19/04/2010	30/04/2010	860.20
APD	13.6	5.0	30/04/2010	26/05/2010	1,768.00
APD	13.6	6.9	26/05/2010	10/08/2010	7,131.84
APD	13.6	5.55	10/08/2010	02/09/2010	1,736.04
APD	13.6	6.9	02/09/2010	29/10/2010	5,348.88
APD	13.6	5.1	29/10/2010	04/11/2010	416.16
APD	22.7	3.4	29/10/2010	04/11/2010	463.08
APD	13.6	6.0	04/11/2010	18/11/2010	1,142.40
APD	22.7	4.0	04/11/2010	18/11/2010	1,271.20
APD	13.6	8.0	18/11/2010	07/03/2011	11,859.20
APD	22.7	2.0	18/11/2010	07/03/2011	4,948.60
APD	13.6	4.0	07/03/2011	19/04/2011	2,339.20
APD	22.7	6.0	07/03/2011	18/04/2011	5,720.40
Glucose exposure in the last year = 45005.20 g					

Table 2.2 Glucose exposure in the last year

An example of method of calculating glucose exposure in one of the patient in the last year before donating PDE sample to the UK EPS and DNA bank study.

2.7 Demographic and clinical characteristics of Cohorts

2.7.1 Demographic and clinical characteristics in SKI-1 patient cohort

Thirty one patients (19 males and 12 females) donated samples in this cohort. Twenty six patients in this cohort had UO ≥ 200 mL/day, while four of the patients had UO < 200 mL/day and it was not recorded for one patient. Episodes of peritonitis for patients in this cohort varied from patients without a history of peritonitis to those who had up to 4 episodes. Twenty nine patients were from a Caucasian background, while one was African, and another Asian. Any patient with diagnosis of peritonitis in the two months period prior to sample collection was excluded from this cohort. The summary of baseline clinical characteristics

for the SKI-1 cohort is shown in (Table 2.3). The primary renal diagnosis for patient in the SKI-1 cohort is shown in (Figure 2.6).

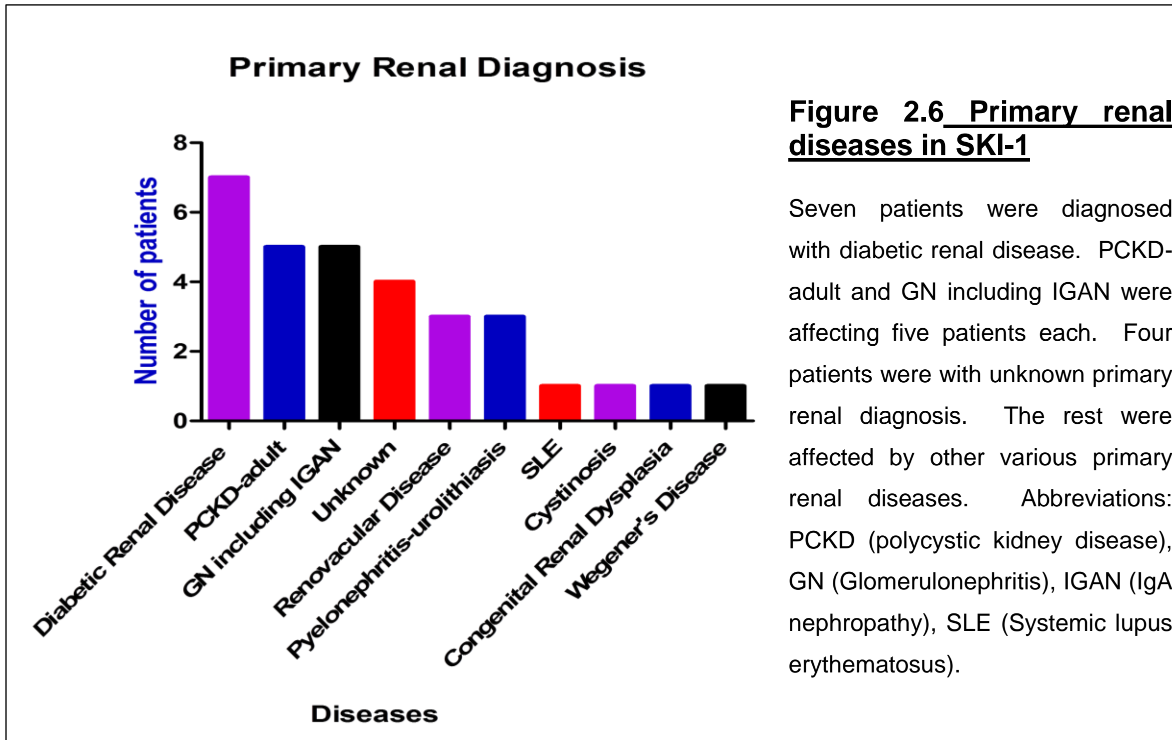


Figure 2.6 <u>Primary renal diseases in SKI-1</u>

Seven patients were diagnosed with diabetic renal disease. PCKD-adult and GN including IGAN were affecting five patients each. Four patients were with unknown primary renal diagnosis. The rest were affected by other various primary renal diseases. Abbreviations: PCKD (polycystic kidney disease), GN (Glomerulonephritis), IGAN (IgA nephropathy), SLE (Systemic lupus erythematosus).

Variables	Number with data available	Mean ± SD	Median (IQR)	Median (Range)
Age at sample (year)	31	56 ± 20	57 (42 - 74)	57 (22 – 88)
Duration on PD (year)	31	2 ± 2	1.3 (0.3 – 3.1)	1.3 (0.003 – 6.9)
Peritonitis episodes	31	0.6 ± 1.1	0 (0 – 1)	0 (0 – 4)
D/Pcr	18	0.72 ± 0.11	0.72 (0.63 – 0.82)	0.72 (0.53 – 0.93)
UF (mL)	17	185 ± 97	200 (113 – 255)	200 (0 – 400)
Peritoneal Kt/V	28	1.26 ± 0.37	1.3 (1.01 – 1.56)	1.3 (0.51 – 2.17)
Renal Kt/V	29	1.02 ± 0.77	1.07 (0.24 – 1.52)	1.07 (0 – 2.97)
Total Kt/V	28	2.32 ± 0.7	2.31 (1.7 – 2.8)	2.31 (1.3 – 4.16)

Table 2.3 <u>Clinical characteristics in the SKI-1 cohort</u>

Clinical parameters recorded in SKI-1 cohort. Abbreviations: peritoneal dialysis (PD), dialysate-to-plasma ratio of creatinine (D/Pcr), ultrafiltration (UF). K - peritoneal clearance of urea, t - dialysis time, and V - volume of distribution of urea (Kt/V).

2.7.2 Demographic and clinical characteristics in SKI-2 cohort

Fifty one patients (29 males and 22 females) were included in this cohort. All patients were from white ethnic backgrounds. The summary of baseline clinical characteristics in the SKI-2 is given in (Table 2.4). Episodes of peritonitis for patients in this cohort were various from patients without history of peritonitis to others who had up to 4 episodes of peritonitis. Sixteen patients were on CAPD during PDE sampling, while thirty five patients were on APD. Any patient with diagnosis of peritonitis in the two months period prior to sample collection was excluded from this cohort. There were wide ranges of primary renal diagnosis in second Sheffield cohort (Figure 2.7).

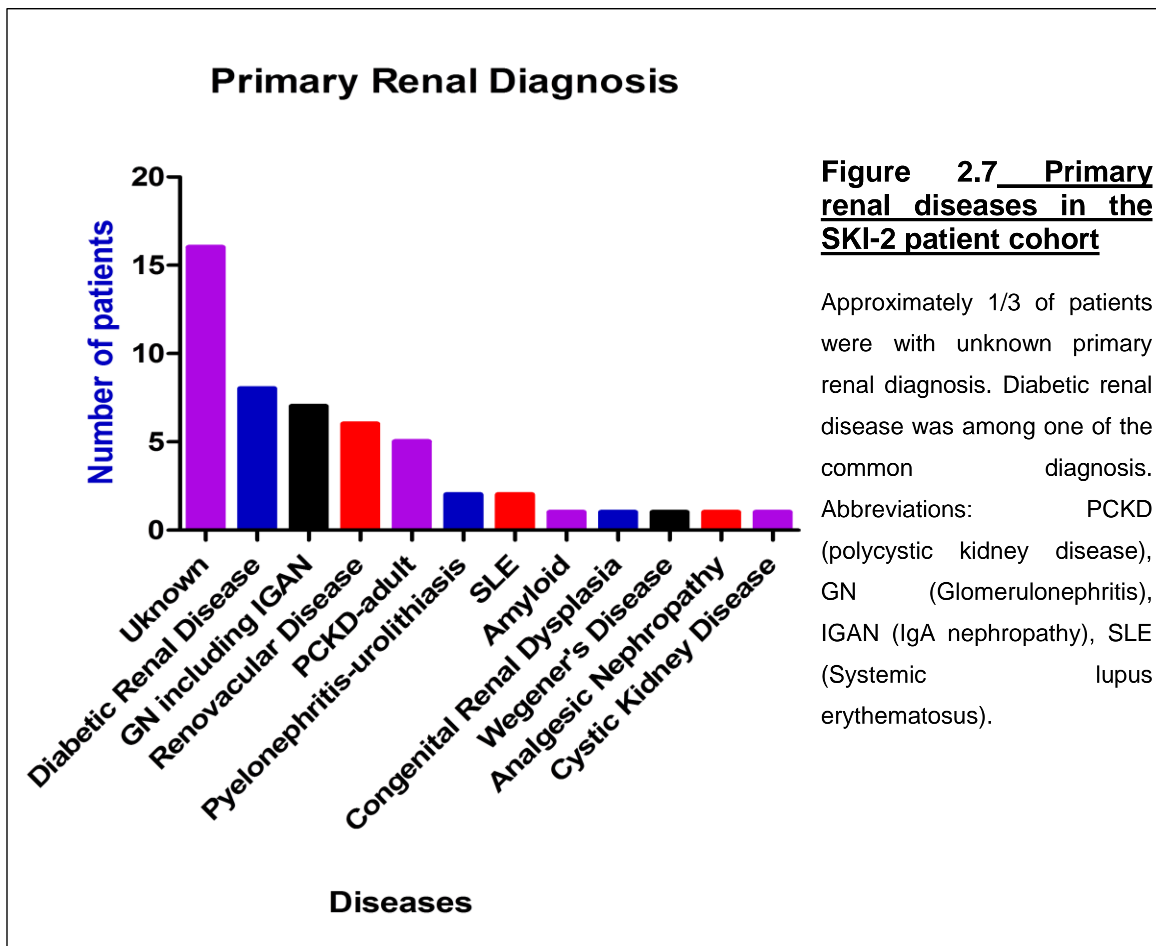


Figure 2.7 Primary renal diseases in the SKI-2 patient cohort

Approximately 1/3 of patients were with unknown primary renal diagnosis. Diabetic renal disease was among one of the common diagnosis. Abbreviations: PCKD (polycystic kidney disease), GN (Glomerulonephritis), IGAN (IgA nephropathy), SLE (Systemic lupus erythematosus).

Variables	Number of patients with data available	Mean ± SD	Median (IQR)	Median (Range)
Age at sample (year)	51	59 ± 16	62 (46 - 72)	62 (20 – 85)
Duration on PD (year)	51	1.8 ± 1.9	1.1 (0.2 – 2.8)	1.1 (0.1 – 9.1)
Peritonitis episodes	51	0.6 ± 1	0 (0 – 1)	0 (0 – 4)
D/Pcr at 2hr	51	0.53 ± 0.1	0.53 (0.46 – 0.6)	0.53 (0.3 – 0.8)
D/Pcr at 4hr	51	0.73 ± 0.1	0.74 (0.66 – 0.79)	0.74 (0.46 – 1)
D/D0 glucose at 2hr	51	0.55 ± 0.1	0.55 (0.48 – 0.6)	0.55 (0.27 – 0.92)
D/D0 glucose at 4hr	51	0.34 ± 0.1	0.34 (0.28 – 0.4)	0.34 (0.12 – 0.62)
UF capacity (mL)	48	73 ± 213	128 (-94 – 200)	128 (-475 – 500)
Peritoneal Kt/V	51	1.15 ± 0.4	1.18 (0.85 – 1.3)	1.18 (0.54 – 2.22)
Renal Kt/V	51	1.41 ± 0.93	1.38 (0.64 – 1.98)	1.38 (0 – 4.6)
Total Kt/V	28	2.56 ± 0.8	2.36 (2.03 – 2.92)	2.36 (1.23 – 5.22)
Peritoneal Crcl (l/wk/1.73 m^2)	51	28.1 ± 11	25.7 (19.5 – 33.8)	25.7 (12.5 – 57.5)
Renal Crcl	51	69.8 ± 43.9	66.5 (32.2 – 101.6)	66.5 (0 – 187.8)
Last year glucose exposure (g)	51	22410 ± 16753	24604 (6178 – 36502)	24604 (1877 – 67767)
Urine output (U.O.) (mL/day)	51	1486 ± 864	1400 (820 – 2130)	1400 (0 – 3840)
drain time (min)	42	14.5 ± 4.1	15 (10 – 20)	15 (10 – 20)
residual volume (RV) (mL)	46	279 ± 120	251 (190 – 359)	251 (80 – 590)
eGFR (mls/min/1.73m^2)	51	8 ± 4	6 (5 – 12)	6 (3 – 15)
Intra peritoneal pressure (cm)	35	17 ± 5	16 (13 – 19)	15.5 (10 – 28)
Serum albumin (g/L)	51	39 ± 4	40 (36 – 42)	40 (28 – 47)

Table 2.4 <u>Clinical characteristics in the SKI-2</u>

Mean ± SD, Median (IQR), and (Range) for clinical parameters recorded in SKI-2 cohort. Abbreviations: peritoneal dialysis (PD), dialysate-to-plasma ratio of creatinine (D/Pcr), ratio of dialysate glucose at 4 hours dwell time to dialysis glucose at 0 dwell time (D/D0 glucose), ultrafiltration (UF), K - peritoneal clearance of urea, t - dialysis time, and V - volume of distribution of urea (Kt/V), creatinine clearance (Crcl), estimated glomerular filtration rate (eGFR).

2.7.3 Demographic and clinical characteristics in the GFS cohort

2.7.3.1 Control group (i.e. none EPS)

Forty two patients (18 males and 24 females) were included in the control group of GFS. A total of 158 PDE samples were collected from these patients. Episodes of peritonitis for patients in this cohort were various from patients without a history of peritonitis to others who had up to 14 episodes of peritonitis. Ninety nine samples were collected during CAPD, while fifty three samples were collected during APD. Termination in the control group was due to peritonitis, transplantation, death, or other technique failure without any signs or symptoms of EPS. Baseline clinical characteristics in the control group/GFS are illustrated in (Table 2.5).

2.7.3.2 EPS group

Twelve patients (6 males and 6 females) were included in the EPS group of the GFS cohort. A total of 43 PDE samples were collected from these patients. Episodes of peritonitis for patients in this cohort were various from patients without history of peritonitis to others who had up to 6 episodes of peritonitis. Thirty samples were collected during CAPD, while eleven samples were collected during APD. Termination of PD in this group was due to EPS, transplant, patient choice, UF failure (type 2) or other technique failure, but all of them developed EPS ultimately. Baseline clinical characteristics in the EPS group/GFS are illustrated in (Table 2.6). Comparison of EPS group (GFS), control group (GFS), SKI-1, and -2 cohorts is given in (Table 2.7). One of the interesting finding was better residual renal function in SKI-2 in comparison to the GFS. The median value of renal Crcl in SKI-2, GFS (control), and GFS (EPS) were 66.5, 23.4, and 11.7 respectively.

Variables	Number of patients with data available	Mean ± SD	Median (IQR)	Median (Range)
Age at sample (year)	42	55 ± 15	54 (44 - 66)	54 (18 – 84)
Duration on PD (year)	42	2.8 ± 3	1.7 (0.5 – 4.8)	1.7 (0.02 – 12.7)
Peritonitis episodes	42	1.3 ± 2.8	0 (0 – 1.3)	0 (0 – 16)
D/Pcr at 4hr	42	0.72 ± 0.14	0.72 (0.62 – 0.81)	0.72 (0.47 – 1.06)
Ultrafiltration (UF) capacity (mL)	42	428 ± 272	432 (279 – 582)	432 (-100 – 1610)
Daily UF (mL)	42	911 ± 655	829 (460 – 1406)	829 (-940 – 2485)
Daily water removal (mL)	42	1780 ± 716	1838 (1303 – 2254)	1838 (224 – 3039)
Peritoneal Kt/V	42	1.64 ± 0.48	1.53 (1.34 – 1.9)	1.53 (0.86 – 3.41)
Renal Kt/V	42	0.71 ± 0.73	0.51 (0.12 – 1.22)	0.51 (0 – 2.59)
Total Kt/V	42	2.4 ± 0.8	2.2 (1.8 – 2.7)	2.2 (1.4 – 4.7)
Peritoneal Crcl (l/wk/1.73 m^2)	42	51.4 ± 14	48.1 (42.3 – 58)	48.1 (31.5 – 101.5)
Renal Crcl	42	38.5 ± 41.5	23.4 (6.7 – 64.1)	23.4 (0 – 175.2)
Glucose exposure rate (g/day)	41	133 ± 39	127 (109 – 145)	127 (61 – 261)
Urine output (U.O.) (mL/day)	42	870 ± 746	742 (177 – 1434)	742 (0 – 2466)
Serum albumin (g/L)	42	36 ± 6	37 (32 – 40)	37 (23 – 46)
Weight (Kg)	42	70 ± 15	70 (57 – 83)	70 (45 – 92.5)
Height (m)	42	1.66 ± 0.08	1.64 (1.61 – 1.71)	1.64 (1.52 – 1.89)
Body mass index (BMI) (Kg/ m2)	42	25.2 ± 4.4	24.2 (21.9 – 29.3)	24.2 (18 – 33.9)

Table 2.5 <u>Baseline clinical characteristics in the control group/GFS</u>

Clinical parameters recorded in the control group/GFS. Abbreviations: peritoneal dialysis (PD), dialysate-to-plasma ratio of creatinine (D/Pcr), K - peritoneal clearance of urea, t - dialysis time, and V - volume of distribution of urea (Kt/V), creatinine clearance (Crcl).

Variables	Number of patients with data available	Mean ± SD	Median (IQR)	Median (Range)
Age at sample (year)	12	50 ± 14	51 (40 - 62)	51 (22 – 71)
Duration on PD (year)	12	3 ± 3.2	2.6 (0.5 – 4.3)	2.6 (0.02 – 11.4)
Peritonitis episodes	11	1 ± 1.5	1 (0 – 1)	1 (0 – 5)
D/Pcr at 4hr	12	0.8 ± 0.16	0.78 (0.7 – 0.92)	0.78 (0.52 – 1.07)
Ultrafiltration (UF) capacity (mL)	12	312 ± 141	345 (165 – 440)	345 (81 – 473)
Daily UF (mL)	12	1124 ± 511	1270 (733 – 1500)	1270 (40 – 1698)
Daily water removal (ml)	12	1532 ± 700	1576 (968 – 1771)	1576 (408 – 2752)
Peritoneal Kt/V	12	1.76 ± 0.35	1.75 (1.43 – 1.94)	1.75 (1.38 – 2.48)
Renal Kt/V	12	0.39 ± 0.45	0.22 (0 – 0.74)	0.22 (0 – 1.27)
Total Kt/V	12	2.15 ± 0.26	2.14 (1.9 – 2.34)	2.14 (1.87 – 2.67)
Peritoneal Crcl (l/wk/1.73 m^2)	11	60.5 ± 17.4	58.1 (48.9 – 78.7)	58.1 (29 – 83.9)
Renal Crcl	12	21.1 ± 24.8	11.7 (0 – 42)	11.7 (0 – 69)
Glucose exposure rate (g/day)	12	175.8 ± 76.7	173.6 (113.4 – 222.3)	173.6 (65.3 – 358.7)
Urine output (U.O.) (mL/day)	12	409 ± 522	179 (0 – 771)	179 (0 – 1482)
Serum albumin (g/L)	10	38 ± 2.3	38 (37 – 39)	38 (32 – 40)
Weight (Kg)	12	71 ± 14	69 (59 – 80)	69 (52 – 95)
Height (m)	12	1.65 ± 0.11	1.67 (1.57 – 1.73)	1.67 (1.44 – 1.78)
Body mass index (BMI) (Kg/ m2)	12	26.1 ± 3.9	26.4 (22.5 – 29.1)	26.4 (19.8 – 33.1)

Table 2.6 Baseline clinical characteristics in the EPS/GFS

Mean ± SD, Median (IQR), and (Range) for baseline clinical parameters recorded in the EPS group/GFS. Abbreviations: peritoneal dialysis (PD), dialysate-to-plasma ratio of creatinine (D/Pcr), K - peritoneal clearance of urea, t - dialysis time, and V - volume of distribution of urea (Kt/V), creatinine clearance (Crcl).

Variables	SKI 1	SKI 2	GFS control	GFS EPS
N (male)	31 (19)	51 (29)	42 (18)	12 (6)
Age (year)	57 (22 – 88), n=31	62 (46 - 72), n=51	54 (18 – 84), n=42	51 (40 - 62), n=12
Duration on PD (year)	1.1 (0.003 – 6.9), n=31	1.1 (0.1 – 9.1), n=51	1.7 (0.02 – 12.7), n=42	2.6 (0.5 – 4.3) , n=12
Peritonitis episodes	0 (0 – 1), n=31	0 (0 – 1), n=51	0 (0 – 1.3), n=42	1 (0 – 1), n=11
D/Pcr at 4hr	0.72 (0.63 – 0.82), n=18	0.74 (0.66 – 0.79), n=51	0.72 (0.62 – 0.81), n=42	0.78 (0.7 – 0.92), n=12
D/D0 gluc at 4hr		0.34 (0.28 – 0.4), n=51		
UF capacity (mL)	200 (113 – 255), n=17	128 (-94 – 200), n=48	432 (279 – 582), n=42	345 (165 – 440), n=12
Daily UF (mL)			829 (460 – 1406), n=42	1270 (733 – 1500), n=12
Daily water removal (mL)			1838 (1303 – 2254), n=42	1576 (968 – 1771), n=12
Peritoneal Kt/V	1.3 (1.01 – 1.56), n=28	1.18 (0.85 – 1.3) , n=51	1.53 (1.34 – 1.9), n=42	1.75 (1.43 – 1.94), n=12
Renal Kt/V	1.07 (0.24 – 1.52) , n=29	1.38 (0.64 – 1.98) , n=51	0.51 (0.12 – 1.22), n=42	0.22 (0 – 0.74), n=12
Total Kt/V	2.31 (1.7 – 2.8)), n=28	2.36 (2.03 – 2.92) , n=51	2.2 (1.8 – 2.7), n=42	2.14 (1.9 – 2.34), n=12
Peritoneal Crcl (l/wk/1.73 m^2)		25.7 (19.5 – 33.8), n=51	48.1 (42.3 – 58), n=42	58.1 (48.9 – 78.7), n=11
Renal Crcl		66.5 (32.2 – 101.6), n=51	23.4 (6.7 – 64.1), n= 42	11.7 (0 – 42), n=12
Last year glucose exposure (g)		24604 (6178 – 36502), n=51		
Urine volume (mL)		1400 (820 – 2130), n=51	742 (177 – 1434), n=42	179 (0 – 771), n=12
drain time (min)		15 (10 – 20), n=42		
RV (mL)		251 (190 – 359), n=46		
eGFR (mls/min/1.73m^2)		6 (5 – 12), n=51		
Hydrostatic intra peritoneal pressure (cm)		16 (13 – 19), n=35		
Serum albumin (g/L)		40 (36 – 42), n=51	37 (32 – 40), n=42	38 (37 – 39), n=10
Weight (Kg)			70 (57 – 83), n=42	69 (59 – 80), n=12
Height (m)			1.64 (1.61 – 1.71) , n=42	1.67 (1.57 – 1.73), n=12
BMI (kg/ m^2)			24.2 (21.9 – 29.3) , n=42	26.4 (22.5 – 29.1), n=12

Table 2.7 Baseline Comparison between the 3 patient cohorts

Comparison of EPS and control groups from GFS with SKI-1 and -2 cohorts. Median (IQR) for all variables, except Age of the patients and duration on PD Median (range).

2.7.3.3 PDE samples distribution in the GFS Cohort

PDE samples from the GFS were not collected at intervals of 3-6 months as planned. Most patients also did not have samples in the last year before diagnosis of EPS. Only two EPS patients had samples within 3 months of EPS diagnosis. Subsequently PDE samples were grouped in intervals of 200 days to developing EPS in EPS group or technique termination of PD in the control group. In the last 200 days, there was further subdivision to every 100 days and this was helpful by facilitating a better look at late changes. The sample distribution is illustrated in (Figure 2.8). It is shows variation in sample numbers in each interval. Twelve EPS patients and forty-two control patients were included. Two samples of the 12 EPS patients were obtained in the last 100 days compared to 10 in the control group.

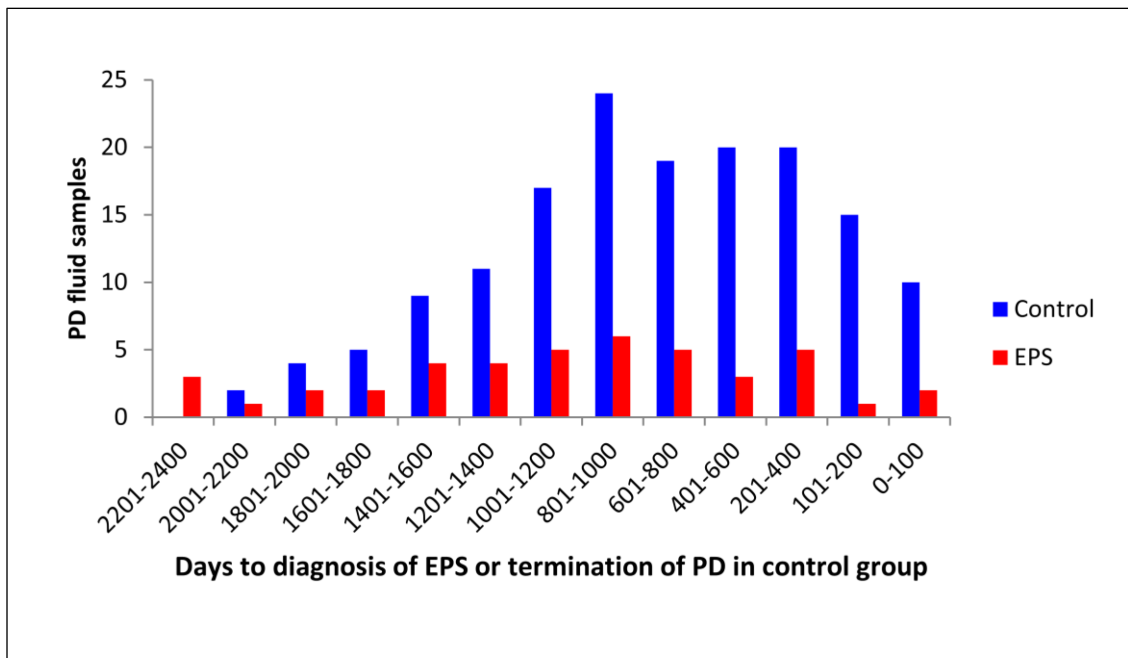


Figure 2.8 <u>PDE samples collection with time in the GFS cohort</u>

GFS sample distribution by time to development of EPS or termination of PD in control group in GFS cohort. PDE samples were grouped into intervals of 200 days (100 days in the last 200 days) either prior to the development of EPS in EPS group or termination of PD without developing EPS in the control group.

2.8 Experimental laboratory Methods

Various experimental methods were applied on peritoneal dialysis effluent (PDE), plasma samples and cell cytospins. Experiments methods performed in this study were:

a) Protein measurement
b) Enzyme activity assays (MMP activity and plasmin activity assays)
c) Enzyme-linked immunosorbent assays (ELISAs)
d) Cell staining

2.8.1 Protein measurement

Protein concentration was measured in PDE fluid samples using the Thermo Scientific™ Pierce™ 660nm protein assay. It is a fast and ready-to-use colorimetric method to measure total protein quantitation. The assay is claimed to more linear and rapid compared to coomassie-based Bradford assays by the manufacturer. Total protein assay methods show some variability toward different proteins. This variable response is due to the variability in amino acid sequence, protein structure, isoelectric point, and the presence of certain side chains or prosthetic groups.

Purified protein for the main protein being assayed is the ideal protein for standard in any protein assay. If there is protein that produces similar assay response to that of the assayed protein, then it provides the most accurate standard. BSA (Bovine Serum Albumin) and BGG (Bovine Gamma Globulin) are the two protein standards used in protein assays.

A standard curve (0, 0.4, 0.8, 1.2, 1.6, and 2 mg/mL) was prepared from a 10 mg/mL BSA stock in PBS (phosphate buffered saline). Each well of a 96 well EIA/RIA plate (Corning Incorporated, NY 14831, USA) was loaded with 10 µl of standard or sample (suitably diluted) in duplicate. 150 µl of Pierce 660 nm

protein assay reagent (Cat# 22660, Thermo Scientifc Pierce Protein assay) was subsequently added to each well. After application of an adhesive plate sealer, the plate was mixed on a Stuart plate shaker at 400 RPM for 1 minute. After 5 a minute incubation at room temperature, optical density of each well was read at 660 nm on 96 well microplate reader using software called Thermo Labsystems Multiskan Ascent plate reader (Labsystems, UK).

2.8.2 Enzyme activity assays

2.8.2.1 MMP activity assay

2.8.2.1.1 Rationale for measuring MMP activity

The ENZchek Gelatinase/Collagenase Assay Kit (E-12055) was used to measure MMP activity in PDE. DQ Gelatin was used as substrate (E-12055, Life Technologies - Molecular Probes) (formerly Invitrogen and Applied Biosystems). The assay was used to determine the effect of the MMP versus TIMP balance on ECM breakdown. E-12055 cleavage provides high sensitivity, fast, and convenient way for measuring gelatinase or collagenase activity in a high-throughput format.

2.8.2.1.2 Principle of assay

DQ labeled gelatin consists of a gelatin molecule (heated Collagen) heavily labeled with both a fluorochrome (FITC) and a quenching agent (QA). The close proximity of the fluorochrome and quencher prevents fluorescence. ECM proteases in the sample will breakdown collagen releasing the fluorophore and quenching molecules. The increased space between the quencher and fluorochrome allows fluorescence which can then be detected and monitored with a fluorescence microplate reader or standard fluorometer (Figure 2.9). ECM proteolytic activity can be assessed by measuring the change in fluorescence over time with the proteolytic activity proportional to increase in fluorescence. While the majority of ECM proteolytic activity is a result of MMP activity, the use

of a broad spectrum but specific MMP inhibitor such as 1,10-Phenanthroline, monohydrate can be used to determine which proportion of the activity relates to MMPs.

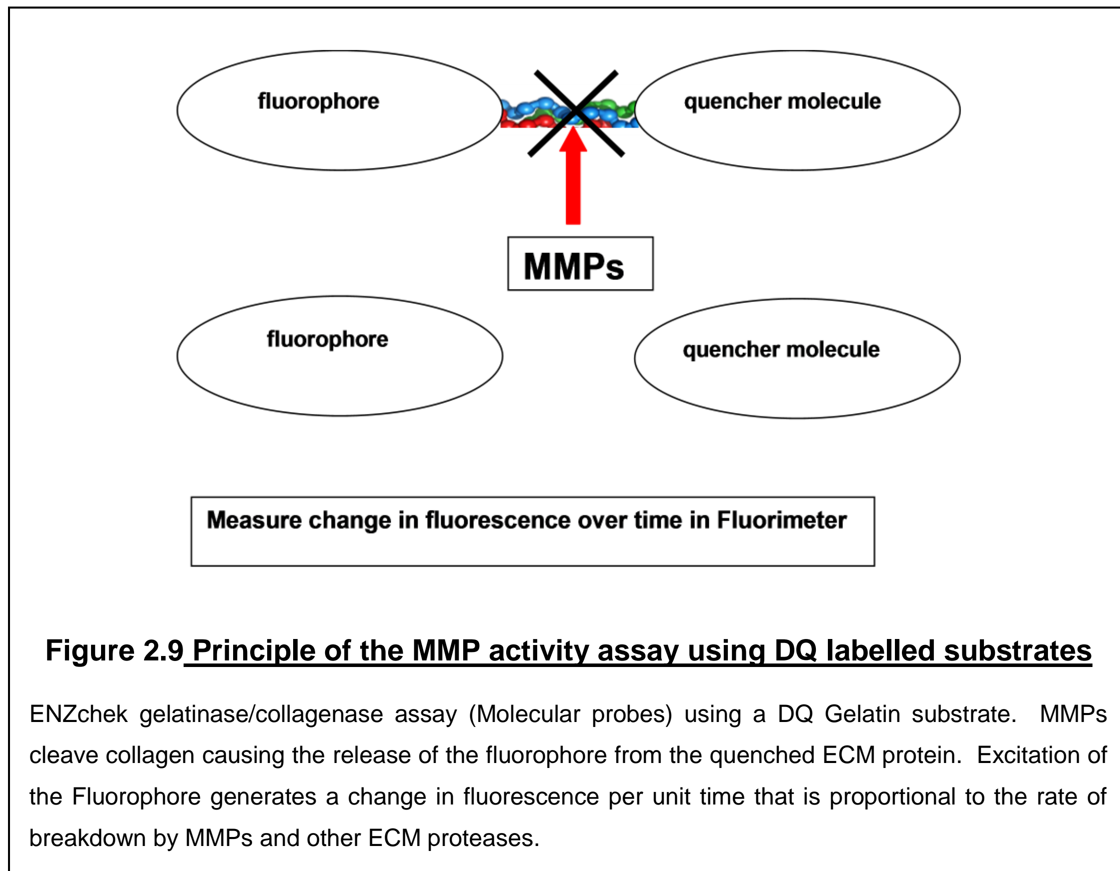


Figure 2.9 Principle of the MMP activity assay using DQ labelled substrates

ENZchek gelatinase/collagenase assay (Molecular probes) using a DQ Gelatin substrate. MMPs cleave collagen causing the release of the fluorophore from the quenched ECM protein. Excitation of the Fluorophore generates a change in fluorescence per unit time that is proportional to the rate of breakdown by MMPs and other ECM proteases.

2.8.2.1.3 Experimental protocol of Gelatinase/Collagenase Activity

2.8.2.1.3.1 Materials

The EnzChek Gelatinase/Collagenase Assay Kit consists of the following components:

1) DQ gelatin: Isolated from pig skin and fluorescein conjugate. Each vial contains 1 mg substrate lyophilized from 1 mL of PBS, pH 7.2.
2) 10X Reaction Buffer: It consists from 50 mL of 0.5 M Tris-HCl, 50 mM $CaCl_2$, 1.5 M NaCl, 2 mM sodium azide, pH 7.6.

3) 1,10-Phenanthroline, monohydrate: MW = 198.2, a general inhibitor for metalloproteinase.
4) Collagenase Type IV: This is from Clostridium histolyticum. One unit is the amount of enzyme which is required to release 1 μmole of L-leucine equivalents from collagen in duration of 5 hours at 37°C with pH 7.5.

EnzChek Gelatinase/Collagenase Assay Kit contains collagenase purified from Clostridium histolyticum to serve as a control enzyme. 100 μg/mL DQ gelatin with 2 hours incubation period, the MMP activity assay can be detected as low as a final concentration of 0.002 U/mL (7 ng protein/mL). One unit is the amount of enzyme which is required to liberate 1 μmole of L-leucine equivalents from collagen in 5 hours at 37°C with pH 7.5. 24 hours incubation can increase the sensitivity approximately 10 fold. Incubation times of 15 minutes are enough for high enzyme concentrations such as a final concentration of 0.2–0.4 U/mL.

2.8.2.1.3.2 Reagent Preparation

1) DQ gelatin: The concentration of the stock solution of DQ gelatin was 1.0 mg/mL. It was prepared by adding 1.0 mL of deionized water (dH_2O) to each vial DQ gelatin. The stock solution was agitated in an ultrasonic water bath for approximately 5 minutes and heated to 50°C to help dissolution. The DQ gelatin stock solution should never be frozen because it may lead to increase background fluorescence of the substrates with freezing and thawing. Reconstituted DQ gelatin may be used fresh or stored in the dark at 4°C. Sodium azide will be added to a final concentration of 2 mM for longer storage.
2) 10X Reaction Buffer: 1X Reaction Buffer was prepared by diluting 1 mL of the 10X Reaction Buffer in 9 mL dH2O.
3) 1,10-Phenanthroline, monohydrate: 9.9 mg of the 1,10-Phenanthroline, monohydrate was dissolved in 25 μL ethanol. After that 10 μL of this solution was added to 2 mL of 1X Reaction Buffer to prepare a 10 mM working solution.

4) Collagenase Type IV: 1000 U/mL of stock solution was prepared by dissolving 500U of Clostridium collagenase in the 0.5 mL dH2O. The stock solution can be used for at least 6 months, if it is aliquoted and stored at -20°C.

2.8.2.1.3.3 Assay procedure

MMP activity was assayed on PDE using the ENZchek gelatinase/collagenase assay kit with a DQ gelatin substrate according to manufactures instruction. Each well of a 96 well of BD Falcon 96-Well Multiwell Micro Plate was loaded with final assay volume of 200 μl as follows:

100 μl of standard or sample was loaded in duplicate. For each assay, the standard curve was (0, 0.003125, 0.00625, 0.0125, 0.0250, 0.05, 0.1 U/mL) of clostridium collagenase using 1X reaction buffer as the diluent. Samples were loaded neat. 80 ul of reaction Buffer was added to each well. After that, 20 ul of a 12.5 μg/mL solution of DQ gelatin was added to each well to complete the final volume of 200 μl. All steps were repeated when assays using Gelatinase/Collagenase inhibitors were run except 70 ul of reaction Buffer was added + 10 ul of 1,10-phenanthroline monohydrate instead of 80 ul of reaction Buffer.. Fluorescence was then measured at room temperature with protection from light. The measurements were taken every 15-20 min in first 3 hours, and then the measurements were less frequent for up to 48 hours. Excitation was set at 495 and emission at 515 nm on a Fluorimeter, typically the Varioskan™ Flash Reader from Thermo Scientific.

All assays was performed with and without the general inhibitor of metalloproteinase (10 μl 1,10-Phenanthroline, monohydrate) to calculate the extent of the proteolytic activity due to MMP activity only. The rate of reaction with MMP inhibitor was subtracted from the rate without to calculate the MMP specific cleavage of the substrate. The reason of measuring fluorescence at multiple points was to get good linear reaction rate for all samples.

2.8.2.1.3.4 Development and validation of EnzCheck assay for PDE

Assay protocol was established by testing PDE at different dilutions (1:1, 1:2, 1:5, 1:10, and 1:20) to determine the best sample dilution to position readings in the middle of the standard curve These test runs showed that no PDE samples dilution was required as PDE did not affect assay activity & because MMP activities were towards the bottom of the standard curve in all samples. The inter assay precision was obtained by repeatedly assaying one of the samples in every plate. The inter-assay coefficient was below 10%.

2.8.2.2 Plasmin activity assay

2.8.2.2.1 Rationale for measuring plasmin activity

Measuring plasmin activity in PDE samples were used to determine the overall effect of the plasmin activity in the samples. A colorimetric substrate V0882 (sigma) was used for measuring the plasmin activity.

2.8.2.2.2 Principle of assay

The plasmin activity assay is a colorimetric activity assay using substrate V0882 (sigma).

N-L-valine-L-leucine-L-lycine (Val-Leu-Lys) peptide structure is the functional unit of V0882. (Val-Leu-Lys) is attached to a p-nitroaniline (pNA) molecule (Figure 2.10). Plasmin willcomplex and cleave the colorimetric substrate V0882 releasing the the pNA from the Val-Leu-Lys peptide. After clevage, free pNA part will give a yellow colour to the sample which can be measured at 405nm which is proportional to its release and thus plasmin activity.

D-Val-Leu-Lys-pNa $\xrightarrow{\text{Plasmin}}$ D-Val-Leu-Lys + p-Nitroanilide (El-Shabouri, Hussein et al. 1989)

The optical density of each well was read at 405 nm on 96 well microplate reader (Thermo Labsystems Multiskan Ascent.) with curve fit and analysis performed using Genesis software.

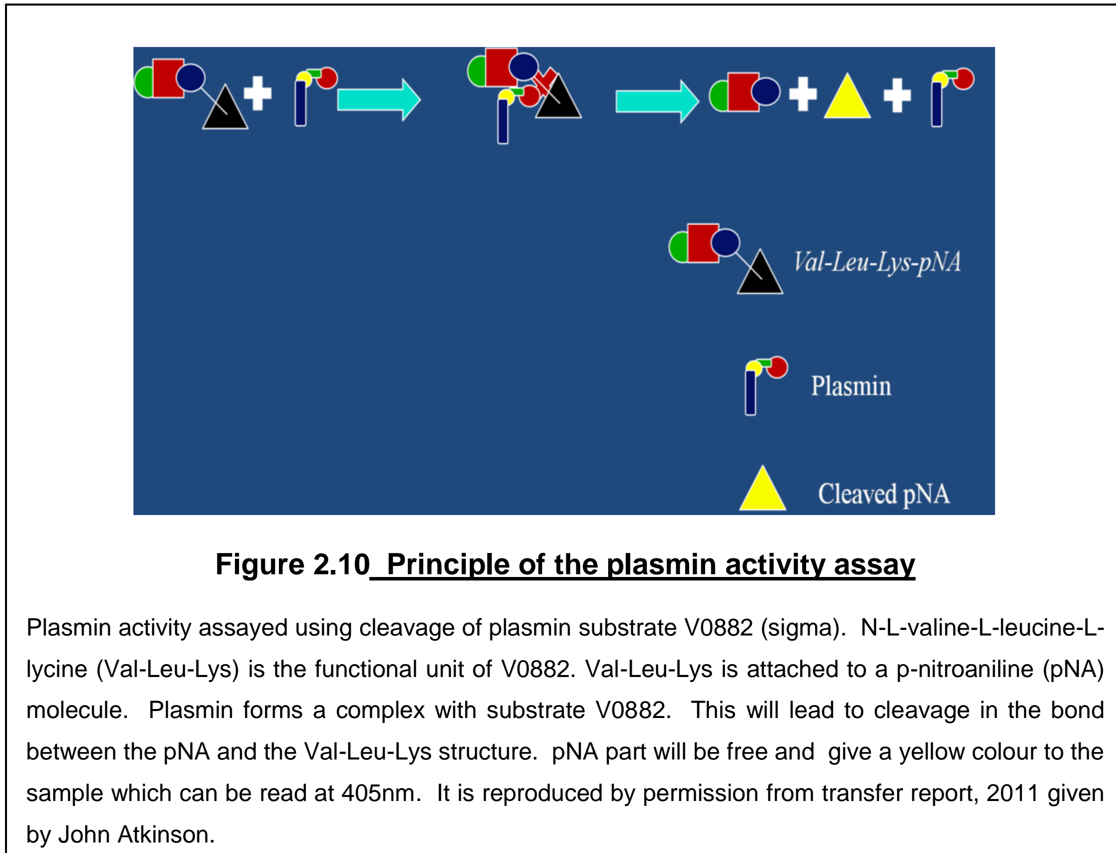


Figure 2.10 Principle of the plasmin activity assay

Plasmin activity assayed using cleavage of plasmin substrate V0882 (sigma). N-L-valine-L-leucine-L-lycine (Val-Leu-Lys) is the functional unit of V0882. Val-Leu-Lys is attached to a p-nitroaniline (pNA) molecule. Plasmin forms a complex with substrate V0882. This will lead to cleavage in the bond between the pNA and the Val-Leu-Lys structure. pNA part will be free and give a yellow colour to the sample which can be read at 405nm. It is reproduced by permission from transfer report, 2011 given by John Atkinson.

2.8.2.2.3 Experimental protocol

Plasmin activity was assayed in peritoneal dialysis effluent (PDE) using cleavageof substrate V0882 (sigma). 100ul of the substrate V0882 (D-Val-Leu-Lys-pNa substrate 3mM) was added to 100ul of standard or PDE samples in a 96 well plate. The absorbances were measured at 405nm at 0,30,60,90 and 120 minutes on the microplate reader using software called Thermo Labsystems Multiskan Ascent plate reader (Labsystems, UK). The plate was incubated in the dark at 37°C during incubation periods. Plasmin from human plasma (P1867 (sigma)) was used to obtain for a standard curve (0, 1.25, 2.5, 5, 10, 20 µg/mL) in which zero was distilled water.

2.8.2.2.4 Development and validation of assay

Similar to the MMP activity above, the assay protocol was established by testing samples at different dilutions. Neat samples produced the optimal assay. The inter-assay coefficient was always below 10%.

2.8.3 ELISAs

ELISAs (Enzyme-linked immunosorbent assays) are commonly used for the quantitative and qualitative assessment of chemokines, cytokines, phosphorylated targets, growth factors, immunoglobulins in addition to the other immunological markers. ELISAs are also used for detecting and quantification of protein analytes from biological sample such as plasma, serum, cell culture supernatants, cell lysates, urine, PDE. ELISAs are valuable tools in drug development because they are widely used for biomarker profiling of healthy individuals versus diseases and in toxicity profiling. Various commercial "sandwich" ELISAs (pre-coated ELISA or coat-It-Yourself ELISA) were used in this study. ELISA kits were bought from different companies according need. The following ELISA kits were used.

1. Human MMP-1,-2,-3,-9 from R/D system. MMP-13 from abcam.
2. Human TIMP-1,-2, and -3 from R/D system.
3. Human MMP-1/TIMP-1 complex from R/D system.
4. Human Transferrin and Albumin from AssayPro
5. Human IgG from Mabtech
6. Human Beta 2 microglobulin from abcam
7. Human intelectin-1 from USCN
8. Human collagen (α1) I from USCN
9. Human dermatopontin I from USCN

2.8.3.1 Human MMP-1,-2,-3,-9, TIMP-1,-2,-3, and MMP-1/TIMP-1 Complex ELISA Kits (R&D systems Duoset system)

2.8.3.1.1 Materials

The standard, capture antibody, and detection antibody are summarized in (Table 2.8.A and B). Streptavidin-HRP (Part 890803, 1 vial) was used same for all ELISA. The streptavidin conjugated to horseradish-peroxidase should be stored at a 2 - 8° C for not more than 6 months after opening and never frozen. Streptavidin-HRP was diluted 1:200 in R&D reagent diluent. Other solutions required are summarised in (Table 2.9). In all assays the blocking solution was same as reagent diluent except in Human MMP-1 /TIMP-1 Complex DuoSet (DY1550) where 1% BSA in PBS. Reagent diluent is 0.15 M NaCl, 10 mM $CaCl_2$, 50 mM Tris, 0.05% Brij 35, pH 7.45-7.55.

ELISA	Standard	Capture Antibody	Detection Antibody
Human TIMP-1 DuoSet (DY970)	80 ng/mL of recombinant human TIMP-1 after adding 0.5 mL of RD to 1 vial. A 7 point standard curve with two-fold serial dilutions in RD with high standard of 2000 pg/mL.	360 µg/mL of mouse anti-human TIMP-1 after reconstituted with 1 mL of PBS. Working concentration is 2 µg/mL in PBS.	9 µg/mL of biotinylated goat anti-human TIMP-1 after reconstituted with 1 mL of RD. Working concentration is 50 ng/mL in RD.
Human TIMP-2 DuoSet (DY971)	70 ng/mL of recombinant human TIMP-2 after adding 0.5 mL of RD to 1 vial. A 7 point standard curve with two-fold serial dilutions in RD with high standard of 2000 pg/mL.	360 µg/mL of mouse anti-human TIMP-2 after reconstituted with 1 mL of PBS. Working concentration is 2 µg/mL in PBS.	9 µg/mL of biotinylated goat anti-human TIMP-2 after reconstituted with 1 mL of RD. Working concentration is 50 ng/mL in RD.
Human TIMP-3 DuoSet (DY973)	90 ng/mL of recombinant human TIMP-3 after adding 0.5 mL of RD to 1 vial. A 7 point standard curve with two-fold serial dilutions in RD with high standard of 4000 pg/mL.	360 µg/mL of mouse anti-human TIMP-3 after reconstituted with 1 mL of PBS. Working concentration is 2 µg/mL in PBS.	360 µg/mL of biotinylated mouse anti-human TIMP-3 after reconstituted with 1 mL of RD. Working concentration is 2 µg/mL in RD.

Table 2.8.A <u>Materials required in TIMP ELISA kits</u>

Standard, capture antibodies, and detection antibodies used for performing R&D ELISAs, for human TIMP-1,-2, and -3 duosets (R&D systems).

ELISA	Standard	Capture Antibody	Detection Antibody
Human MMP-1 DuoSet (DY901)	180 ng/mL of recombinant human MMP-1 after adding 0.5 mL of diH2O to 1 vial. A 7 point standard curve with two-fold serial dilutions in RD, with high standard of 10,000 pg/mL.	360μg/mL of goat anti-human MMP-1 after reconstituted with 1 mL of PBS. Working concentration is 2 μg/mL in PBS.	18 μg/mL of biotinylated goat anti-human MMP-1 after reconstituted with 1 mL of RD. Working concentration is 100 ng/mL in RD.
Human MMP-2 DuoSet (DY902)	2100 ng/mL of recombinant human MMP-2 after adding 0.5 mL of diH2O. A 7 point standard curve with two-fold serial dilutions in RD with high standard of 20 ng/mL.	360 μg/mL of mouse anti-human MMP-2 after reconstituted with 1 mL of PBS. Working concentration is 2 μg/mL in PBS.	90 μg/mL of biotinylated mouse anti-human MMP-2 after reconstituted with 1 mL of RD. Working concentration is 500 ng/mL in RD.
Human MMP-3 DuoSet (DY513)	90 ng/mL of recombinant human MMP-3 after adding 0.5 mL of diH2O. A 7 point standard curve with two-fold serial dilutions in RD with high standard of 2000 pg/mL.	144 μg/mL of goat anti-human MMP-3 after reconstituted with 1 mL of PBS. Working concentration is 0.8 μg/mL in PBS.	36 μg/mL of biotinylated goat anti-human MMP-3 after reconstituted with 1 mL of RD. Working concentration is 100 ng/mL in RD.
Human MMP-9 DuoSet (DY911)	50 ng/mL of recombinant human MMP-9 after adding 0.5 mL of diH2O. A 7 point standard curve with 2-fold serial dilutions in RD with high standard of 2000 pg/mL.	180 μg/mL of mouse anti-human MMP-9 w after reconstituted with 1 mL of PBS. Working concentration is 1 μg/mL in PBS.	36 μg/mL of biotinylated goat anti-human MMP-9 after reconstituted with 1 mL of RD. Working concentration is 100 ng/mL in RD with 2% heat inactivated normal goat serum (prepare 1-2 hrs prior to use).
Human MMP-1/TIMP-1 Complex DuoSet (DY1550)	90 ng/mL of recombinant human TIMP-1 after adding 0.5 mL of RD. A 7 point standard curve with 2-fold serial dilutions in RD with high standard of 4000 pg/mL.	288 μg/mL of goat anti human MMP-1 after reconstituted with 1 mL of PBS. Working concentration is 1.6 μg/mL in PBS.	18 μg/mL of biotinylated goat anti-human TIMP-1 after reconstituted with 1 mL of RD. Working concentration is 100 ng/mL in RD.

Table 2.8.B <u>Materials required in MMP and MMP-1 /TIMP-1 complex ELISA kits</u>

Standard, capture antibodies, and detection antibodies used for performing R&D ELISAs, for human MMP-1,-2,-3, -9, and MMP-1 /TIMP-1 complex duosets (R&D systems).

Solution	Composition
PBS	1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 137 mM NaCl, pH 7.2 - 7.4.
Wash Buffer	0.05% Tween 20 in PBS
Reagent Diluent	1% BSA in PBS
Substrate Solution	Colour Reagent A (H_2O_2) & Colour Reagent B (Tetramethylbenzidine) 1:1
Stop Solution	2 N H_2SO_4

Table 2.9 Solutions required in Human MMPs and TIMPs ELISAs

Solutions and their composition which are required for MMPs and TIMPs ELISAs

2.8.3.1.2 Assay procedure

Specific ELISA kits were used to measure different MMPs, TIMPs, and the MMP-1/TIMP-1 complex in PDE fluid. The assays were performed according to manufacturer's instructions in all cases. Commercial DuoSet ELISA kits (R&D systems) were used in all assays except MMP-13. MMP-13 was from abcam (MMP-13 Human ELISA/ab100605).

In all commercial DuoSet ELISA kits, the capture antibodies were coated and covered with an adhesive strip overnight at room temperature prior to running the assay. Next day, aspiration and washing of each well was performed using washing buffer for three times (0.05% Tween 20 in PBS). After that, 300 μL of reagent diluent was added to each well and covered with a new adhesive strip to block the plate at room temperature for a minimum of 1 hour. Then, aspirate and wash 3x with 0.05% Tween 20 in PBS. After that, the plates were ready for the addition of samples and standards. One hundred μL of samples or standards in reagent diluent were added per well and covered with a new adhesive strip and incubated for 2 hours at room temperature.

After that, repeat the aspiration & washing step prior to 100 µL of the detection antibody diluted in reagent diluent being added to each well. The plate was covered with a new adhesive strip and incubated for 2 hours at room temperature. Following a repeat aspiration & wash, 100 µL of the working dilution of Streptavidin-HRP was added to each well and covered with a new adhesive strip and incubated for 20 minutes at room temperature in a dark place. Then, repeat the aspiration & wash step prior to 100ul of Substrate Solution [1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine)] being added to each well and incubated for 20 minutes at room temperature avoiding direct light. After that, 50 µL of Stop Solution (2 N H_2SO4) was added to each well. Gently tap the plate to ensure thorough mixing. The optical density of each well was read at 450 nm on 96 well microplate reader using Ascent Genesis software on a Thermo Labsystems Multiskan Ascent plate reader.

2.8.3.2 Human MMP13 ELISA Kit (abcam)

Unlike the rest of the MMP and TIMP ELISA, the MMP13 Human Elisa Kit was bought from abcam (ab100605).

2.8.3.2.1 Materials and solutions

The materials and solutions of (ab100605) should be at room temperature prior to use (Table 2.10). There are other materials which are not provided with kits, but they are required including microplate reader with ability to measure at 450 nm, precision pipettes, Adjustable 1-25 mL pipettes as this required for reagent preparation, cylinders, tubes for standard and sample preparation, distilled water, absorbent paper, and computer and software for ELISA data analysis.

Item	Amount	Notes
MMP13 Microplate (pre-coated)	1 X 96 wells	
Recombinant Human MMP13 Standard	2 vials	A 7 point standard curve was prepared according to manufacturer's procedure (0, 8.2, 24.69, 74.1, 222.2, 666,7, 2000 pg/mL)
Biotinylated anti-Human MMP13	2 vials	1X Biotinylated MMP13 Detection Antibody (Ab) was prepared by spining the vial of Biotinylated anti-Human MMP13 for few seconds. Then, add 100 µL of 1X Assay Diluent into the vial and mix it gently. The detection Ab was diluted 80-fold by 1X assay diluent before use in the Assay.
20X Wash Solution	25 mL	It was diluted to 1 X wash solution in diH2O
5X Assay Diluent	15 mL	It was diluted to 1 X assay diluent in diH_2O
HRP-Streptavidin Concentrate	200 µL	It should be diluted 35000-fold by 1X assay diluent
Substrate Reagent (TMB One-Step)	12 mL	
Stop Solution	8 mL	

Table 2.10 Materials and solutions required in MMP13 human ELISA Kit

Materials and solutions with their amounts, dilutions and preparations according to the manufacturer's instructions (ab100605).

2.8.3.2.2 Assay procedure

An antibody pre-coated 96-well plate is used for this assay. All materials are brought to room temperature prior to use. 100 µL of each standard and PDE samples (neat) were loaded in wells. The plate was covered and incubated for 2.5 hours with gentle shaking at room temperature. If MMP13 is present in any samples, then it will bound to the wells via the immobilized antibody. The samples/standards were then discarded & wash with 1X Wash Solution (4 times). 100 µL of 1X Biotinylated MMP13 Detection Ab was added to each well & incubated for 1 hour with gentle shaking at room temperature.

After that unbound biotinylated Ab was washed away. 100 µL of 1X HRP-Streptavidin solution was added to each well & incubated for 45 minutes with gentle shaking at room temperature. Post washing, 100 µL of TMB One-Step Substrate Reagent was added to each well. It was incubated for 30 minutes in the dark with gentle shaking at room temperature. 50 µL of stop Solution was added to each well. Finally, optical density of each well was read at 450 nm on 96 well microplate reader as previously.

2.8.3.3 Human albumin and transferrin ELISA Kits (AssayMax)

Commercially available sandwich ELISAs were used for measuring albumin and transferrin in samples. These kits were AssayMax Human Albumin ELISA Kit, catalog No. EA3201-1, Lot No. 02021212 and AssayMax Human Transferrin ELISA Kit, catalog No. ET3105-1, Lot No. 04721227.

2.8.3.3.1 Principal of the Assays

Human albumin and transferrin ELISA kits were used for quantifying albumin and transferrin respectively. Each was a quantitative sandwich enzyme immunoassay. Specific polyclonal antibodies for human albumin or transferrin had been pre-coated onto a 96-well plate allowing albumin or transferrin to be sandwiched between the capture antibody and biotinylated secondary polyclonal antibodies. Secondary antibody was detected by a streptavidin-peroxidase conjugate using a TMB substrate. The developed colour was stopped. After that, the colour intensity was measured.

2.8.3.3.2 Materials

All reagents should be brought to the room temperature before use. The standard, antibodies, and other materials are summarized in (Table 2.11). The kit components were either stored at -80^{0}C or -20^{0}C upon arrival.

Materials	Albumin	Transferrin
96-well polystyrene microplate	Coated with polyclonal antibodies for human albumin	Coated with polyclonal antibodies for human transferrin
Sealing Tapes	3 pre-cut tapes	3 pre-cut tapes
Standard	Human albumin in a buffered protein base (800 ng, lyophilized)	Human transferrin in a buffered protein base (1 µg, lyophilized)
Biotinylated Antibody	Biotinylated Human Albumin Antibody. It is 80-fold concentrated antibody (100 µL)	Biotinylated Human Albumin Antibody. It is 100-fold concentrated antibody (80 µL)
MIX Diluent Concentrate	10-fold concentration of the buffered protein base (30 mL)	10-fold concentration of the buffered protein base (30 mL)
Wash Buffer	20-fold concentration of the buffered surfactant (60 mL)	20-fold concentration of the buffered surfactant (60 mL)
Streptavidin-Peroxidase Conjugate	100-fold concentration (80 µL)	100-fold concentration (80 µL)
Chromogen Substrate	Tetramethylbenzidine (8 mL). It is a stabilized peroxidase chromogen.	Tetramethylbenzidine (8 mL). It is a stabilized peroxidase chromogen.
Stop Solution	0.5 N hydrochloric acid (12 mL)	0.5 N hydrochloric acid (12 mL)

Table 2.11 Materials and solutions required in human albumin and transferrin ELISA Kits

Materials and solutions with their amounts and dilutions according to the manufacturer instructions from AssayMax.

2.8.3.3.3 Reagent Preparation

1) Diluent Concentrate (10-fold): Mix the diluent concentrates gently to dissolve any crystals that have been formed prior to dilution in 1:10 with reagent grade water after which it can be stored for up to 1 month at 2-8 ^{o}C.2).

2) Standard Curve: Reconstitute the 800 ng of Albumin or 1 µg of Transferrin standard with 4 and 2.5 mL of diluent respectively to make a standard

solution of 200 ng/mL of albumin and 400 ng/mL of transferrin (stock solutions). The standards were agitated gently for 10 minutes before making dilutions. For both albumin and transferrin, duplicate standard points were prepared by serially diluting of the stock solutions. Eight points standard curve for albumin were made. These were 200, 100, 50, 25 12.5, 6.25, 3.125 and 0 ng/mL, while the standard curve for transferrin were 100, 50, 25, 12.5, 6.25, 3.125, 1.563 and 0 ng/mL. The mix diluents were used as diluents.

3) Biotinylated human albumin antibody (80-fold) or biotinylated transferrin antibody (100-fold): The antibodies were spun down briefly and then they were diluted to the desired amount of the antibody (1:80 for albumin and 1:100 for transferrin) with diluent. The remaining solutions were stored at -20 C.

4) Wash Buffer Concentrate (20-fold): If crystals have been formed in the wash buffer concentrate, then mix gently until the crystals have been dissolved completely. It was diluted by 1:20 with reagent grade water.

5) Streptavidin-Peroxidase Conjugate (100-fold): It was spun down briefly and then diluted 100 times with diluent. The remaining solutions were stored at -20 C.

2.8.3.3.4 Assay Procedure

The assay procedures were similar for albumin and transferrin. All reagents, working standards and solutions were prepared as instructed. All reagents (either albumin or transferrin reagents) were brought to the room temperature before use in the experiments and the assays were performed at room temperature between 20-30 °C. 50 µL of standard or sample were loaded per well. The wells were covered with a sealing tape. The incubation period was 1 hour and 2 hours in albumin and transferrin assays respectively.

The solutions were discarded from the wells and washed out manually using 1X wash buffer (5 times). 50 μL of biotinylated human albumin or transferrin antibodies were loaded into each well for an incubation period of 30 minutes. After that, the plate was washed 5 times and 50 μL of streptavidin-peroxidase conjugate added & incubated for 30 minutes at. Wash the microplate again as described above and add 50 μL of chromogen substrate for 15 minutes for albumin and 10 minutes for transferrin. The plates were tapped gently to ensure thorough mixing50 μL of the stop solutions was added to each well and the optical density read at 450 nm as previously.

2.8.3.4 Human beta 2 microglobulin (B2M) ELISA kit (abcam)

The kit must be stored immediately at the -20°C upon the receipt, (product code ab99977).

2.8.3.4.1 Materials and reagent preparation

The contents of the kit and methods of the reagent preparation are illustrated in (Table 2.12).

2.8.3.4.2 Assay procedure

All reagents, working standards and solutions were prepared as instructed. All reagents were brought to the room temperature before use in the experiment and the assay was performed at room temperature. Antibody specific for Human B2M were pre-coated onto a 96-well plate. 100 μL of standards and samples were loaded into the wells for two and half hours. The solutions were discarded from the wells and washed out manually using 1X wash buffer (4 times). 100 μL of biotinylated B2M detection antibody were loaded to each well for an incubation period of 1 hour. After washing away unbound biotinylated antibody in a similar to the previous wash step (4 times), HRP-conjugated streptavidin (100 μL) was loaded into each well and incubated for 45 minutes. The wells were again washed, and then a TMB substrate solution (100 μL) was added for 30 minutes.

The Stop solution (50 µL) was added. Optical density of each well was read at 450 nm on 96 well microplate reader as previously.

Materials	Reagent preparation
B2M microplate (96 wells) (1 plate)	
Assay diluent A (2 x 30 mL)	
Assay diluent B (5-fold) (15 mL)	Assay diluent B was diluted 1:5 with distilled water.
Wash buffer concentrate (20-fold) (25 mL)	If the crystals have been formed in the wash buffer concentrate, then mix gently until the crystals have been dissolved completely. It was diluted by 1:20 with distilled water.
Recombinant human B2M standard (2 vials)	Standard was prepared immediately before using in the assay (fresh preparation). The vial was spun briefly and 400 µL Assay Diluent A (for plasma) was added or 400 µL 1X Assay Diluent B (for PDE) was added to prepare 50 ng/mL B2M Stock Standard. Eight points standard curve for B2M were made. These were 1000, 500, 250, 125, 62.5, 31.25, 15.6 and zero pg/mL.
Biotinylated anti-human B2M (2 vials)	A vial of biotinylated anti-Human B2M was spun briefly. 100 µL of 1X Assay Diluent B was added into the vial to make a detection antibody oncentrate. Then, pipette up and down to mix gently. It is important to store the concentrate either at 4°C for 5 days or it needs to be aliquoted and frozen at -20°C for up to 2 months). The detection antibody concentrate was diluted 1:80 with 1X assay diluent B before using it in the assay procedure.
HRP-streptavidin concentrate (300-fold) (200 µL)	A vial of the 300X HRP-streptavidin concentrate was spun briefly. HRP-streptavidin concentrate were diluted 1:300 with 1X assay diluent B before using it in the assay procedure.
TMB (substrate reagent) (12 mL)	
Stop solution (8 mL)	

Table 2.12 <u>Materials and solutions required in human beta 2 microglobulin ELISA Kit</u>

Materials and solutions with their amounts and dilutions according to the manufactures instructions.

2.8.3.5 Human IgG ELISA kit (Mabtech)

The standard vial was stored at the -20°C upon receipt, while the capture and detection antibodies were stored at 4-8 ^{0}C. (Product code 3850-1AD-6).

2.8.3.5.1 Materials and reagent preparation

The kit consists of the following:

- Incubation buffer: PBS with 0.05% Tween 20 (PBS-Tween) containing 0.1% BSA. It is used not only as block solution, but also as diluent. The same buffer was used for dilution and blocking.
- Monoclonal capture antibody (MT145): Supplied in sterile-filtered PBS (0.2 µm) with sodium azide (0.02%). The volume was 300 µL at a, concentration of 0.5 mg/mL.
- ALP-conjugated detection antibody (MT78-ALP). Supplied in Tris-buffer (0.1 M) with 0.15% Kathon and 1% BSA.
- Lyophilized human IgG standard. 11 points for a standard curve were prepared ranging from 0.2 to 100 ng/mL using incubation buffer as diluent to prepare the curve. The preparation of the standard was started by adding 500 µl PBS to the vial which made up a stock solution of 50 µg/mL. This stock solution must be used immediately or stored in aliquots at -20°C for any future uses.
- Substrate were not included and bought separately. High sensitivity pNPP substrate was used because it is suitable for ELISA assays with alkaline phosphatase (ALP). The substrate was supplied in diethanolamine buffer and it is ready-to-use solution. A soluble yellow product was produced after adding the substrate which is measured at 405 nm. The substrate can be bought from Sigma-Aldrich or Mabtech.
- Washing buffer PBS-Tween: 0.05% Tween 20 in PBS, pH 7.2-7.4.

2.8.3.5.2 Assay procedure

The ELISA plate was coated with MT145 antibody after diluting the antibody to 2 µg/mL in PBS pH 7.4. 100 µl of this antibody was placed in each well and incubated overnight at 4-8°C. The following day, the plate was washed twice with washing buffer (200 µl/well) & 200 µL of block solution (Incubation buffer) added & incubated for 1 hour at room temperature. After 1 hour, the plate was washed out five times with washing buffer & 100 µL of standard or samples diluted in incubation buffer loaded into each well followed by incubation for 2 hours at room temperature. After the incubation period, the plate was washed out five times with washing buffer & 100 µL of MT78-ALP diluted 1:1000 in incubation buffer added. After incubation for 1 hour at room temperature, the plate was washed out five times with washing buffer. 100 µL p-nitrophenyl-phosphate (pNPP) was loaded on each well. After a suitable development time (30 minutes), optical density of each well was read at 405 nm on 96 well microplate reader as before.

2.8.3.6 ELISAs from USCN for the detection of intellectin-1, dermatopontin and collagen α1(I)

1. E90933Hu 96 Tests, Enzyme-linked Immunosorbent Assay Kit For Omentin, Organism: Homo sapiens (Human), USCN
2. E92432Hu 96 Tests, Enzyme-linked Immunosorbent Assay Kit For Dermatopontin, Organism: Homo sapiens (Human), USCN
3. E90350Hu 96 Tests, Enzyme-linked Immunosorbent Assay Kit For collagen (α1) I, Organism: Homo sapiens (Human), USCN

The 96-well plate must be stored at -20 C, while the rest of the kit must be stored at 4 ^{O}C.

2.8.3.6.1 Materials and reagents preparation

The contents of the kits and methods of the reagent preparations are given in (Table 2.13).

Materials	Reagent preparation
Pre-coated and ready to use 96-well plate for either intelectin-1, COL1a1 or DPT	
Standard Diluent	
Assay Diluent A (2-fold) for either intelectin-1, COL1a1 or DPT	6mL of Assay Diluent A concentrate (2×) was diluted with 6mL of distilled water to made 12 mL of Assay Diluent A.
Assay Diluent B (2-fold) for either intelectin-1, COL1a1 or DPT	6mL of Assay Diluent B concentrate (2×) was diluted with 6mL of distilled water to made 12 mL of Assay Diluent B.
Wash buffer (30-fold)	Distilled water was used to dilute wash buffer concentrate to get wash buffer (1×)
Lyophilized standard (lyophilized) for either intelectin-1, COL1a1 or DPT	Standard for intelectin-1, COL1a1 or DPT were reconstituted with 1.0mL of standard diluent and it was kept for 10 minutes at room temperature with shake gently. The concentrations of the standard in the stock solution were 500pg/mL for intelectin-1, 80ng/mL for COL1a1 and 40ng/mL for DPT. Series concentration of standard were produced for intelectin-1 (500, 250, 125, 62.5, 31.2, 15.6, 7.8 and 0 pg/mL), for COL1a1 (20, 10, 5, 2.5, 1.25, 0.625,0.312 and 0 ng/mL), and for DPT (10, 5, 2.5, 1.25, 0.625,0.312, 0.156 and 0 ng/mL).
Detection Reagent A for either intelectin-1, COL1a1 or DPT	Stock of detection A was spun briefly prior to use. It was diluted to the working concentration (1:100) using working Assay Diluent A.
Detection Reagent B for either intelectin-1, COL1a1 or DPT	Stock of detection B was spun briefly prior to use. It was diluted to the working concentration (1:100) using working Assay Diluent B.
Plate sealer for 96 wells	
TMB Substrate	
Stop Solution	

Table 2.13 Materials and solutions required in human intelectin-1, collagen type I alpha 1, and dermatopontin ELISA Kits

Materials & solutions with their amounts and dilutions according to the manufactures instructions from USCN.

2.8.3.6.2 Assay procedure

All reagents, working standards and solutions were prepared as instructed & brought to the room temperature before use. 100µL standard (intelectin-1, COL1a1 or DPT) or sample was added to each well with incubation for 2 hours at 37^{0}C. After 2 hours, the liquid in the wells were removed, but not washed. 100µL of prepared detection reagent A (detection reagent A for intelectin-1, COL1a1 or DPT) was added to each well & incubated for 1 hour at 37^{0}C. After, the plate was washed out three times with washing buffer. 100µL of prepared detection reagent B was added to each well & incubated for 30 minutes at 37^{0}C. The plate was washed out three times with washing buffer. 90µL of substrate solution was added to each well and incubated for 20 minutes at 37^{0}C. The plate was protected from light. 50µL of stop solution was added to each well & optical density of each well was read at 450 nm as previously

2.8.3.7 Precision for all ELISA kits

Inter-assay Precision (Precision between assays): 1-3 samples were always repeat tested in all ELISAs to calculate CV. The target inter-Assay CV was always below 10%.

2.8.3.8 Technical hints for all ELISA kits

When performing ELISAs, the use of high quality BSA for the reagent diluent is important for the ideal performance of the assays. Impurities in BSA preparations can interfere with the detection of certain analytes. The wash technique is crucial for proper assay performance. Wash buffer should be removed forcefully and completely from the wells via decanting or aspiration. Any remaining wash buffer can be removed by inverting and blotting the plate against clean paper towels. Microbial contamination of buffers and reagents should be avoided because it may interfere with the assay sensitivity. Bubbling and foaming should be avoided during reconstituting or mixing components. The

plate should be properly covered in incubation period. It is very important to briefly spin down the standard vial because the powder may fall from the cap during opening the standard vial, if it is not spun down. Lyophilizedilised materials must be dissolved thoroughly and gently during reconstituting without vortex. Vortex may lead to protein destabilization of the standard. It is also important to keep special care about the microplate strips. It is important to remove and store the excess microplate strips from the plate frame immediately by returning them to the foil pouches provided by company which contain desiccant inside. Then, reseal the pouch tightly to minimize exposure to water vapor and store in -20 C. It is important to avoid mixing reagents or materials between the ELISA kits even if they are all for detecting same protein and they were purchased from same company. The other important technical hint is that residual substrate solution should not be dumped back into the main vial. Any solution with crystals needs to be warmed at room temperature by mixing the solution vial gently until the crystal dissolved completely. Another important technical point is to be ensured that calibration of the pipettes is accurate. TMB Substrate must be protected from light. It is also advised to remove fingerprint or drop of water on the bottom of the 96 wells plate and ensure there are no bubbles on the liquid surface. Finally, it is essential to run the microplate reader immediately after stopping the substrate in the plates.

2.8.4 Cell staining

Immunohistchemistry (IHC) is a staining technique used for identification of protein expression or antigen in cell samples by using a specific antibody that binds to it. The antibody allows visualization of the protein under a microscope. With IHC it is possible to determine specific cell component localisation and distribution. IHC has many applications including diagnosis of illnesses, development of medications and for biological research purposes.

The processes of PD cytospin for slide preparation are described in (section 2.3). In the SKI-2 patient cohort, overnight bags were used for slide preparation. PD fluids with suspended cells were centrifuged using a centrifuge for 8 minutes, at 2000 g and 4ºC. The pellet was suspended in 2mL of PD fluid and 300 µL loaded onto a cytospin. Cytospin was performed at 1200g for 10 minutes. Eight slides were obtained for each patient. Cells were fixed in acetone for 10 minutes at -20°C. Slides were then left to dry at room temperature for 15 minutes. Slides were labelled with a pencil & stored at -20°C until used for immunocytochemistry.

In the day of cell staining, the endogenous peroxidase activity was quenched by immersing in a solution of 3% hydrogen peroxide in methanol for 20 minutes after that rinsed in dH2O. Slides were rinsed in PBS, then blocked with serum from the Impress Kit (catalog number was MP-7500 from Vector laboratories). The slides were incubated with primary antibody for 1 hour at room temperature (Type of primary antibody is different according to the cell of interest to be stained). Trial staining runs were conducted to find the optimum dilution. Dilutions were made up in sterile PBS. Slides were washed again with stirring in PBS for 5 minutes (x2). Slides were incubated with the secondary biotinylated antibody from the Impress Kit. Slides washed for 5 minutes with stirring in PBS. Meanwhile, diaminobenzidine (DAB) was prepared as per kit instructions (5 mL dH2O plus 2 drops of buffer, 4 drops of DAB solution and 2 drops of peroxide). Slides were incubated in chromagen, viewing the colour development under microscope. The reaction was stopped by dH2O. After that, counterstain for 15-30 seconds in Haematoxylin, blue with Scotts' Tap water. Slides were coversliped in consul mount and dried overnight.

2.8.4.1 Primary antibody

All four primary antibodies used were bought from Dako. Monoclonal mouse anti-human CD68 Clone PG-M1 was used for macrophages, monoclonal mouse anti-human CD45 leukocyte common antigen (Clone 2B11 + PD7/26) was used for leukocyte, monoclonal mouse anti-human mesothelial cell clone HBME-

1 was used for mesothelial cells, and polyclonal rabbit anti-human S100A4 was used for fibroblasts as shown in (Table 2.14).

Targeted cell (cell marker)	Primary antibody (Dilution)	Source
Macrophages (CD68)	Monoclonal mouse Anti-Human CD68 Clone PG-M1, (1:100)	(Dako, M0876, UK)
Leukocytes (CD45)	Monoclonal Mouse Anti-Human CD45 Leukocyte Common Antigen (Clone 2B11 + PD7/26), (1:50)	(Dako, M0701, UK)
Mesothelial cells	Monoclonal Mouse Anti-Human Mesothelial Cell Clone HBME-1, (1:75)	(Dako, M3505, UK)
Fibroblasts (FSP-1)	Polyclonal Rabbit Anti-Human S100A4, (1:200)	(Dako, A5114, UK)

Table 2.14 Primary antibodies used for various cell staining

Primary antibodies used in cell staining with sources and dilutions.

2.8.4.2 Secondary antibody

ImmPRESS™ universal reagent, anti-mouse/rabbit IgG was used as secondary antibody in the protocol (catalog number was MP-7500 from Vector laboratories) being stored at 2 - 8 °C prior to use. Vector ImmPRESS™ reagent is ready-to-use and needs no dilution. The ImmPRESS™ polymerized reporter enzyme staining system. The ImmPRESS™ reagents have a "micro-polymer" of very active peroxidase with a mixture of anti-mouse IgG as well as anti-rabbit IgG secondary antibodies. The ImmPRESS™ reagent is provided pre-diluted. Ready-to-use 2.5% of normal horse serum blocking solution was also included. ImmPRESS™ universal kit consisted of the 50 mL ImmPRESS™ Universal anti-mouse/rabbit IgG Reagent and 50 mL Ready-to-use normal horse serum blocking solution. The peroxidase substrate which is used is Diaminobenzidine (DAB). It produced a brown colour.

2.8.4.3 DAB Peroxidase Substrate

Vector® DAB Substrate (3,3'-diaminobenzidine, catalog number- SK-4100) yields a brown reaction product when oxidized by HRP. If nickel chloride is added to the substrate working solution, this results in a grey-black colouration. Vector® DAB Substrate can be used on cells or tissue sections, or even on membranes such as nitrocellulose. The Vector® DAB Substrate Kit has all of the reagents essential to prepare either a DAB or a DAB/nickel substrate working solution. These reagents were supplied in convenient dropper bottles (6 mL DAB Stock Solution, 6 mL Hydrogen Peroxide Solution, 6 mL Buffer Stock Solution, 6 mL Nickel Solution). The substrate was stored at 2 - 8 °C in dark place.

2.8.4.4 Photomicrography

An Olympus BX61 microscope with 2CC-12 FW colour digital camera was used for taking photographs. Neutral density filters were used when required. 400X was used as an objective magnification. The capture software was Olympus Cell^F image analysis version.

2.8.4.5 Cell counting

Fifteen random non-overlapping fields at 400x magnification were captured for each slide. The total number of cells for each slide was counted manually. Positive stained cells from the fifteen slides images were counted and divided by total cell count (positive and negative cells) for each image to obtain the percentage of positive stained cells. The means were calculated for 15 images per slide using Microsoft Excel 2010.

2.9 Statistical analysis

Analysis was performed using Microsoft Excel 2010 software, SPSS Statistics 21, and Graphpad prism (prism 6.01 for windows). $P<0.05$ was

considered statistically significant. Continuous data with normal distribution were expressed as means±SD or SE, while those with skewed distribution were expressed as median (IQR) or (range). Various tests had been used according to the need (Table 2.15). G*Power 31.3 was used for calculating sample size. The correlations run between various data and they were examined by Pearson correlation coefficient. Guidelines for interpreting correlations (Pearson's r) were:

r = +.70 or higher. It means very strong positive relationship
r= +.40 to +.69. It means strong positive relationship
r= +.30 to +.39. It means moderate positive relationship
r= +.20 to +.29. It means weak positive relationship
r= +.01 to +.19. It means no or negligible relationship
r= -.01 to -.19. It means no or negligible relationship
r=-.20 to -.29. It means weak negative relationship
r= -.30 to -.39. It means moderate negative relationship
r= -.40 to -.69. It means strong negative relationship
r= -.70 or higher. It means very strong negative relationship

2.10 Copyright permission

Permission was obtained for figures obtained from other sources as shown in the legend of the figures and appendix 7.

Statistical tests	Reasons of use
Unpaired t test	Unpaired data with Gaussian distribution, and equal SDs (two groups)
Unpaired t test with Welch's correction	Unpaired data with Gaussian distribution, and unequal SDs (two groups)
Mann Whitney test	Unpaired data with non-Gaussian distribution (two groups).
One way ANOVA	Unpaired data with Gaussian distribution (three or more groups)
Kruskal-Wallis test	Unpaired data with non-Gaussian distribution (three or more groups)
Friedman test	Each row represents matched data with non-Gaussian distribution (three or more groups).
Post-hoc comparisons using the Tukey's multiple comparisons test	Compare the mean of each column with the mean of every other column
The correlation test (Compute Pearson correlation coefficient).	Gaussian distribution of the data

Table 2.15 Statistical tests

Various statistical tests had been used with the reasons of using them.

CHAPTER THREE

Changes in ECM proteolytic systems in peritoneal dialysis effluent and plasma

3.1 Introduction

Numerous fibrotic disease mechanisms are known to be conserved across multiple organ systems. It is therefore reasonable to assume that systems involved in normal extracellular matrix (ECM) homeostatic balance that are altered in fibrotic disorders in other organs, may well also be implicated in PS and EPS. This would include the down regulation of enzyme systems involved in ECM breakdown (eg. matrix metalloproteinases (MMPs) and plasmin), the over expression of the natural inhibitors of these enzymes (eg. tissue inhibitors of matrix metalloproteinases (TIMPs) and plasminogen activator inhibitors (PAIs)), or excess production of ECM components.

MMPs are shut down in numerous fibrotic states resulting in the failure to clear ECM in the face of either continuing or accelerated deposition. MMPs and TIMPs are thought to play an important role in fibrosis in many organs such as the kidney (Johnson, Haylor et al. 2002), lung (Selman, Ruiz et al. 2000) and liver (Nie, Duan et al. 2004). It is possible that one or many members of the MMP family may have reduced expression or activation in PS & EPS. More likely is that there is over expression of the natural inhibitors of these enzymes such as TIMPs. TIMP-1 is particularly elevated in renal scarring (Johnson, Haylor et al. 2002) as well as other types of tissue scarring (Wang, Tu et al. 2011). There is study which showed that MMP-2 may be useful as a marker of increased solute transport, peritoneal injury or progression to EPS (Hirahara, Inoue et al. 2007). This serves to demonstrate the importance of understanding the ECM processing profile on an individual disease basis.

MMPs and TIMPs are produced by many cells (Figure 3.1) (Gibson, Cullen et al. 2009) including monocytes, (Zhang, McCluskey et al. 1998). Connective tissue destruction in chronic inflammatory lesions is due to infiltration by Monocyte/macrophages which secrete huge amounts of various MMPs degrading the connective tissue (Borden and Heller 1997). MMPs are also

produced by other inflammatory cells such as neutrophils and wound cells such as fibroblasts, endothelial and epithelial cells (Gibson, Cullen et al. 2009). It is already known that MMP-2 is produced by macrophages, endothelial cells, and myofibroblasts in the peritoneum (Hirahara, Umeyama et al. 2002, Hirahara, Ogawa et al. 2004, Hirahara, Kusano et al. 2006, Hirahara, Inoue et al. 2007).

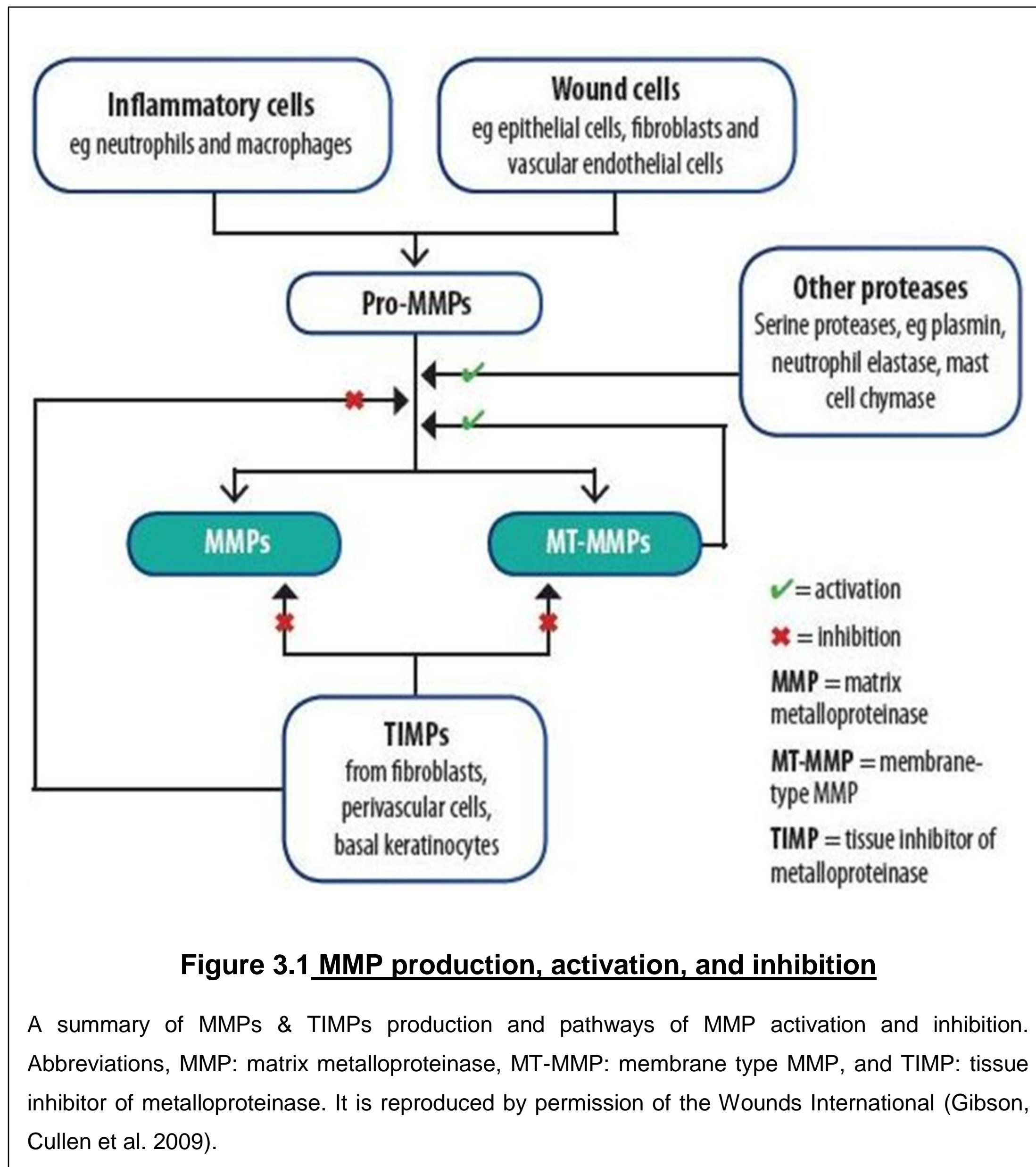


Figure 3.1 MMP production, activation, and inhibition

A summary of MMPs & TIMPs production and pathways of MMP activation and inhibition. Abbreviations, MMP: matrix metalloproteinase, MT-MMP: membrane type MMP, and TIMP: tissue inhibitor of metalloproteinase. It is reproduced by permission of the Wounds International (Gibson, Cullen et al. 2009).

Human peritoneal mesothelial cells (HPMC) have a role in controlling ECM accumulation by secreting MMPs such as MMP-2, MMP-3, and MMP-9. In Culture, TIMPs are also produced through HPMC. MMP and TIMP secretion by HPMC is mainly regulated by inflammatory cytokines. This process is important in the maintenance of the peritoneal membrane integrity in healthy individual and in the changes that happen after prolonged PD (Martin, Yung et al. 2000).

Proteolytic enzymes have various roles in inflammation such as ECM and basement-membrane proteins degradation. The MMP and plasmin systems are the two most important ECM proteolytic enzyme systems. TIMPs and plasmin activator inhibitors are key regulators of proteolytic activity, moderating MMP and plasmin activities respectively. The MMP to TIMP balance plus the regulation of tPA and uPA by plasmin activator inhibitors regulate ECM turnover.

In wound healing, MMPs play important and beneficial roles. They can remove of bacteria and damaged ECM (inflammation healing phase), degrade capillary basement membrane allowing angiogenesis (proliferation healing phase), encourage epidermal cells migration (proliferation healing phase), and regulate ECM scar contraction and remodeling (remodeling healing phase). All these are significant roles of the MMPs in normal or aberrant wound healing such as fibrosis. Importantly, high MMP activity for prolonged periods can create problems by causing excessive protein destruction leading to tissue damage or impairment of the healing process (Gibson, Cullen et al. 2009). For example, MMP-2 activation in rats can cause peritoneal injury during PD (Hirahara, Ogawa et al. 2004).

TIMP-1 and MMP-2 levels in the peritoneal effluent are known to mirror the small solute transport rate (Hirahara, Inoue et al. 2007). Regression analysis showed that serum levels of TIMP-1 and MMP-7 are predictors of heat shock protein (Hsp) 27 concentrations in patients on chronic dialysis (automated peritoneal dialysis and hemodialysis). Hsp27 is a marker of cellular stress in

children on long term dialysis (Musial and Zwolinska 2012). Mast cells are increased in number in PD patients (Alscher, Braun et al. 2007). Mast cell tryptase is defined as a serine protease which is involved in fibrosis and angiogenesis (Kondo, Kagami et al. 2001, Alscher, Braun et al. 2007)

The Plasmin system also plays an important role in fibrosis alongside the MMP system. They work together to breakdown most ECM components. In addition to that several pro-MMPs are activated by the plasmin system (Galis and Khatri 2002) such as MMPs-1, 3, 7, 9, 10 and 13 (Amalinei, Caruntu et al. 2007). MMP and Plasmin system are described in detail in section 1.8 in chapter one.

Taken together, it appears that the MMP and plasmin systems either directly or through their endogenous inhibitors of activity (TIMP) or activation (PAI) have a major role in ECM remodeling and homeostasis. Further those perturbations in either would have a dramatic effect on normal function in any organ especially when under stress.

3.2 Hypothesis

Given the importance of the MMP and plasmin systems in ECM regulation and their known role in other fibrotic diseases it is likely that similar changes in their normal function also underlie the development of PS and the switch to EPS. This chapter therefore proposes to test the hypothesis that changes in the MMP and plasmin system activity in the peritoneum underlies PS &/or the switch to EPS. Further that changes in these systems may be detectable in the PD effluent and that they may have values as non-invasive prognostic markers or as diagnostic tools for PS and EPS.

3.3 Aims

To test this hypothesis this chapter aims to gain an insight into the contribution of the MMP and plasmin systems to the pathology of PS and EPS. To do this, the following studies will be undertaken:

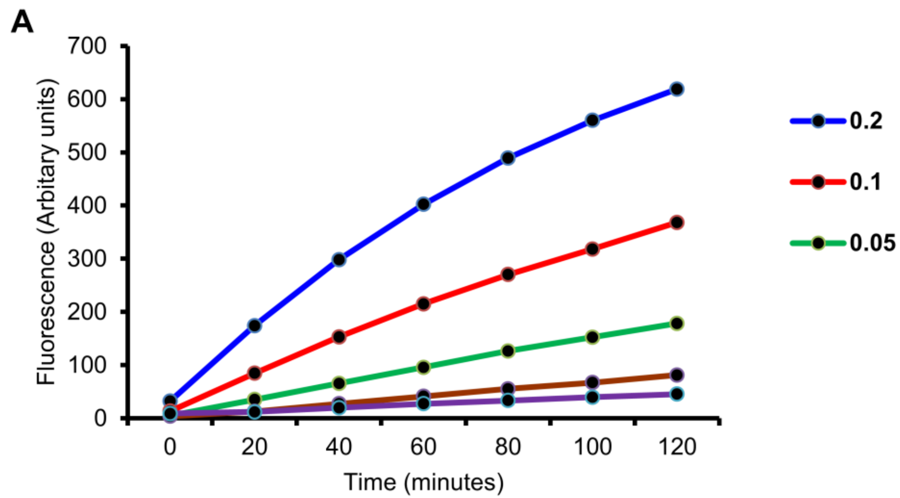
1. Measure MMP and plasmin activity in PDE samples from PD patients to establish any abnormal levels of ECM proteolysis
2. If there are changes in activity, determine the contributions of individual proteolytic enzymes and their natural inhibitors by ELISA kits
3. Determine the value of any identified enzyme or inhibitor in predicting EPS development

3.4 Methods

3.4.1 Activity assays

MMP activity in PDE samples was measured using the ENZchek Gelatinase/Collagenase Assay Kit (E-12055) (section 2.8.2.1). Various concentrations of MMP activity were used and then changes of fluorescence were measured with time at room temperature. From this, standard curve was created for fluorescence changes/hr to MMP units/mL (Figure 3.2). All PDE samples were loaded neat.

Plasmin activity in PDE samples was assessed by using cleavage of the V0882 substrate (sigma) (section 2.8.2.2). Various concentrations of plasmin activity were used and then changes of optical density at 405 nm were measured with time at room temperature. From this, standard curve was created for optical density changes/hr to plasmin activity µg/mL (Figure 3.3). All PDE samples were loaded neat or 1:2 /1.3.



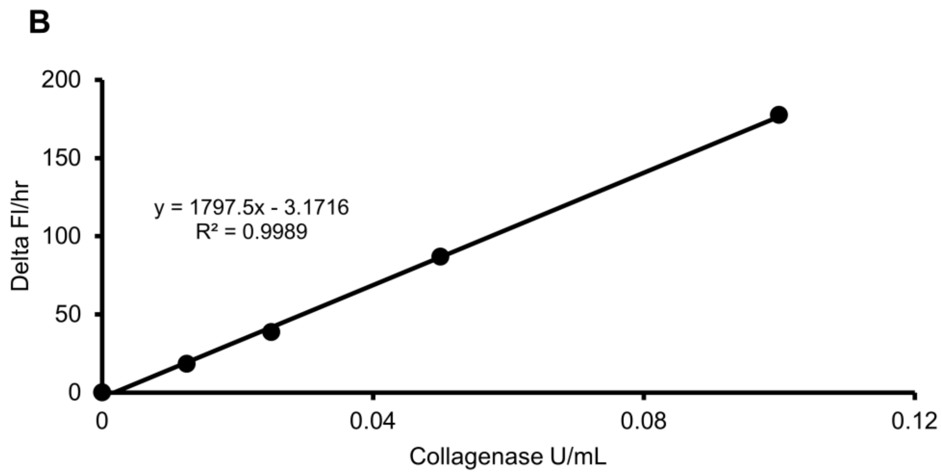


Figure 3.2 Rate of reaction and standard curve of MMP activity assay

Rate of reaction (A) is showing changes of fluorescence within first 120 minutes for various concentrations of MMP activity. Standard curve (B) is showing fluorescence changes/hr to MMP activity U/mL.

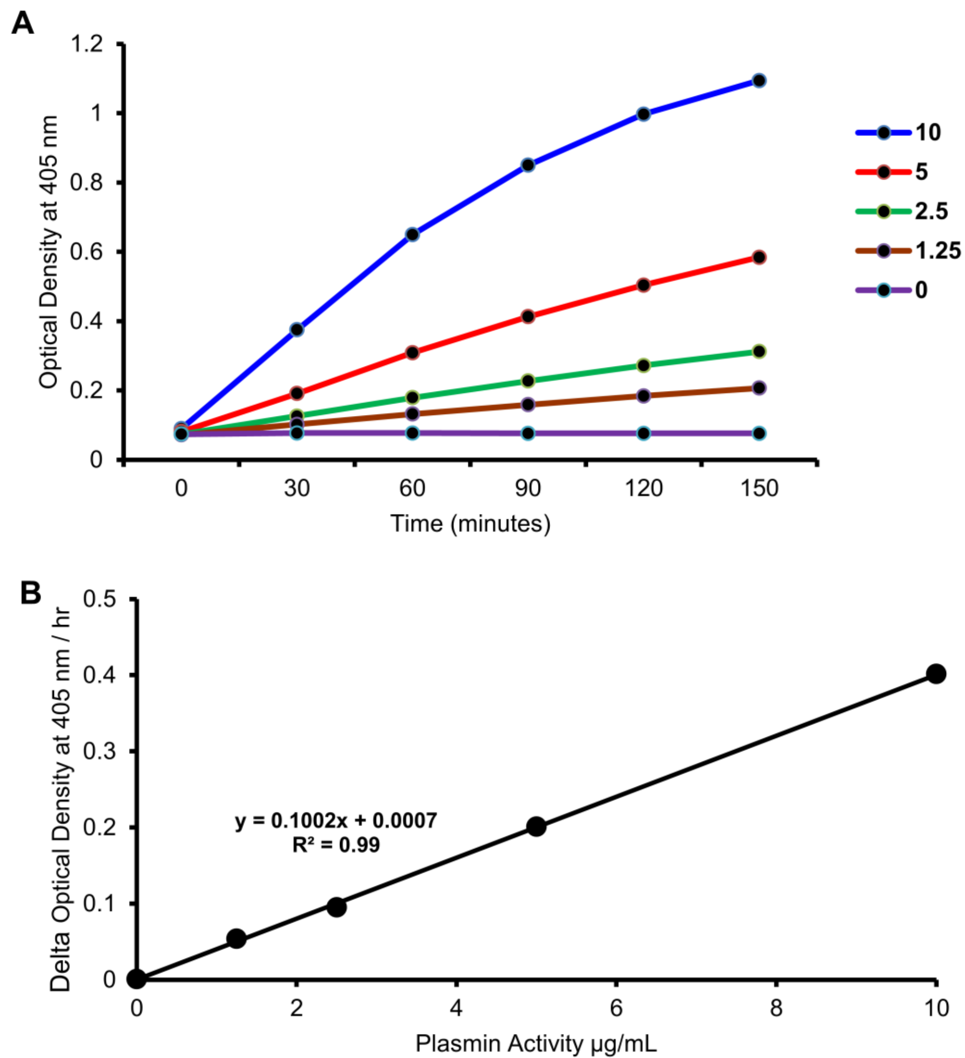


Figure 3.3 Rate of reaction and standard curve of plasmin activity assay

Rate of reaction (A) is showing changes of optical density at 405 nm within first 150 minutes for various concentrations of plasmin activity. Standard curve (B) is showing optical density at 405 nm changes/hr to plasmin activity µg/mL.

3.4.2 Protein assay

Protein in PDE samples was measured using Thermo Scientific™ Pierce™ 660nm protein assay (section 2.8.1) because protein concentration was used for correcting TIMPs and MMPs in SKI-1. All PDE samples were loaded neat or 1:2 /1.3. Various concentrations of BSA (Bovine Serum Albumin) were prepared from a stock concentration of BSA in PBS (phosphate buffered saline). Each of the standard curve point was calculated from the average value of the duplicate readings. Then the curve was generated by plotting the measured protein concentrations on the x–axis, while the corresponding mean 660 nm absorbance on the y-axis. After that either straight line or the best fit curve was obtained from the points. The standard curve and the calculating equation can be performed either by using Excel or Thermo Lab systems Multiskan Ascent plate reader software (Figure 3.4).

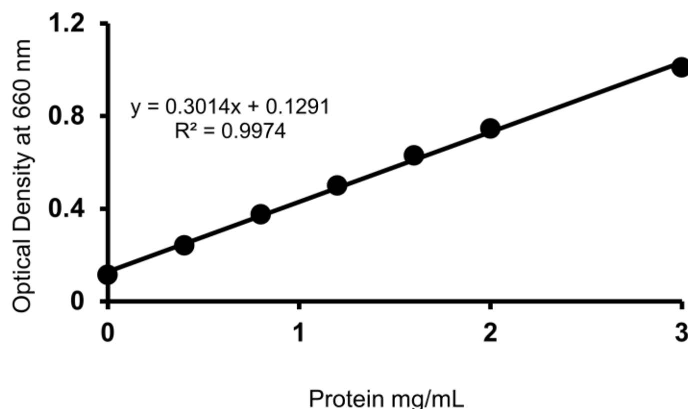


Figure 3.4 Standard curve for protein

The protein concentrations are plotted on the x-axis, while the absorptions are plotted on the y-axis. Optical density changes were measured in the 96 well microplate reader at 660 nm. Standard curve is made for protein either by using Microsoft Excel or Thermo Labsystems Multiskan Ascent plate reader software.

3.4.3 ELISAs

Individual TIMPs and MMPs in these 2 cohorts and the GFS patients was measured using standard commercial ELISAs (sections 2.8.3.1 and 2.8.3.2). Two examples of standard curves (TIMP-1 and MMP-2) are given as examples for TIMPs and MMPs standard curve (Figure 3.5). The SKI-1 cohort consists of overnight PDE samples only. The GFS cohort consists of 4 hour dwell time PDE samples only. The SKI-2 cohort has PDE samples obtained from patients undergoing PET at 0, 2, 4 hour, and overnight with a plasma samples at 2 hour. During ELISA, samples were diluted to ensure measurements were within the most accurate range of the ELISA. MMP-1, MMP-9, MMP-13, TIMP-3, and MMP-1/TIMP-1 complex were measured in PDE samples in a dilution of 1:1 (neat samples).

TIMP-1, TIMP-2, MMP-2, and MMP-3 were measured in PDE and plasma samples. They required dilutions as the concentrations of TIMP-1, TIMP-2, MMP-2, and MMP-3 are high in PDE samples. The dilutions were very variable depending on subtype of MMP or TIMP; for example TIMP-1 needed more dilution than the rest of TIMPs and MMPs. Sample dilutions were also affected by dwell times as overnight samples required higher dilution than others. Flushed samples needed less dilution because they are diluted. All efforts were made to ensure measurements were within the most accurate range of the ELISA. TIMP-1, TIMP-2, MMP-2, and MMP-3 concentrations were higher in plasma samples in comparison to PDE samples, requiring a higher dilution. The dilutions were approximately 1:400, 1:700, 1:75, and 1:80 for measuring plasma levels for TIMP-1, TIMP-2, MMP-2, and MMP-3 respectively.

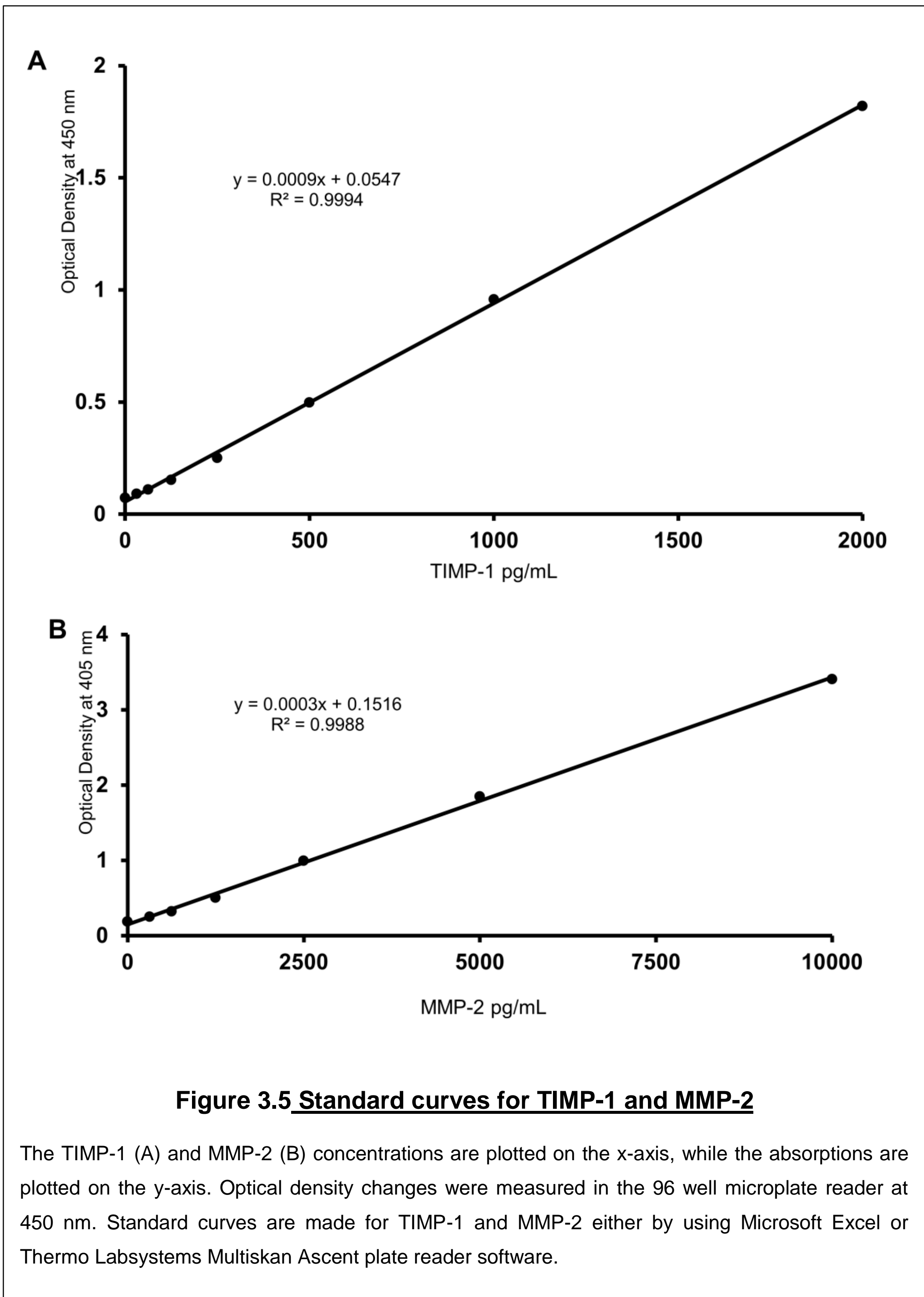


Figure 3.5 Standard curves for TIMP-1 and MMP-2

The TIMP-1 (A) and MMP-2 (B) concentrations are plotted on the x-axis, while the absorptions are plotted on the y-axis. Optical density changes were measured in the 96 well microplate reader at 450 nm. Standard curves are made for TIMP-1 and MMP-2 either by using Microsoft Excel or Thermo Labsystems Multiskan Ascent plate reader software.

3.5 Results

3.5.1 MMP activity in SKI-1 and SKI-2

MMP activity (i.e. MMP-TIMP balance) was measured in SKI-1 and SKI-2 patient cohorts. To see if there was any change in MMP activity with time on PD, SKI-1 and SKI-2 patient cohort samples were divided into 3 groups based on time on PD (<1 year, 1-3 years, and >3 years). In SkI-1, there was one EPS patient (SKI-120), but this sample was collected over 9 months after an EPS diagnosis was made.

There was no or very little MMP activity in any of Sheffield PDE samples tested from either SKI-1 or SKI-2 cohorts irrespective of duration on PD or EPS development even after 48 hours incubation (Figure 3.6). Given the low values, to validate the assay was working correctly, MMP activity was measured in a CKD patient urine sample and was found to be high as predicted (25.5 u/L). During statistical analysis, PDE sample from one EPS patient and urine sample from one CKD patient were excluded from analysis.

The finding of such low MMP activity compared to other biological fluids was marked and could clearly be associated with a pro-fibrotic phenotype. Thus to establish the reasons behind this low or undetected MMP activity in PDE samples, ELISA for various TIMPs and MMPs was performed on PDE samples to understand the relative MMP-TIMP balance.

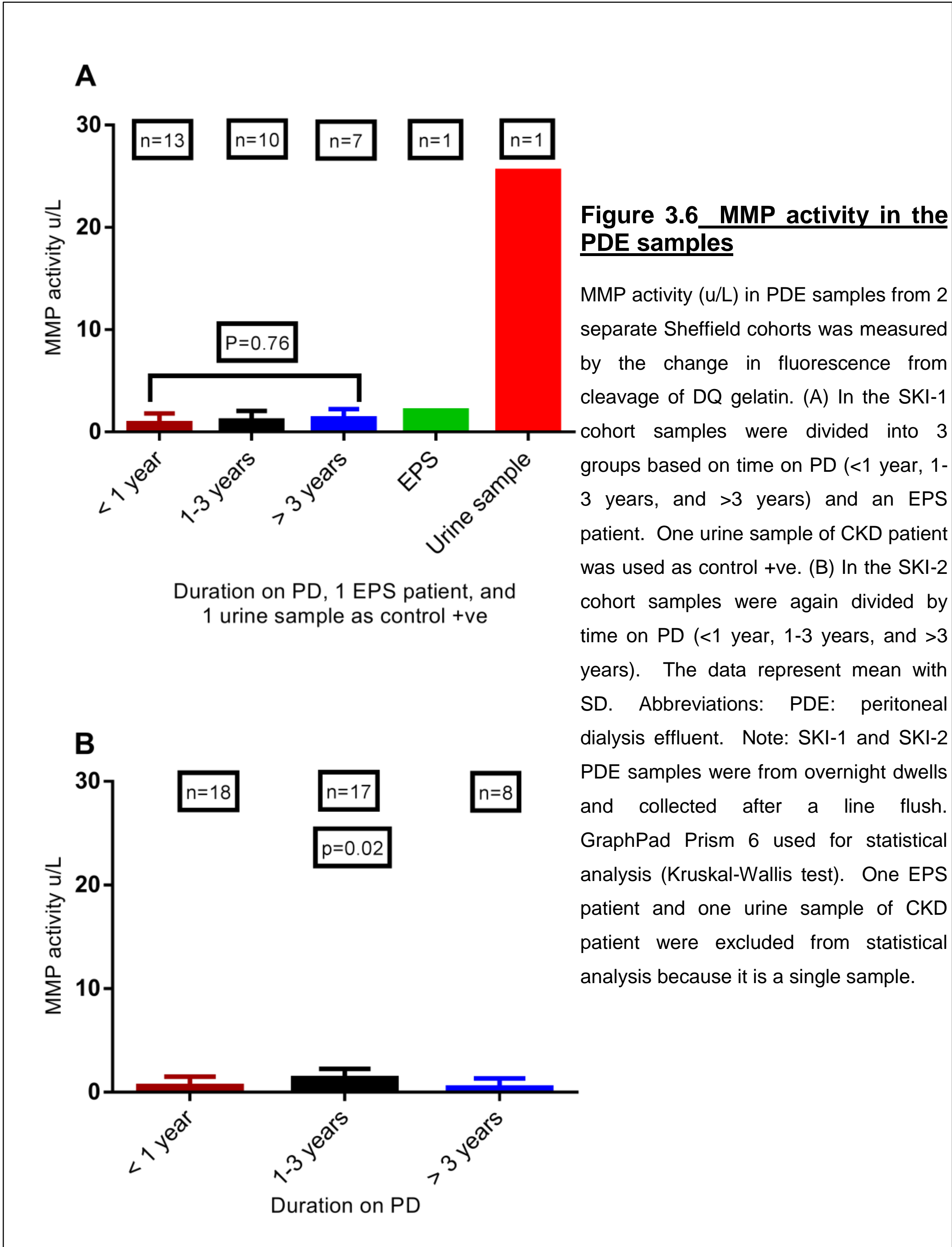


Figure 3.6 MMP activity in the PDE samples

MMP activity (u/L) in PDE samples from 2 separate Sheffield cohorts was measured by the change in fluorescence from cleavage of DQ gelatin. (A) In the SKI-1 cohort samples were divided into 3 groups based on time on PD (<1 year, 1-3 years, and >3 years) and an EPS patient. One urine sample of CKD patient was used as control +ve. (B) In the SKI-2 cohort samples were again divided by time on PD (<1 year, 1-3 years, and >3 years). The data represent mean with SD. Abbreviations: PDE: peritoneal dialysis effluent. Note: SKI-1 and SKI-2 PDE samples were from overnight dwells and collected after a line flush. GraphPad Prism 6 used for statistical analysis (Kruskal-Wallis test). One EPS patient and one urine sample of CKD patient were excluded from statistical analysis because it is a single sample.

3.5.2 Plasmin activity in SKI-1 and SKI-2 cohorts

In addition to the MMP activity assay, plasmin activity assay was measured in PDE samples. Plasmin plays an important role in activating pro-MMPs to MMPs and MT-MMPs (Figure 3.1) and in ECM degradation. The gradient (Δ optical density at 405 nm/hr) for each PDE sample was calculated to show the plasmin activity with time. A plot of change in optical density for SKI-125 with time is shown as an example (Figure 3.7).

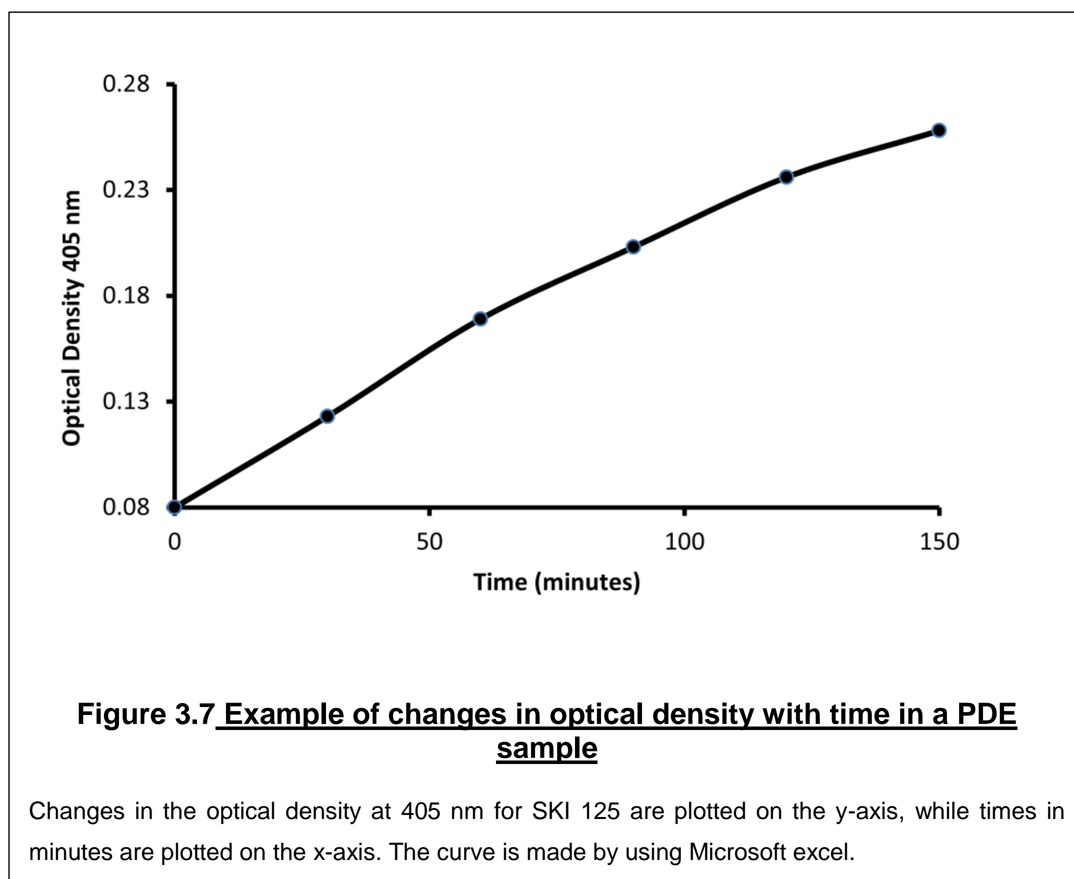


Figure 3.7 <u>Example of changes in optical density with time in a PDE sample</u>

Changes in the optical density at 405 nm for SKI 125 are plotted on the y-axis, while times in minutes are plotted on the x-axis. The curve is made by using Microsoft excel.

In comparison to MMP activity, plasmin activity in PDE samples in both SKI-1 and SKI-2 cohorts was readily detectable using changes in the optical density due to cleavage of substrate V0882 (Figure 3.8). Overall there seemed to be a decrease in plasmin activity in patients on PD for long duration (3 years or more). In the SKI-1 patient cohort the mean plasmin activity was lower in the 3 years PD

group compared to shorter PD periods but never reached significance. However in the SKI-2 cohort, mean plasmin activity for those on PD for more than 3 years was 50% lower than the less than 1 year group and 33% lower than the 1 to 3 year group, with significant difference between the three groups (p=0.004).

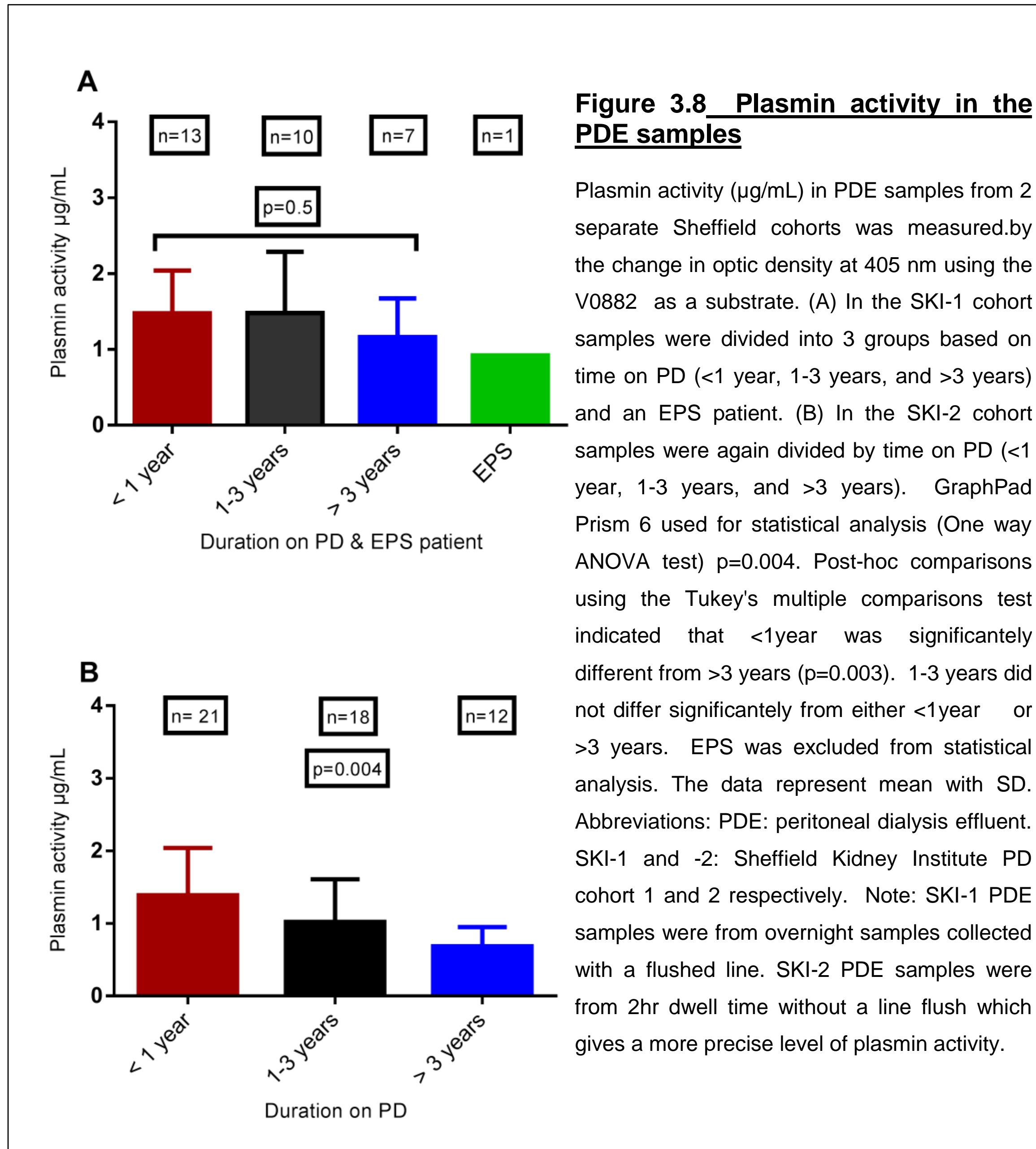


Figure 3.8 Plasmin activity in the PDE samples

Plasmin activity (µg/mL) in PDE samples from 2 separate Sheffield cohorts was measured.by the change in optic density at 405 nm using the V0882 as a substrate. (A) In the SKI-1 cohort samples were divided into 3 groups based on time on PD (<1 year, 1-3 years, and >3 years) and an EPS patient. (B) In the SKI-2 cohort samples were again divided by time on PD (<1 year, 1-3 years, and >3 years). GraphPad Prism 6 used for statistical analysis (One way ANOVA test) p=0.004. Post-hoc comparisons using the Tukey's multiple comparisons test indicated that <1year was significantely different from >3 years (p=0.003). 1-3 years did not differ significantely from either <1year or >3 years. EPS was excluded from statistical analysis. The data represent mean with SD. Abbreviations: PDE: peritoneal dialysis effluent. SKI-1 and -2: Sheffield Kidney Institute PD cohort 1 and 2 respectively. Note: SKI-1 PDE samples were from overnight samples collected with a flushed line. SKI-2 PDE samples were from 2hr dwell time without a line flush which gives a more precise level of plasmin activity.

In the SKI-2 cohort, the plasmin activity at the 2 hour dwell time was negatively correlated to the duration on PD (r= -0.4, p=0.004, n=51 samples), and to the last year glucose exposure during PD (r= -0.51, p=0.0001, n=51 samples) (Figure 3.9).

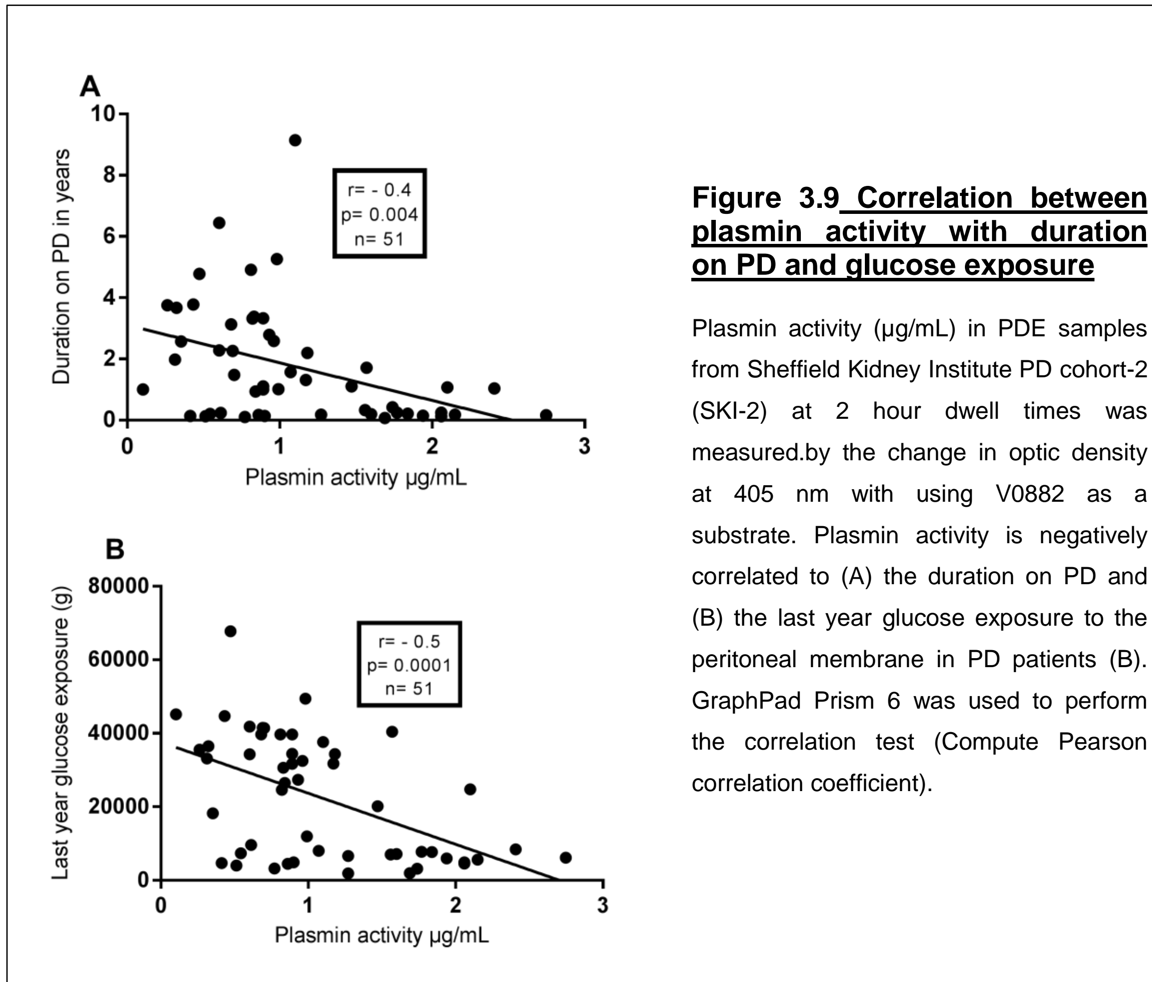


Figure 3.9 Correlation between plasmin activity with duration on PD and glucose exposure

Plasmin activity (μg/mL) in PDE samples from Sheffield Kidney Institute PD cohort-2 (SKI-2) at 2 hour dwell times was measured.by the change in optic density at 405 nm with using V0882 as a substrate. Plasmin activity is negatively correlated to (A) the duration on PD and (B) the last year glucose exposure to the peritoneal membrane in PD patients (B). GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient).

To ascertain the most representative dwell time for measuring plasmin activity in, PDE samples from the same patient with increasing dwell times were analysed. This was to allow identification of the earliest time plasmin activity could be assayed and the linearity in the changes of plasmin activity with dwell time. PDE samples from 6 patients were collected prior to tube flushing during a PET at all-time points (zero, 2hr, 4hr, and overnight). All the 6 samples showed an increase of plasmin activity with dwell time (Figure 3.10). 2 hour dwell times

appear suitable for analysis for all 51 PDE samples in the SKI-2 cohort because samples were collected prior to a line flush at 2 hour dwell time. In 4 hour dwell times, due to the dilution of collected samples with a line flush, samples are more diluted so less plasmin activity was present. This lead to inaccurate and variable results; for example plasmin activity in PDE sample SKI-143 (4hr) dwell was measured before and after a line flush were 2 and 1.5 µg/mL respectively. The presence of plasmin activity in the time zero samples may have been due to residual overnight plasmin activity in the samples.

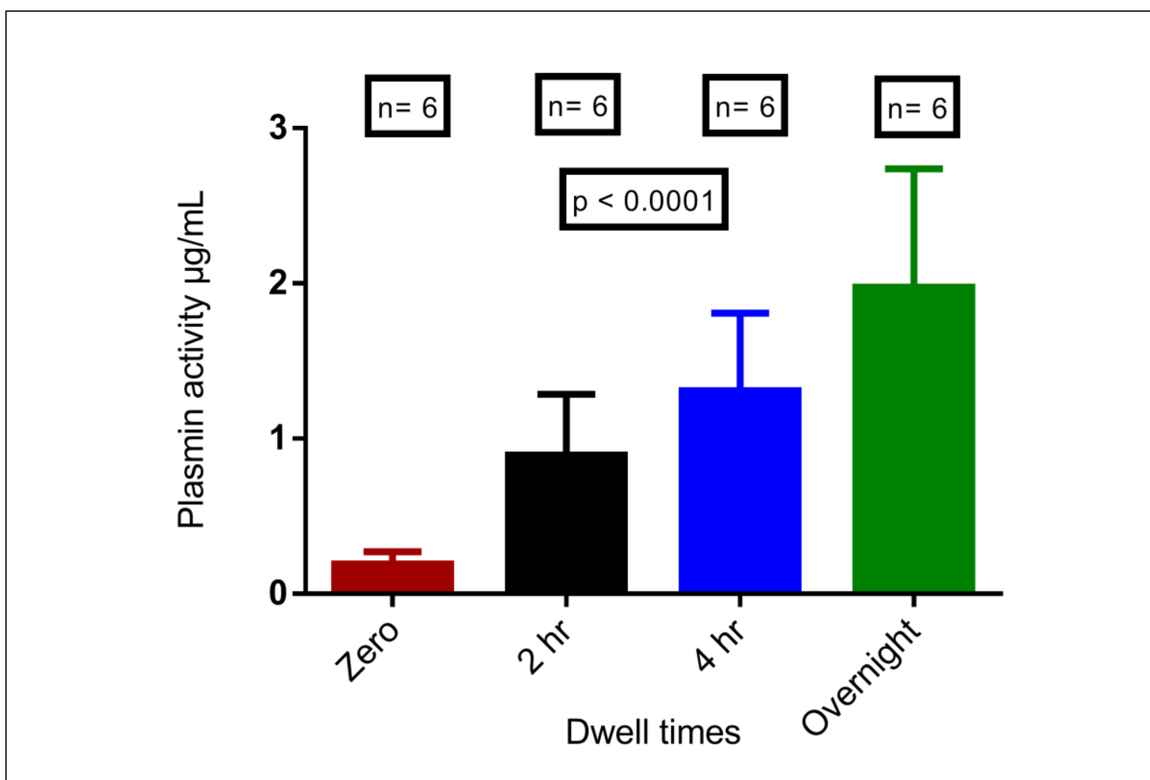


Figure 3.10 <u>Effect of dwell times on measuring plasmin activity</u>

Plasmin activity level was measured in PDE samples of six PD patients in the SKI-2 cohort with each having samples collected at 0, 2 and 4 hour and overnight dwell times. GraphPad Prism 6 used for statistical analysis (One way ANOVA) (P<000.1). Post-hoc comparisons using the Tukey's multiple comparisons test indicated that zero was significantely different from 4hr and overnight with p=0.005 and <0.0001. 2hr was also significantely different from overnight with p=0.006. The rest comparison did not differ significantely from eachothers. The data represent mean with SD. Abbreviations: PDE: peritoneal dialysis effluent. SKI-2: Sheffield Kidney Institute PD cohort 2.

3.5.3 Measurement of individual MMPs and TIMPs in PDE samples from patient cohort SKI-1

Given the lack of MMP activity in PDE, then it is reasonable to assume that this would affect ECM clearance and thus lead to a pro-fibrotic environment in the peritoneum. It was therefore important to ascertain why there was this lack of activity; a failure to make MMPs, an overproduction of TIMPs or combinations of the two are potential options.

3.5.3.1 Measurement of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13

The exact dwell times for SKI-1 samples are unknown and the degree of line flushing that can lead to sample dilution was also unknown. To compensate correction has been performed to total protein concentration in PDE samples. MMP-1, -9, and -13 were low or undetected in most samples in the SKI-1 cohort (Figure 3.11). In comparison, MMP-2 level was considerably higher and detectable in all PDE samples with a cohort mean (±SD) of 33±24 ng/mL. MMP-2 level in the single EPS patient was higher than other groups. MMP-3 was also detectable but the level was lower than MMP-2 (Figure 3.12).

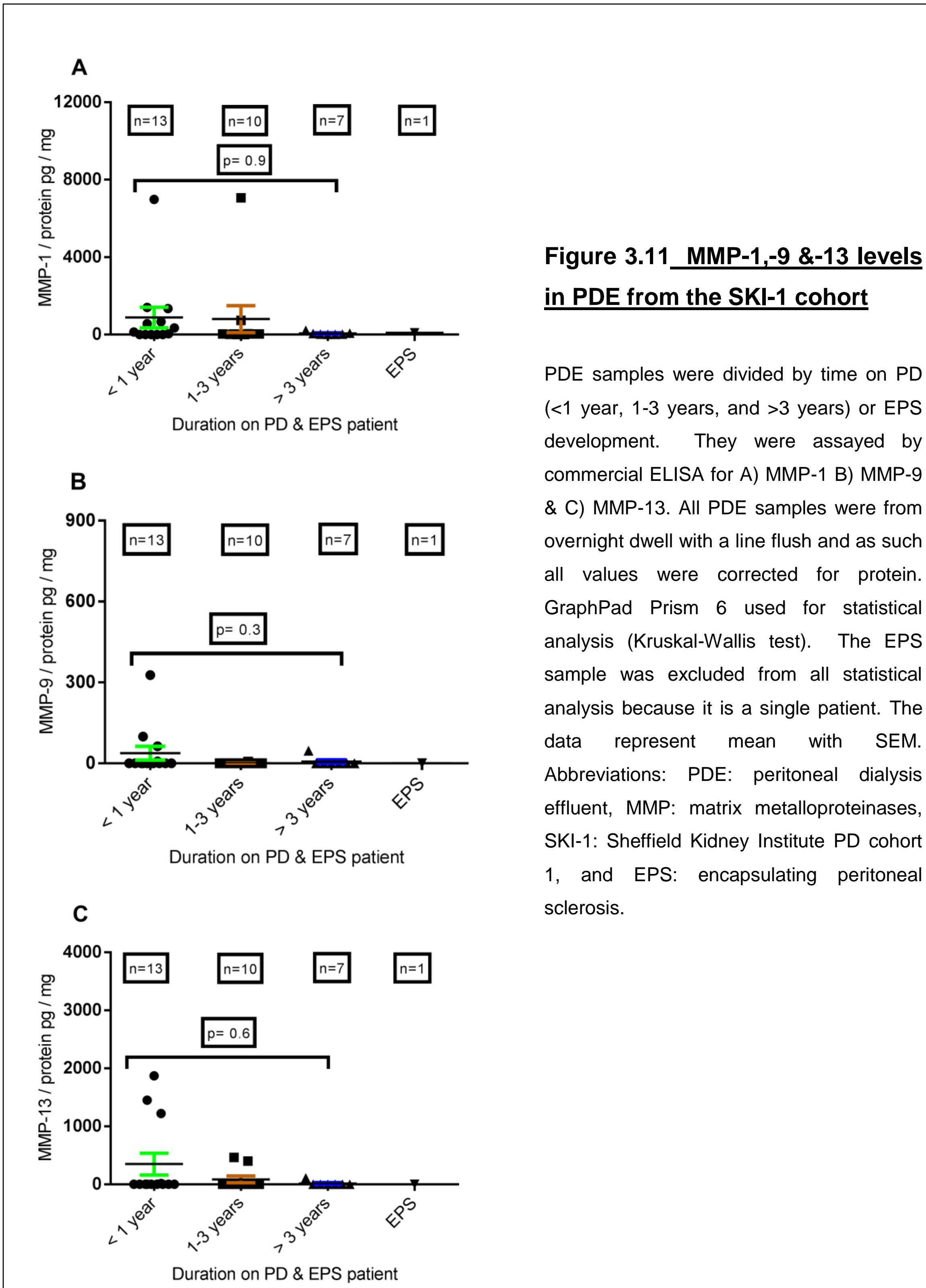


Figure 3.11 MMP-1,-9 &-13 levels in PDE from the SKI-1 cohort

PDE samples were divided by time on PD (<1 year, 1-3 years, and >3 years) or EPS development. They were assayed by commercial ELISA for A) MMP-1 B) MMP-9 & C) MMP-13. All PDE samples were from overnight dwell with a line flush and as such all values were corrected for protein. GraphPad Prism 6 used for statistical analysis (Kruskal-Wallis test). The EPS sample was excluded from all statistical analysis because it is a single patient. The data represent mean with SEM. Abbreviations: PDE: peritoneal dialysis effluent, MMP: matrix metalloproteinases, SKI-1: Sheffield Kidney Institute PD cohort 1, and EPS: encapsulating peritoneal sclerosis.

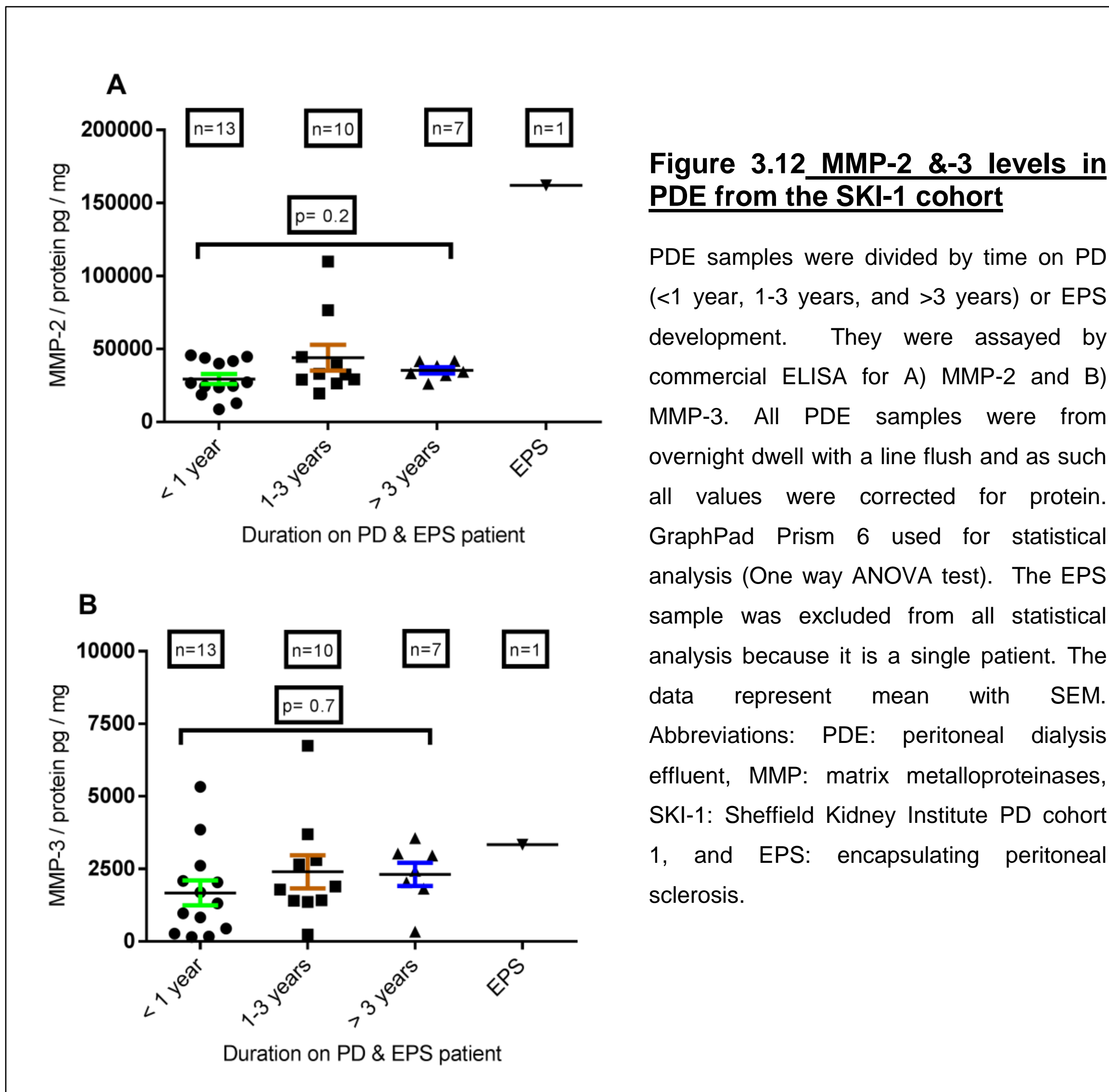


Figure 3.12 MMP-2 &-3 levels in PDE from the SKI-1 cohort

PDE samples were divided by time on PD (<1 year, 1-3 years, and >3 years) or EPS development. They were assayed by commercial ELISA for A) MMP-2 and B) MMP-3. All PDE samples were from overnight dwell with a line flush and as such all values were corrected for protein. GraphPad Prism 6 used for statistical analysis (One way ANOVA test). The EPS sample was excluded from all statistical analysis because it is a single patient. The data represent mean with SEM. Abbreviations: PDE: peritoneal dialysis effluent, MMP: matrix metalloproteinases, SKI-1: Sheffield Kidney Institute PD cohort 1, and EPS: encapsulating peritoneal sclerosis.

3.5.3.2 Measurement of TIMP-1, and the MMP-1/TIMP-1 complex

TIMP-1 was high in all Sheffield PDE samples from the start of PD. TIMP-1 in the SKI-1 cohort (Mean±SD) was 110±89 ng/mL. TIMP-1 in the single EPS patient in SKI-1 was particularly high (224 ng/mL), which when corrected to the amount of protein in the sample was clearly elevated (Figure 3.13.A). MMP-1/ TIMP1 complex was also measured, Like MMP-1, the MMP-1/TIMP-1 complex was low or undetected in Sheffield PDE samples most likely due to very little or no MMP-1 in PDE samples.(Figure 3.13.B).

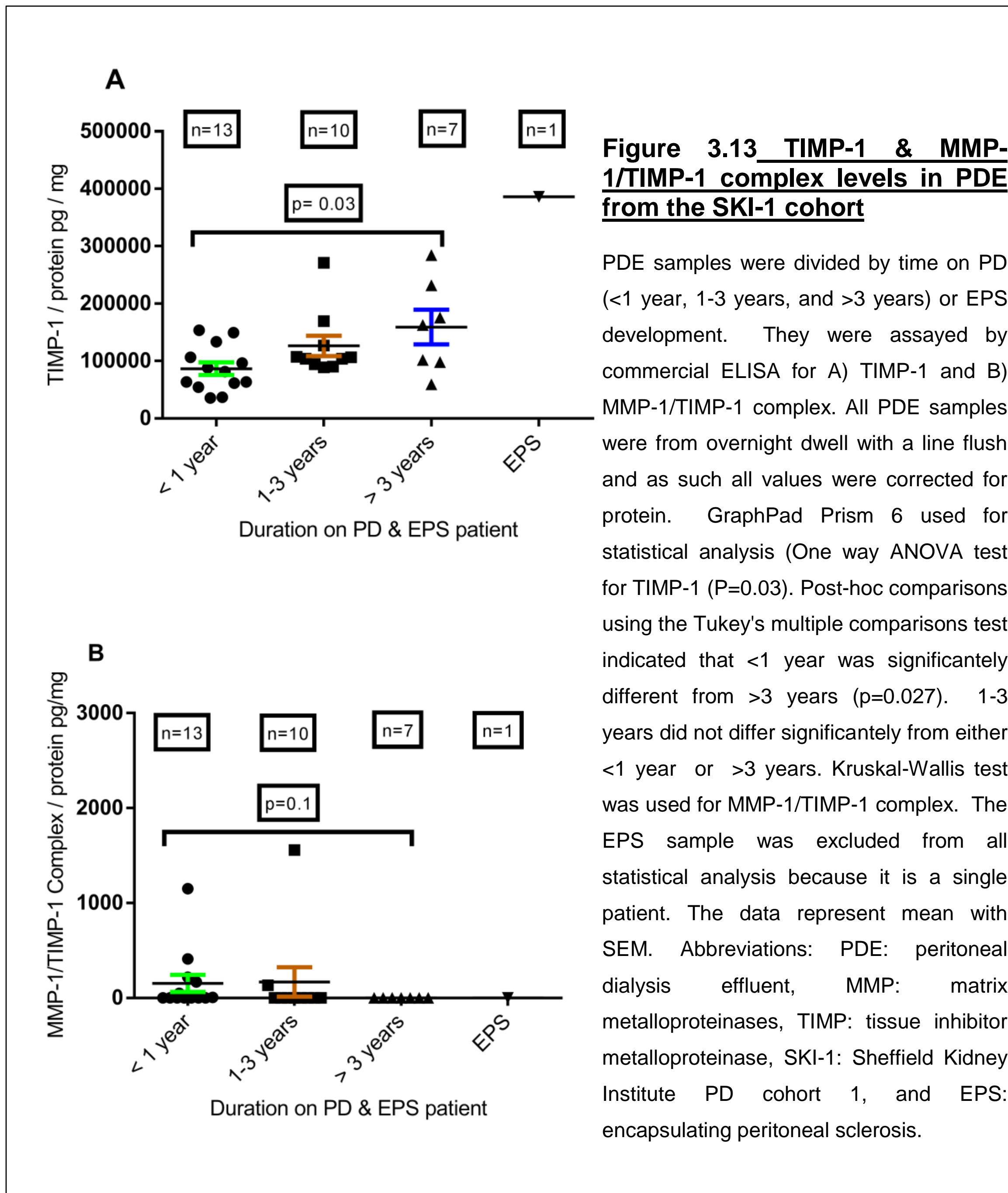


Figure 3.13 TIMP-1 & MMP-1/TIMP-1 complex levels in PDE from the SKI-1 cohort

PDE samples were divided by time on PD (<1 year, 1-3 years, and >3 years) or EPS development. They were assayed by commercial ELISA for A) TIMP-1 and B) MMP-1/TIMP-1 complex. All PDE samples were from overnight dwell with a line flush and as such all values were corrected for protein. GraphPad Prism 6 used for statistical analysis (One way ANOVA test for TIMP-1 (P=0.03). Post-hoc comparisons using the Tukey's multiple comparisons test indicated that <1 year was significantely different from >3 years (p=0.027). 1-3 years did not differ significantely from either <1 year or >3 years. Kruskal-Wallis test was used for MMP-1/TIMP-1 complex. The EPS sample was excluded from all statistical analysis because it is a single patient. The data represent mean with SEM. Abbreviations: PDE: peritoneal dialysis effluent, MMP: matrix metalloproteinases, TIMP: tissue inhibitor metalloproteinase, SKI-1: Sheffield Kidney Institute PD cohort 1, and EPS: encapsulating peritoneal sclerosis.

3.5.3.3 Measurement of TIMP-2 and TIMP-3

TIMP-2 was detectable in all in Sheffield PDE samples in the SKI-1 cohort, from the start of PD with a mean±SD value of 17.2±12.4 ng/mL, which is 7 fold less than TIMP-1 in the same PDE samples. Of note was that the single EPS

patient reached 39.3 ng/mL, which was the maximum value seen. TIMP-2 was corrected for protein in the PDE samples to normalize for dilution, with the level in the EPS sample was clearly elevated (67.8 ng/mg) (Figure 3.14 A). TIMP-3 had the lowest level among the three TIMPs measured in this cohort averaging 244 pg/mL in none EPS patients (Figure 3.14 B). However TIMP-3 in the EPS patient was also high (1392 pg/mL) compared to all other samples. After correction for protein, TIMP-3 in the EPS PDE remained higher than any other sample measured.

TIMP-2 was very strongly positively correlated to TIMP-1 ($r=0.8$, $p < 0.0001$). TIMP-2 was less strongly positively correlated to TIMP-3 ($r=0.65$, $p < 0.0001$) with the correlation between TIMP-1 and TIMP-3 weak ($r=0.37$, $p= 0.04$). Together this demonstrates differential regulation of the TIMPs in PDE and low level of TIMP-3 in PDE may be due to binding to ECM.

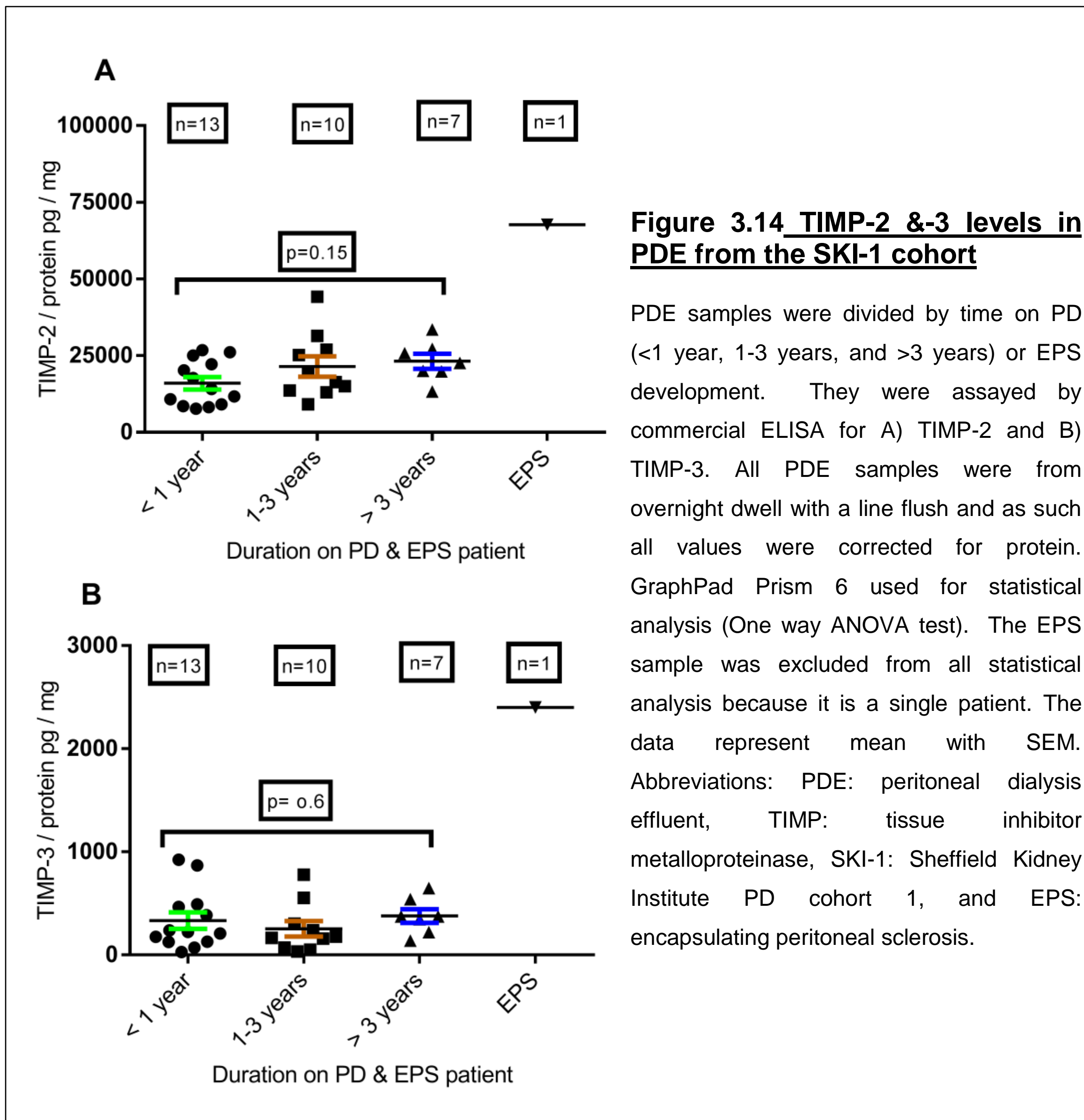


Figure 3.14 TIMP-2 &-3 levels in PDE from the SKI-1 cohort

PDE samples were divided by time on PD (<1 year, 1-3 years, and >3 years) or EPS development. They were assayed by commercial ELISA for A) TIMP-2 and B) TIMP-3. All PDE samples were from overnight dwell with a line flush and as such all values were corrected for protein. GraphPad Prism 6 used for statistical analysis (One way ANOVA test). The EPS sample was excluded from all statistical analysis because it is a single patient. The data represent mean with SEM. Abbreviations: PDE: peritoneal dialysis effluent, TIMP: tissue inhibitor metalloproteinase, SKI-1: Sheffield Kidney Institute PD cohort 1, and EPS: encapsulating peritoneal sclerosis.

3.5.4 MMPs and TIMPs in PDE and plasma samples in SKI-2

MMPs and TIMPs measured again in Plasma and PDE samples in the second Sheffield Kidney Institute PD cohort 2 (SKI-2) for two reasons. Firstly, to validate the data above in a more rigoursly collected cohort and and secondly, to compare plasma levels of TIMP-1, TIMP-2, MMP-2, and MMP-3 in PD patients versus their levels in plasma of 18 healthy individuals.

TIMP-1, TIMP-2, MMP-2, and MMP-3 were easily detectable in SKI-1, and thus it seemed logical to restrict to these 4 proteins & confirm these findings in the second Sheffield cohort, SKI-2, where there were known dwell times, the line flush controlled and detailed clinical information available. TIMP-1, TIMP-2, MMP-2, and MMP-3 were also measured in plasma of healthy individuals and & PD patients to ascertain if any changes in PDE were simply related to systemic levels.

3.5.4.1 Correlations of protein, MMPs and TIMPs in PDE samples with clinical data in the SKI-2

A set of correlations were run between protein, MMP-2, MMP-3, TIMP-1, and TIMP-2 against various clinical data (D/Pcr, D/D0 glucose and UF).

3.5.4.1.1 Correlations of protein, MMPs and TIMPs in PDE samples against D/Pcr and D/D0 glucose.

Peritoneal solute transport rate may be useful for evaluating peritoneal membrane injury. This is assessed during PET by D/Pcr and D/D0 glucose. These clinical data were analysed to establish any link to the MMP family proteins. D/D0 glucose at 2hr was negatively correlated to overall protein in PDE samples at 2hr ($r= -0.57$, $p < 0.0001$). It was also negatively correlated to TIMP-1 ($r= -0.57$, $p < 0.0001$), TIMP-2 ($r= -0.58$, $p < 0.0001$), MMP-2 ($r= -0.61$, $p < 0.0001$) and MMP-3 ($r= -0.57$, $p < 0.0001$) in PDE samples at 2 hr, n=51. D/Pcr at 2hr was positively correlated to protein in PDE samples at 2hr ($r=0.7$, $p < 0.0001$), n=51. It was also positively correlated to TIMP-1, TIMP-2, MMP-2 and MMP-3 in PDE samples at 2hr (Figure 3.15).

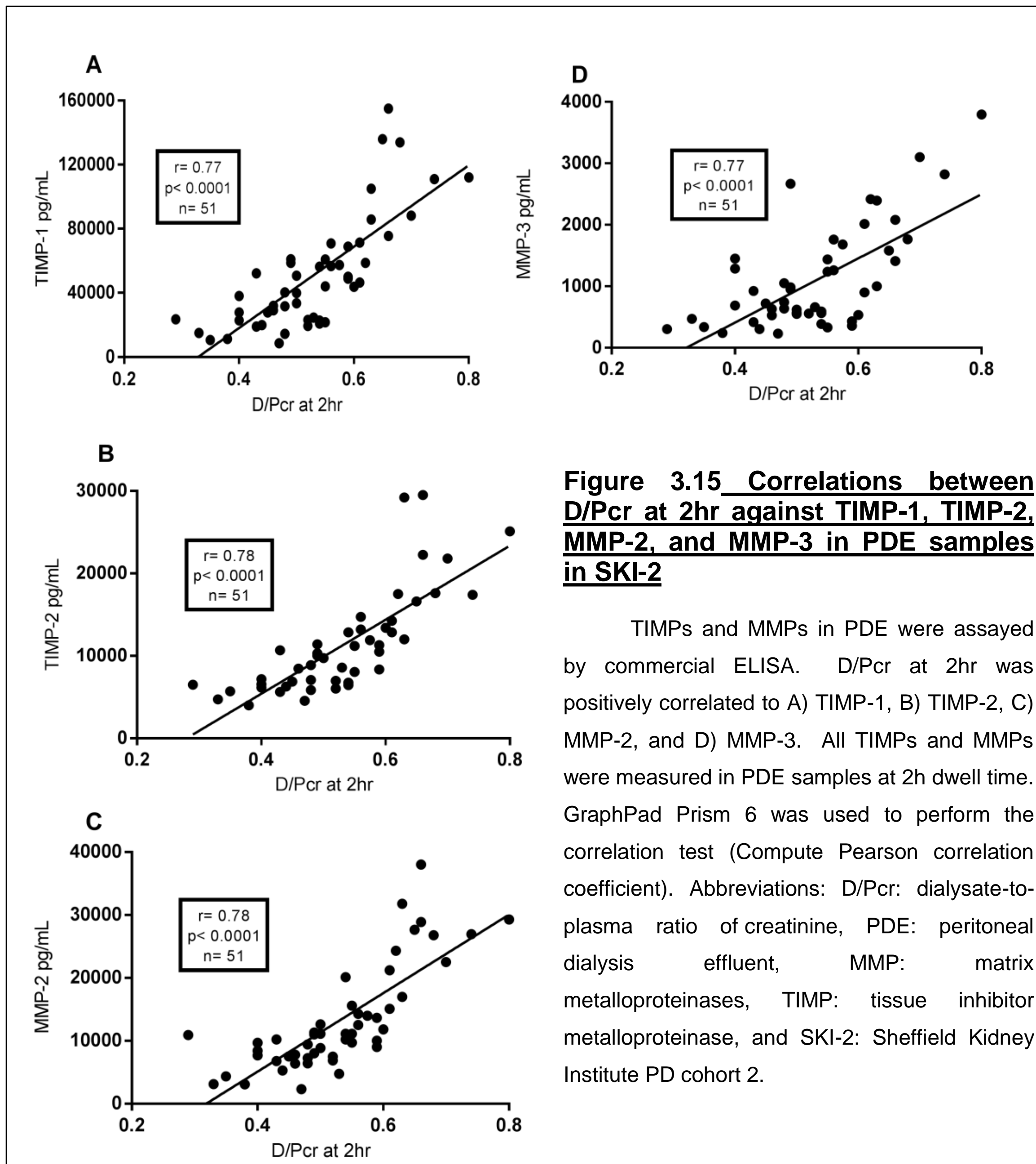


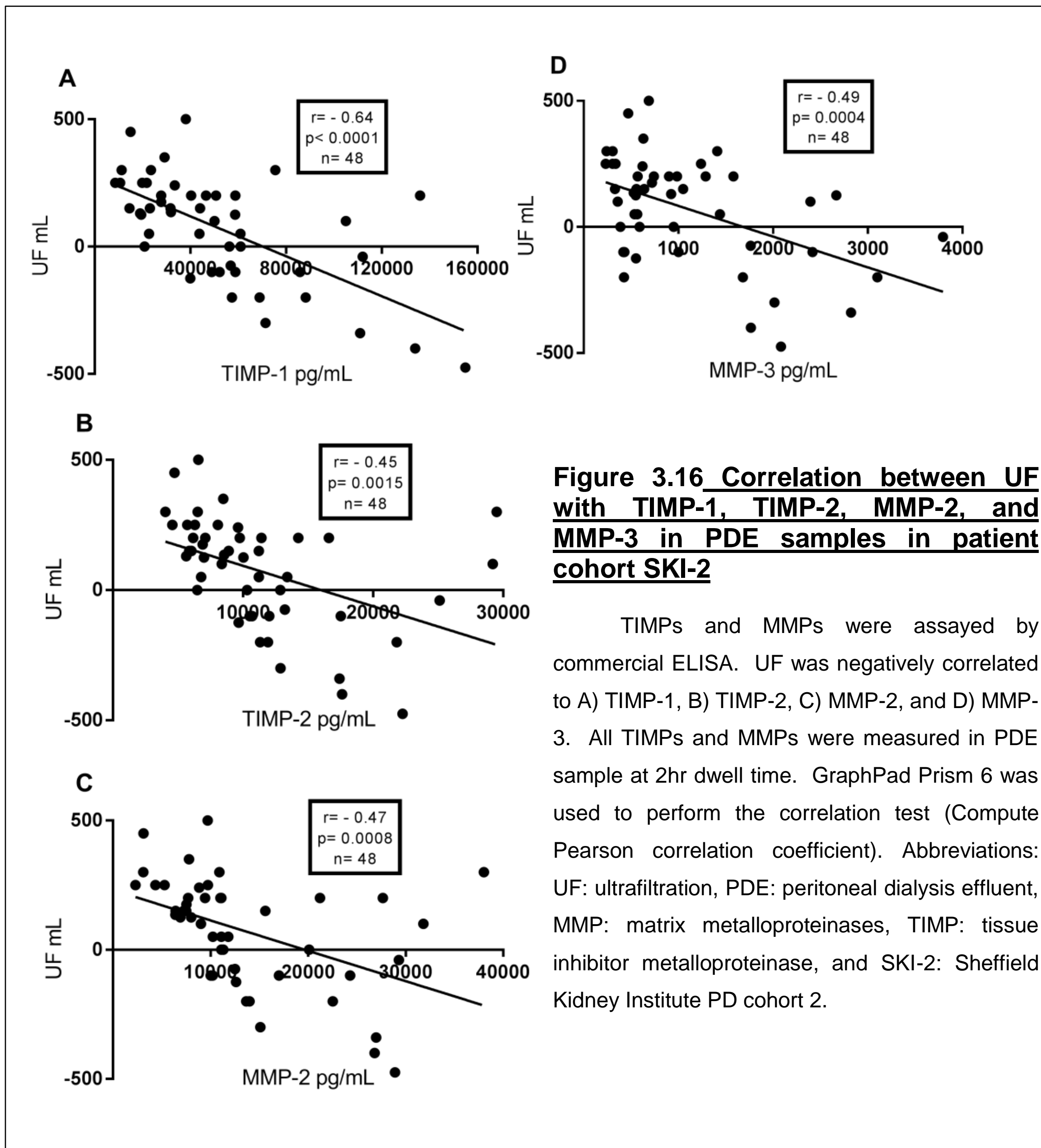
Figure 3.15 Correlations between D/Pcr at 2hr against TIMP-1, TIMP-2, MMP-2, and MMP-3 in PDE samples in SKI-2

TIMPs and MMPs in PDE were assayed by commercial ELISA. D/Pcr at 2hr was positively correlated to A) TIMP-1, B) TIMP-2, C) MMP-2, and D) MMP-3. All TIMPs and MMPs were measured in PDE samples at 2h dwell time. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient). Abbreviations: D/Pcr: dialysate-to-plasma ratio of creatinine, PDE: peritoneal dialysis effluent, MMP: matrix metalloproteinases, TIMP: tissue inhibitor metalloproteinase, and SKI-2: Sheffield Kidney Institute PD cohort 2.

3.5.4.1.2 Correlation of protein, MMPs and TIMPs in PDE samples against UF in PDE samples in the SKI-2 patient cohort.

Ultrafiltration failure (UFF) is an important cause of long-term failure of PD. UF was negatively correlated to protein in PDE samples at 2hr (r= -0.46, p = 0.001), n=48. It was also negatively correlated to TIMP-1, TIMP-2, MMP-2 and

MMP-3 in PDE samples at 2hr specially to TIMP-1 which shows the best negative correlation (r= -0.64, p < 0.0001) (Figure 3.16).



<u>Figure 3.16 Correlation between UF with TIMP-1, TIMP-2, MMP-2, and MMP-3 in PDE samples in patient cohort SKI-2</u>

TIMPs and MMPs were assayed by commercial ELISA. UF was negatively correlated to A) TIMP-1, B) TIMP-2, C) MMP-2, and D) MMP-3. All TIMPs and MMPs were measured in PDE sample at 2hr dwell time. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient). Abbreviations: UF: ultrafiltration, PDE: peritoneal dialysis effluent, MMP: matrix metalloproteinases, TIMP: tissue inhibitor metalloproteinase, and SKI-2: Sheffield Kidney Institute PD cohort 2.

3.5.4.2 Effect of dwell time on TIMP and MMP levels in PDE samples from patient cohort SKI-2

Increases in dwell times had an effect on the TIMP-1,-2 and MMP-2,-3 levels in PDE samples. In SKI-2 cohort, most of the samples had multiple dwell times. 18 of them were collected prior to tube flushing in PET at zero, 2hr, and 4hr and they showed an increase in TIMPs and MMPs levels in the samples with longer dwell times (Figure 3.17) in what appeared to be a linear manner.

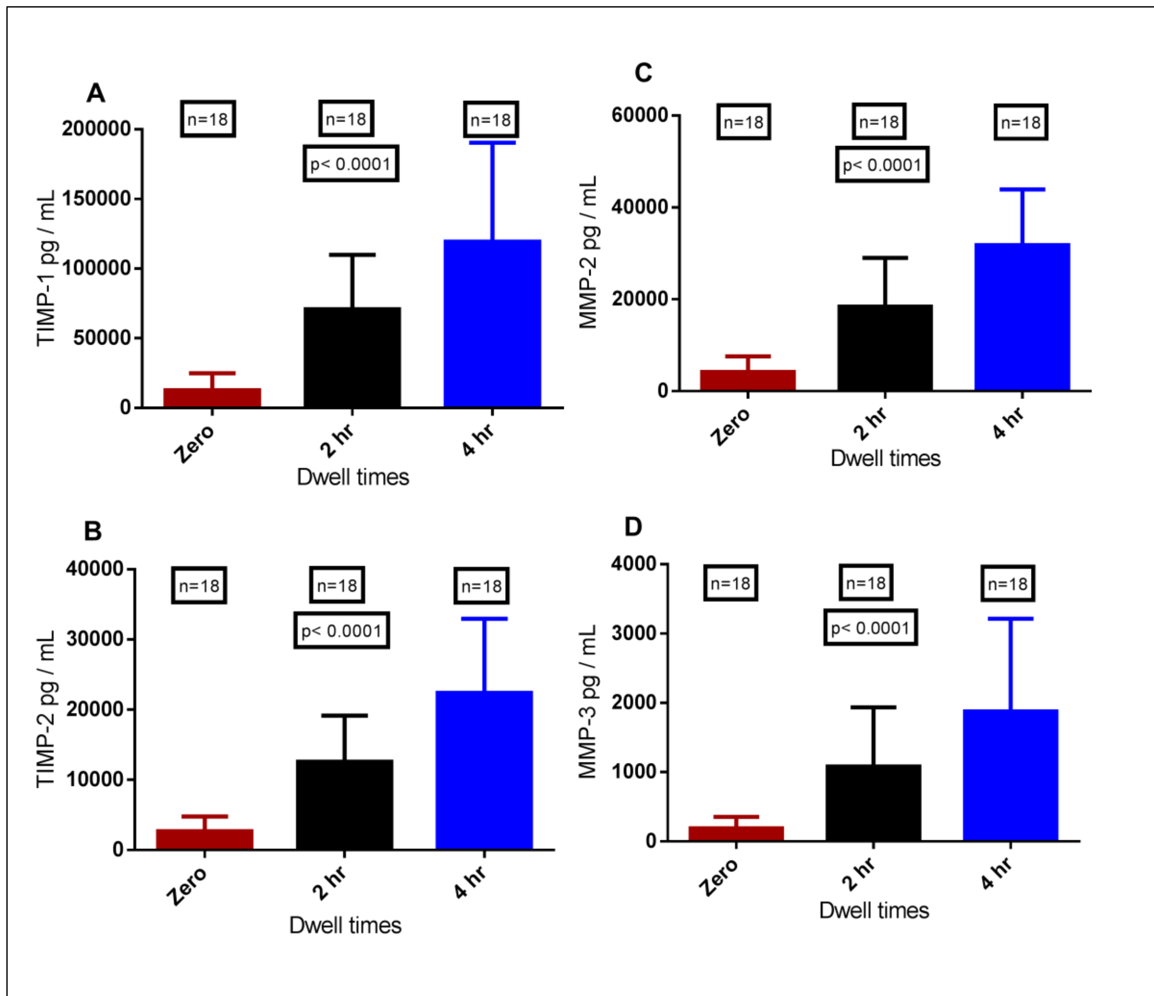


Figure 3.17 <u>Effect of dwell times on TIMP & MMP levels in PDE samples/SKI-2 cohort</u>

PDE samples were collected during a PET test after 0, 2 & 4hr dwell times and assayed by commercial ELISA for TIMPs and MMPs. Graph Pad Prism 6 was used for statistical analysis (Friedman test). The data represent mean with SD.

3.5.4.3 Plasma level in healthy volunteers compared to PD patients in SKI-2

To understand if there was more systemic MMP and TIMPs in PD patients than healthy individuals, MMP & TIMPs levels in plasma were measured in both populations. Healthy volunteers had an age ranging from 22 – 49 years old (mean±SD was 35±9. The female : male ratio was 7 : 11). PD patients had an age ranging from 26 – 78 years old (mean±SD was 60±14 The female : male ratio was 19 : 27). TIMP-1, TIMP-2, MMP-2, and MMP-3 were lower in plasma of healthy volunteers compared to PD patients. TIMP-1 median (IQR) in plasma was 126 (107–144) ng/mL in healthy group, but the median is more than double in PD patients and median (IQR) was 276.5 (253.8–347) ng/mL (Figure 3.18 A). TIMP-2 median (IQR) in plasma was 79.5 (74.7–93.8) ng/mL in healthy individuals, but it rises to approximately double at 150.5 (135.5–172.3) ng/mL in patients on PD (Figure 3.18 B). MMP-2 Median (IQR) in plasma was 165 (150.7–208.6) ng/mL in healthy, but it is higher at 302.1 (242.5–370.8) ng/mL in patients on PD (Figure 3.18 C). MMP-3 median (IQR) in plasma was 10.8 (6.9–13.2) ng/mL in healthy individuals, but it rises to triple at 32.7 (25–48.8) ng/mL in patients on PD (Figure 3.18 D).

3.5.5 TIMPs and MMPs in PDE samples (SKI-1 and SKI-2) in patient with various history of peritonitis

Patients were grouped in SKI-1 (Figure 3.19) and SKI-2 (Figure 3.20) according to the history of peritonitis to (0, 1-2, and 3-4 attacks). Most patients were without history of peritonitis. Even those with history of peritonitis, PDE samples dates were not close to the peritonitis attacks dates as it is clarified in method that patients with history of peritonitis in the two months period prior to sample collection were excluded from this cohort. It seems from figure 3.19 and figure 3.20 that there is no constant relationship between TIMPs and MMPs versus history of peritonitis.

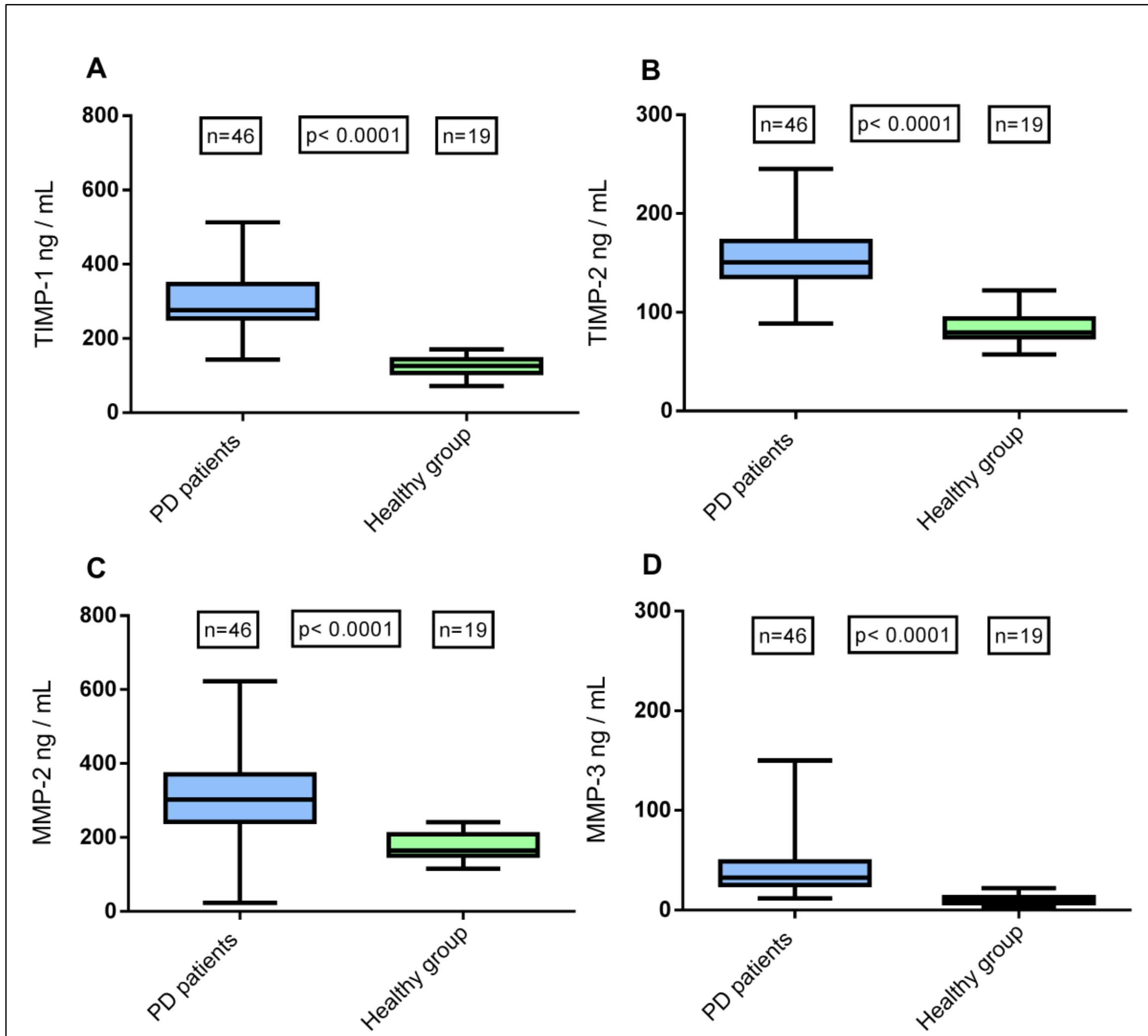


Figure 3.18 <u>Plasma level of TIMPs and MMPs in healthy volunteers and patients on PD</u>

TIMPs and MMPs were assayed in plasma samples by commercial ELISA. Plasma levels of A) TIMP-1, B) TIMP-2, C) MMP-2, and D) MMP-3 in healthy volunteers and PD patients from SKI-2. GraphPad Prism 6 used for statistical analysis (Unpaired t test with Welch's correction). Box and Whisker Plots show median, 25th Percentile, 75th Percentile, and the range. Abbreviations: MMP: matrix metalloproteinases, TIMP: tissue inhibitor metalloproteinase, and SKI-2: Sheffield Kidney Institute PD cohort 2.

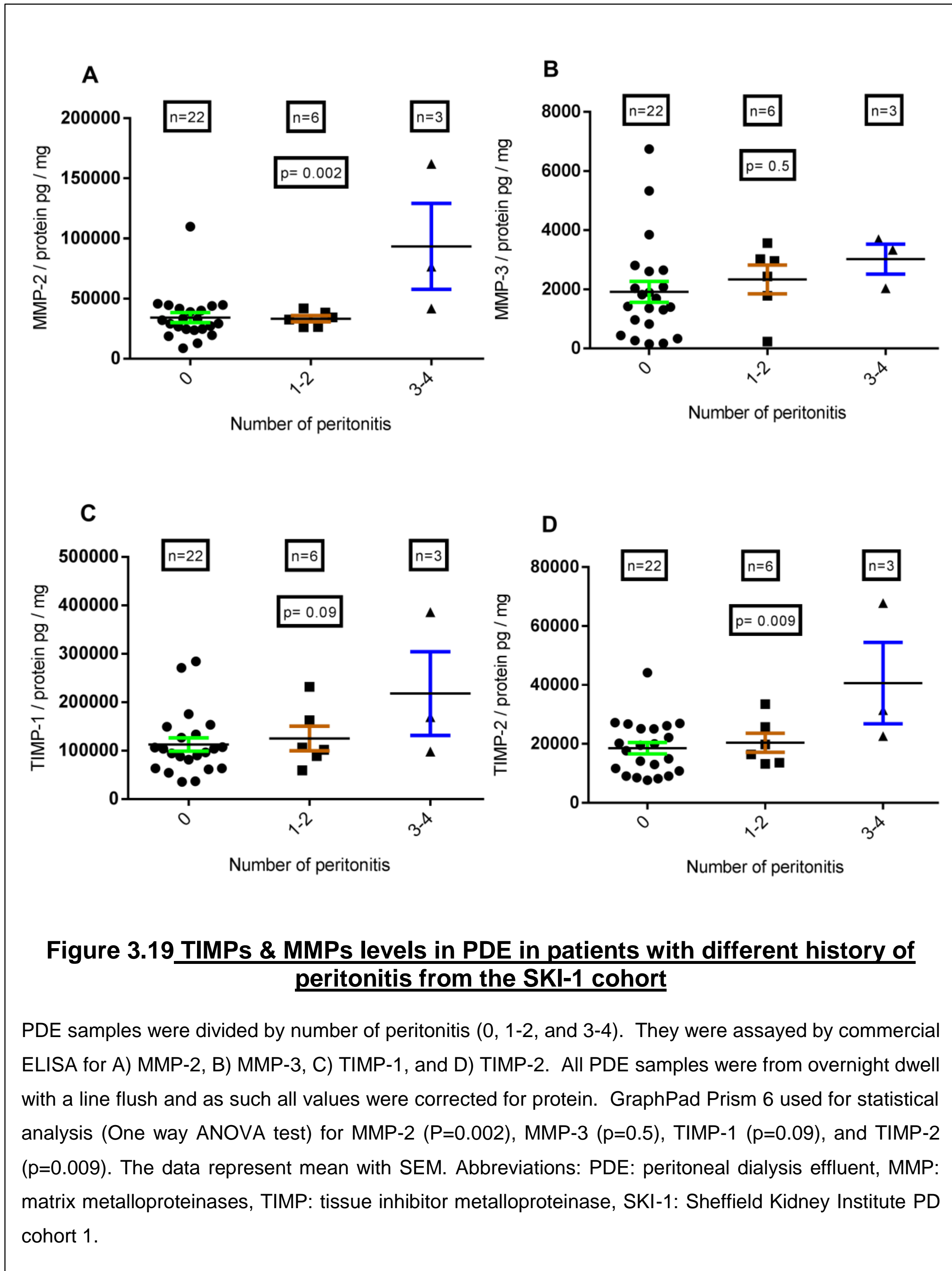


Figure 3.19 <u>TIMPs & MMPs levels in PDE in patients with different history of peritonitis from the SKI-1 cohort</u>

PDE samples were divided by number of peritonitis (0, 1-2, and 3-4). They were assayed by commercial ELISA for A) MMP-2, B) MMP-3, C) TIMP-1, and D) TIMP-2. All PDE samples were from overnight dwell with a line flush and as such all values were corrected for protein. GraphPad Prism 6 used for statistical analysis (One way ANOVA test) for MMP-2 (P=0.002), MMP-3 (p=0.5), TIMP-1 (p=0.09), and TIMP-2 (p=0.009). The data represent mean with SEM. Abbreviations: PDE: peritoneal dialysis effluent, MMP: matrix metalloproteinases, TIMP: tissue inhibitor metalloproteinase, SKI-1: Sheffield Kidney Institute PD cohort 1.

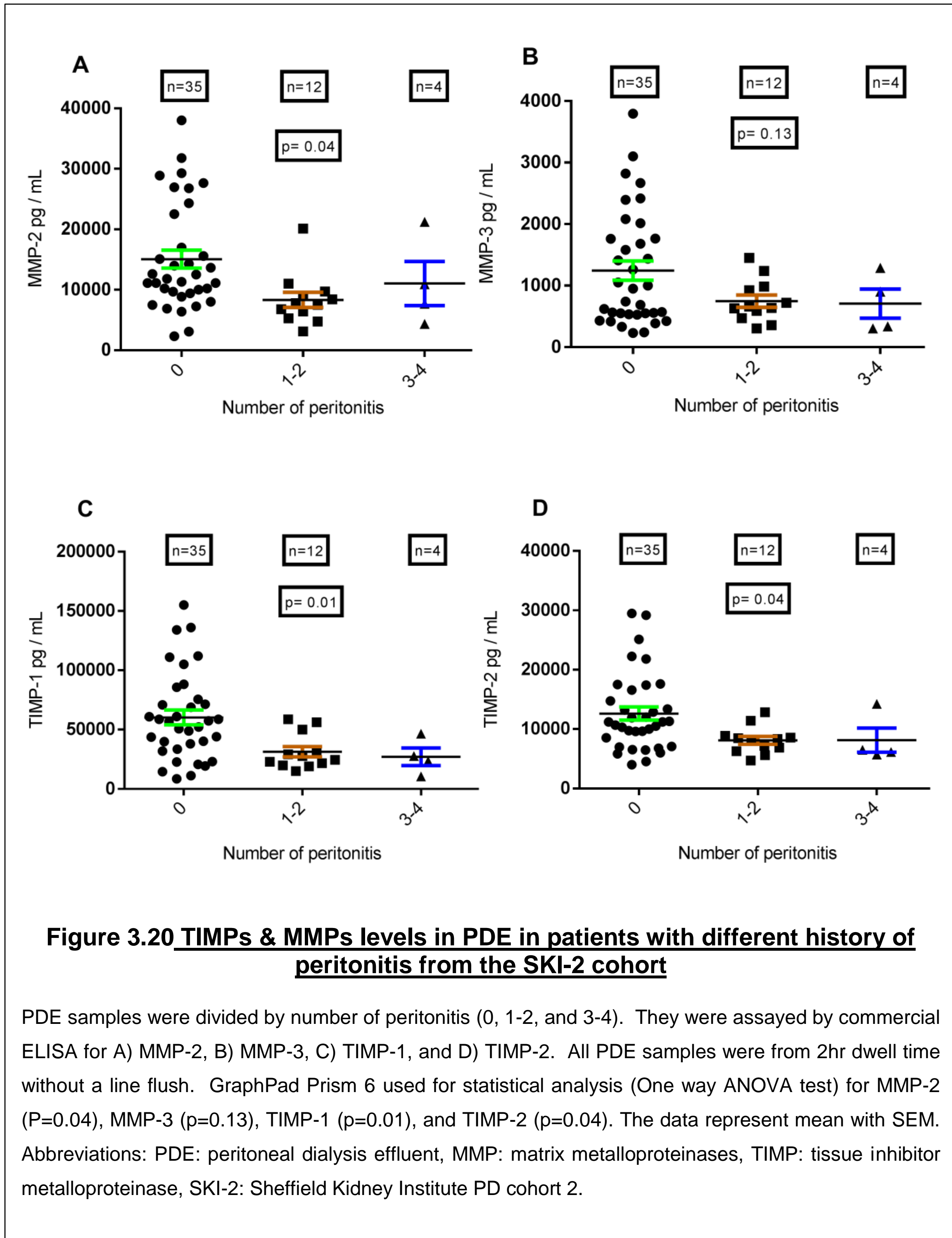


Figure 3.20 <u>TIMPs & MMPs levels in PDE in patients with different history of peritonitis from the SKI-2 cohort</u>

PDE samples were divided by number of peritonitis (0, 1-2, and 3-4). They were assayed by commercial ELISA for A) MMP-2, B) MMP-3, C) TIMP-1, and D) TIMP-2. All PDE samples were from 2hr dwell time without a line flush. GraphPad Prism 6 used for statistical analysis (One way ANOVA test) for MMP-2 (P=0.04), MMP-3 (p=0.13), TIMP-1 (p=0.01), and TIMP-2 (p=0.04). The data represent mean with SEM. Abbreviations: PDE: peritoneal dialysis effluent, MMP: matrix metalloproteinases, TIMP: tissue inhibitor metalloproteinase, SKI-2: Sheffield Kidney Institute PD cohort 2.

3.5.6 Global Fluid Study (GFS) Samples

Data from SKI cohorts 1 and 2 demonstrated low MMP activity in PDE that appeared to be due to high TIMP levels with only MMP2 having significant presence. Further the high level of TIMP-1, TIMP-2 and MMP-2 in the 1 EPS patient in SKI-1 and the positive correlation of TIMP-1, TIMP-2 and MMP-2 with D/Pcr and negative correlation with UF suggested they may be involved in PS or in the switch to EPS. To explore this, TIMPs and MMPs required to be measured in the sequential samples and material from those developing EPS such as those present in the GFS. However, limited materials were available from the GFS and as this is an extremely valuable resource, only proteins where we easily detect proteins in a very small volume of PDE could be investigated. TIMP-1, TIMP-2 and MMP-2 were chosen to be measured in PDE samples. In the GFS, individual patients had their TIMP-1, TIMP-2, and MMP-2 levels measured in 4 hr dwell samples and these were plotted with time on PD for control and EPS groups. In the control group, there were 158 samples that were taken from 43 patients (periodic sampling from each patient). In the EPS group, there were 43 samples that were taken from 12 patients.

3.5.6.1 TIMP-1

TIMP-1 level was plotted for individual control and EPS patients as spaghetti plots (Figure 3.21). In the control group, nearly all of the 158 samples remained bunched and below 155 ng/mL with only 5 (or 3%) with values higher than155 ng/mL at some point randomly on PD that always returned to within this “normal” range. These 5 samples (3%) had D/Pcr at 4hr (0.8 or more). Importantly in the EPS group, all of the samples stayed within the range of 25-155ng/mL with the exception about the EPS samples within last 100 days of EPS diagnosis because they had a rapid increase in the level of TIMP-1. The median (IQR) (ng/mL) for the control was 48.8 (36.7 - 67.2) with a range (10.1 - 339.5), and for the EPS was 62.6 (42.6 – 94) with a range (15.3 – 364).

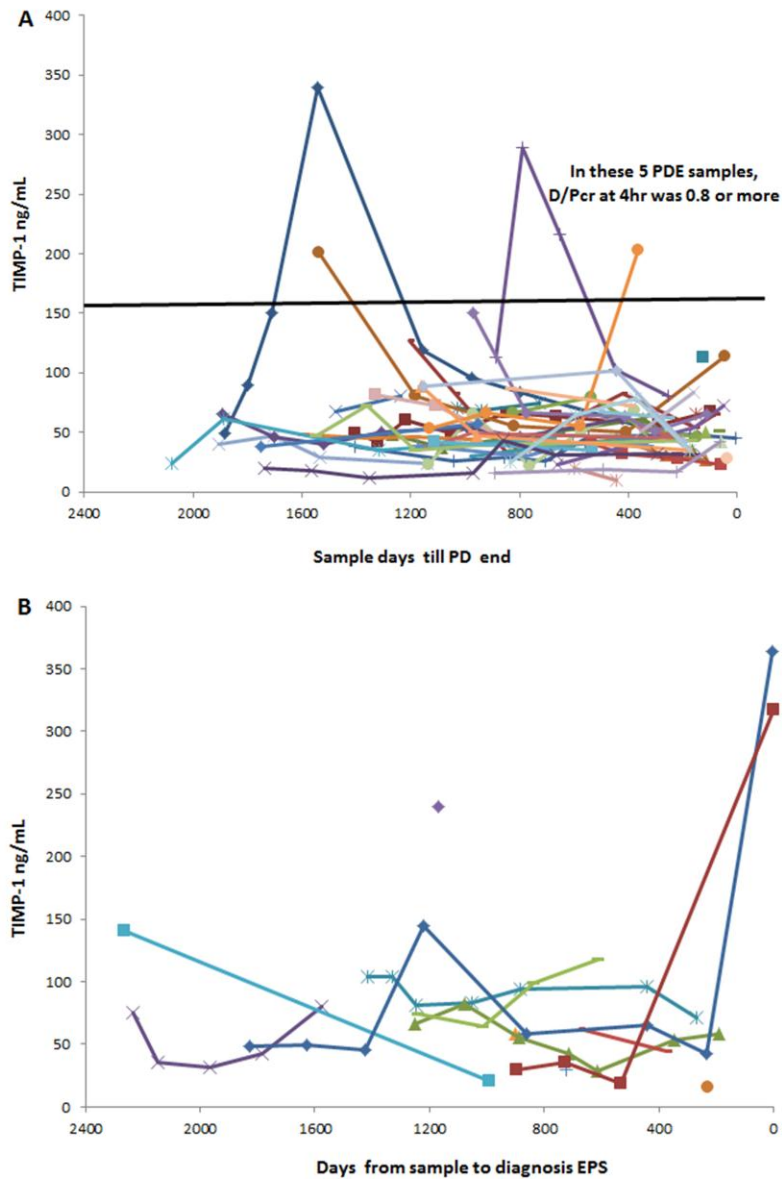


Figure 3.21 Spaghetti plots of TIMP-1 level in PDE samples (control and EPS)

TIMP-1 was assayed by commercial ELISA in the PDE samples from the GFS in control (i.e. not developing EPS) and EPS samples. (A) TIMP-1 plotted against sample days till PD end in those not developing EPS and (B) TIMP-1 plotted against days from sample to EPS diagnosis in the EPS group. Abbreviations: TIMP: tissue inhibitor metalloproteinase, PDE: peritoneal dialysis effluent, GFS: global fluid study, and EPS: encapsulating peritoneal sclerosis.

Mean TIMP-1 level from non-EPS & EPS developing patients with time on PD were subsequently plotted (Figure 3.22). This clearly demonstrated the late divergence in TIMP-1 level between control and EPS in the last 100 days in the EPS group. That said, in patients that ultimately developed EPS, TIMP1 level were not dissimilar to controls until this late period where a significant 6 fold increase in mean TIMP-1 level occurred. However this only included samples from 2 of the 13 EPS patients being followed compared to 10 in the control group at this point (last 100 days).

This late increase in TIMP-1 could potentially point towards its use as a late diagnostic marker as well as a potential pathogenic mechanism. A cutoff of 3 x average of control group (59.4 x 3=178.2) was established to assess how TIMP-1 could be used diagnostically. Five samples from control group (158 samples) fall above this normal range (i.e. 3% false positive if using this as a diagnostic for EPS) but with both EPS samples in the last 100 days higher than this cutoff (plus the high TIMP-1 level in the EPS patient from the SKI-1 cohort) then there is a high detection rate.

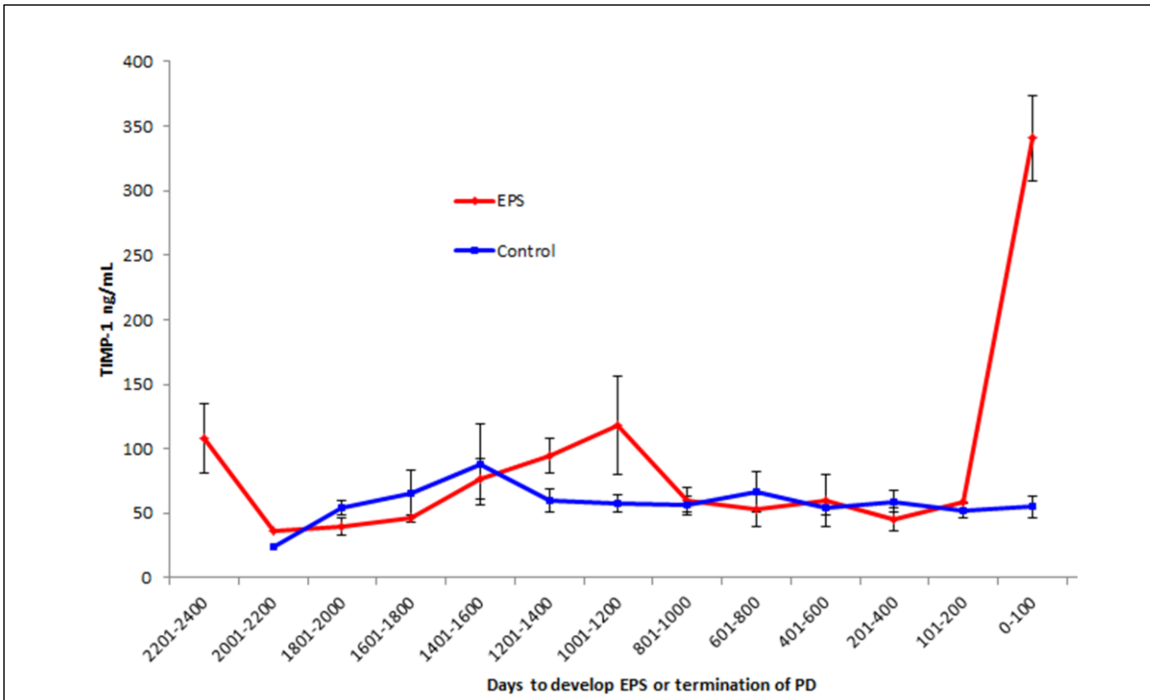


Figure 3.22 Mean TIMP-1 concentrations in control and EPS groups (GFS)

TIMP-1 concentration in PDE samples were grouped into 200 day segments for time before PD stop or EPS diagnosis for both EPS and control groups. Greater resolution was achieved in the last 200 days before cessation by refining to 100 day groupings. Mean TIMP-1 concentration was then calculated within each segment and plotted as mean with SEM with time to EPS diagnosis or PD termination. Abbreviations: TIMP: tissue inhibitor metalloproteinase, PDE: peritoneal dialysis effluent, GFS: global fluid study, and EPS: encapsulating peritoneal sclerosis.

3.5.6.2 TIMP-2

TIMP-2 level was much lower in PDE samples compared to TIMP-1 level. TIMP-2 level was below 31 ng/mL in all PDE samples (Figure 3.23). The median (IQR) (ng/mL) for TIMP-2 in the control group was 9.2 (6.3 - 13.1) with a range (2.5 – 30.5), and for the EPS group was 11 (8.4 – 13.5) with a range (3.9 – 30.7).

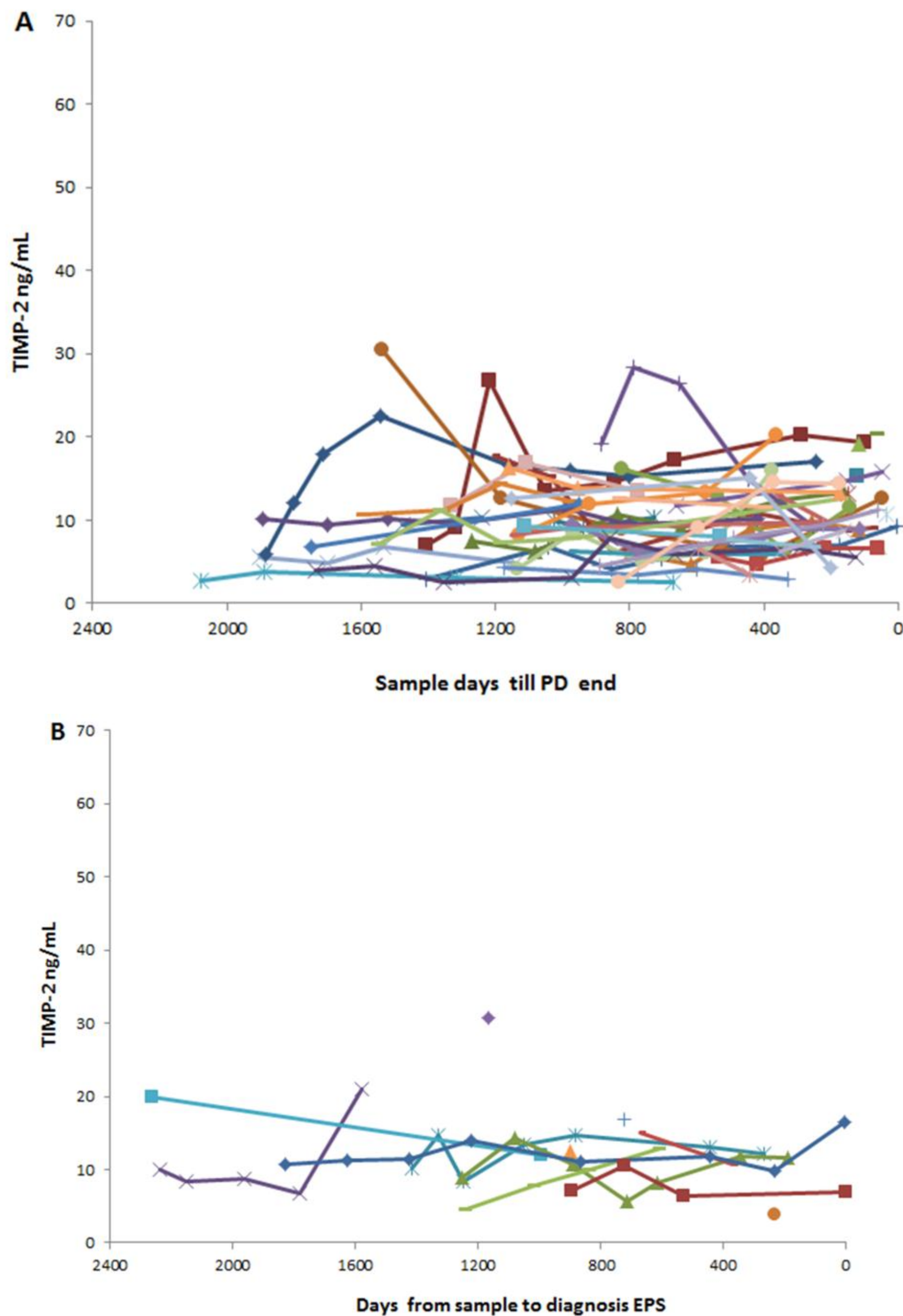


Figure 3.23 Spaghetti plots of TIMP-2 level in PDE samples (control and EPS)

TIMP-2 was assayed by commercial ELISA in the PDE samples from the GFS in control (i.e. not developing EPS) and EPS samples. (A) TIMP-2 plotted against sample days till PD end in those not developing EPS and (B) TIMP-2 plotted against days from sample to EPS diagnosis in the EPS group. Abbreviations: TIMP: tissue inhibitor metalloproteinase, PDE: peritoneal dialysis effluent, GFS: global fluid study, and EPS: encapsulating peritoneal sclerosis.

The mean TIMP-2 level with time on PD was also plotted and showed a relatively consistent level throughout the dialysis period irrespective of the patients' outcome (Figure 3.24).

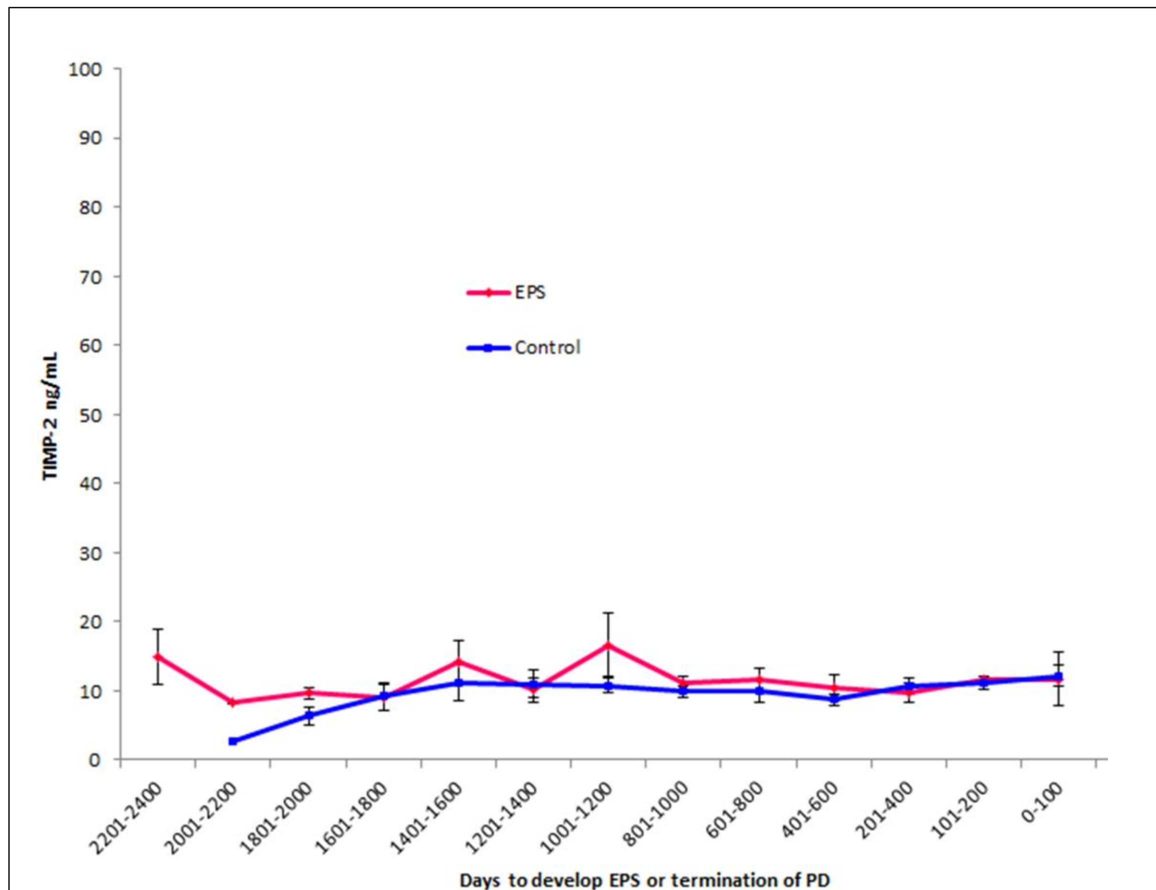


Figure 3.24 Mean TIMP-2 concentrations in control and EPS groups (GFS)

Means TIMP-2 in PDE samples were plotted for every 200 days and every 100 days in the last 200 days for EPS and control groups. There is relatively stable level of TIMP-2 in PDE samples with average of 10.9 ng/mL. Data represents mean with SEM. Abbreviations: TIMP: tissue inhibitor metalloproteinase, PDE: peritoneal dialysis effluent, GFS: global fluid study, and EPS: encapsulating peritoneal sclerosis.

3.5.6.3 TIMP-1/TIMP-2 ratio

Of note was that the few samples that were sporadically high in TIMP-1 were also sporadically high for TIMP2 except those close to EPS diagnosis. Thus this raised the concept that if TIMP-1 could have value as a diagnostic tool

or even late predictive biomarker then could TIMP-2 be used as internal reference for TIMP-1 to minimise the number of "false positive" TIMP-1 in identifying EPS onset. TIMP-2 used because it was relatively stable and it was minimising the effect of high transport status in the control group by dividing TIMP-1 over TIMP-2. In addition to that, GFS samples are from 4 hour dwell time, but it is not recorded whether the samples are diluted or not due to flushed line. Further, could it correct for any variability in sample dilution due to line flushing to further tighten the data.

Individual patients therefore had their TIMP-1/TIMP-2 ratios plotted with time on PD (Figure 3.25). When TIMP-1 level were normalised to TIMP-2 level "spikes" in the TIMP1 baseline were minimised. The maximum level of TIMP-1/TIMP-2 ratio in the non-EPS control group was 18.1, while the TIMP-1/TIMP-2 ratios in the 2 EPS patients who had samples within last 100 days were 22 and 46 respectively. This therefore clearly defined a phenotype in the late EPS samples where the TIMP-1/TIMP-2 ratio was higher than those not developing EPS. The median (IQR) (ng/mL) for the control was 5.6 (4.3 - 7.3) with a range (1.8 – 18.1), and for the EPS was 5.5 (4.2 – 7.5) with a range (1.7 – 46).

Mean TIMP-1/TIMP-2 ratio with time on PD was also calculated & plotted which unsurprisingly also showed a stable level with a clear late elevation in EPS patients (Figure 3.26). If a cutoff of 3 x of the mean value in the non-EPS control group (6.1 x 3=18.3) is used, none of the sample from this (158 samples) rose above this cutoff, while just the 2 EPS samples in the last 100 days were higher.

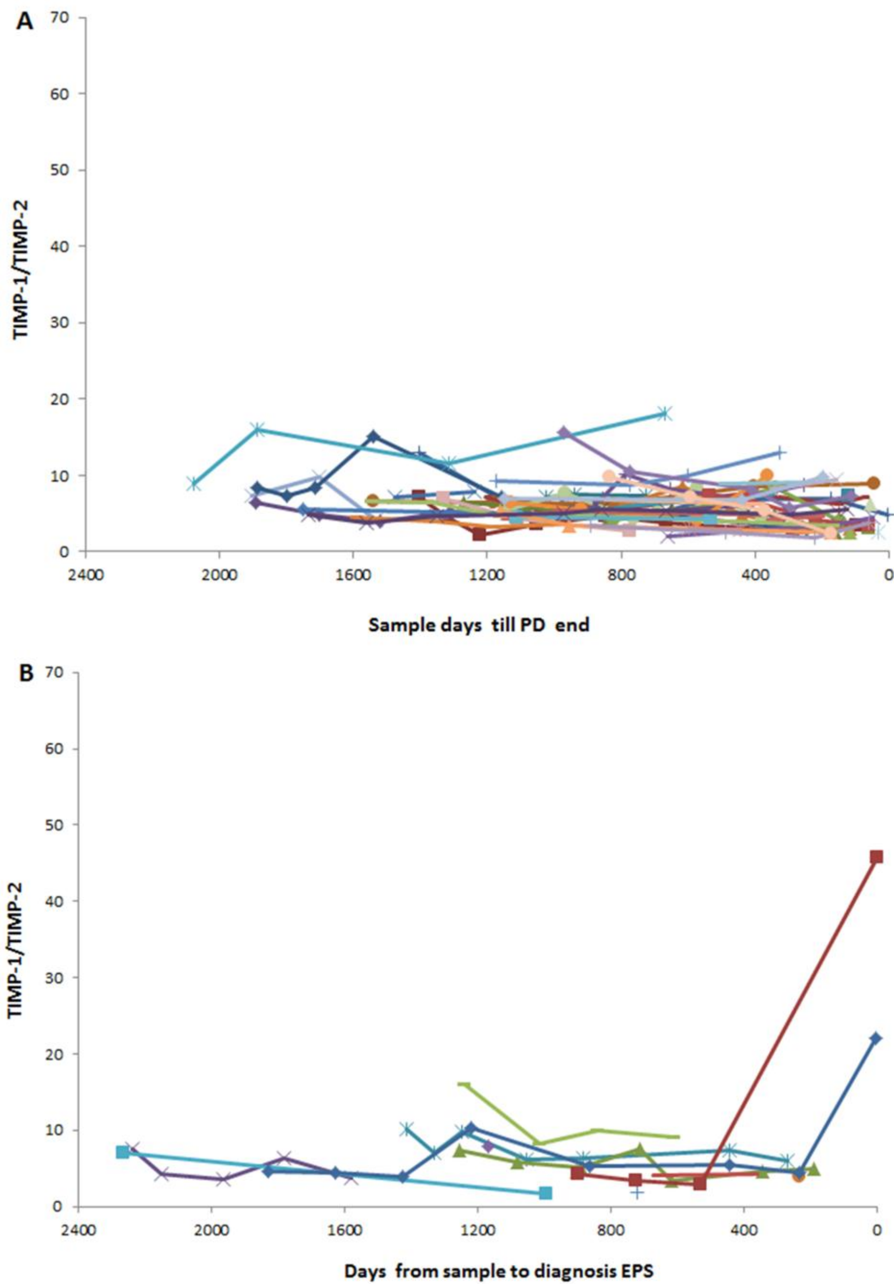


Figure 3.25 <u>Spaghetti plots of TIMP-1/TIMP-2 level in PDE samples (control and EPS)</u>

TIMP-1 &-2 was assayed by commercial ELISA in the PDE samples from the GFS in control (i.e. not developing EPS) and EPS samples. (A) TIMP-1/Timp-2 ratio plotted against sample days till PD end in those not developing EPS and (B) TIMP-1/Timp-2 plotted against days from sample to EPS diagnosis in the EPS group. Abbreviations: TIMP: tissue inhibitor metalloproteinase, PDE: peritoneal dialysis effluent, GFS: global fluid study, and EPS: encapsulating peritoneal sclerosis.

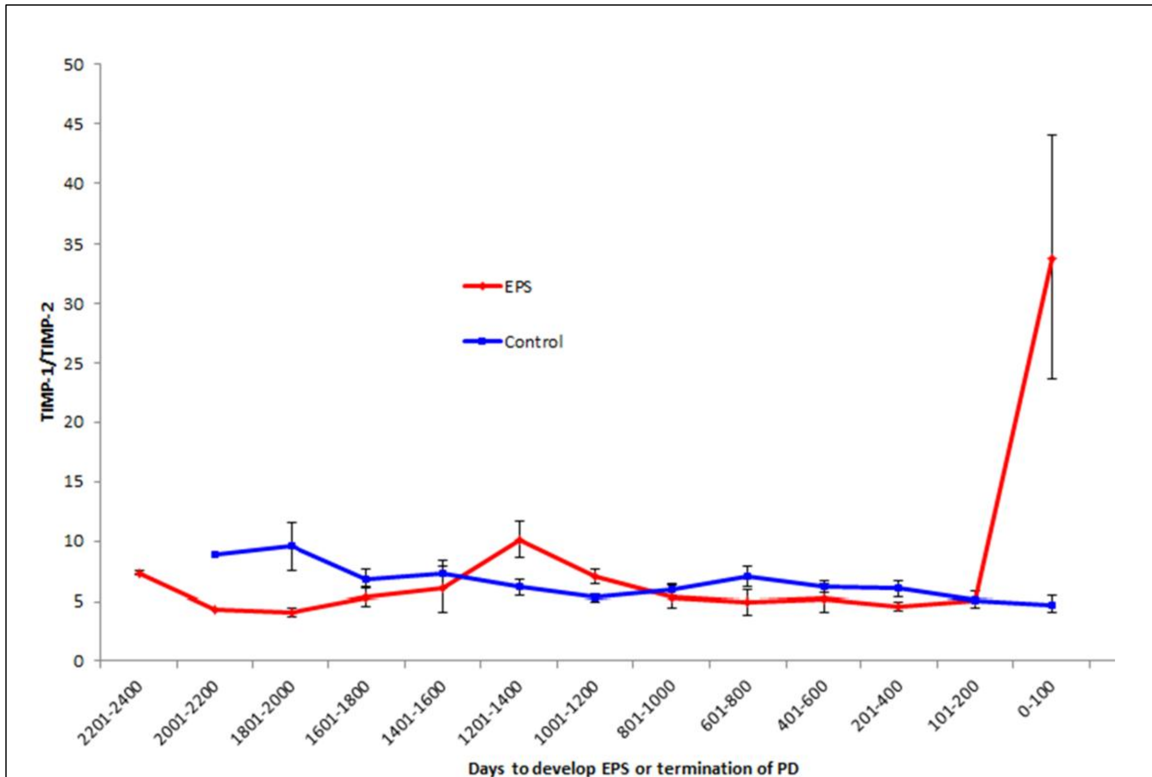
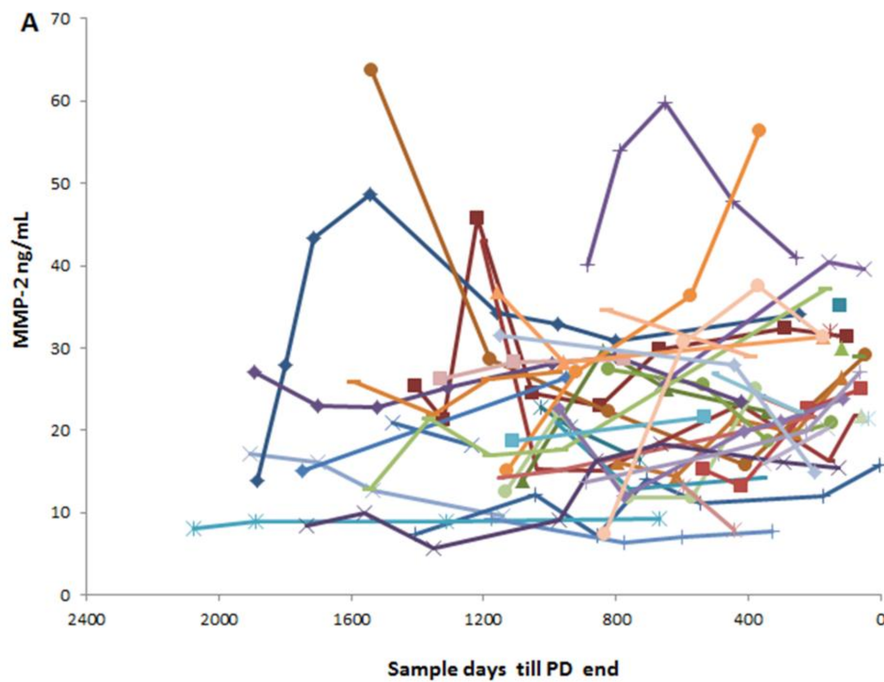


Figure 3.26 Mean TIMP-1/TIMP-2 concentrations in control and EPS groups (GFS)

Samples were grouped by time to EPS diagnosis or stopping PD using a 200 day windows with 100 days close to stopping PD or EPS diagnosis. The mean TIMP-1/TIMP-2 ratio in all PDE samples within each 200 or 100 day window was calculated and plotted chronologically. Data represents mean with SEM. Abbreviations: TIMP: tissue inhibitor metalloproteinase, PDE: peritoneal dialysis effluent, GFS: global fluid study, and EPS: encapsulating peritoneal sclerosis.

3.5.6.4 MMP-2

Individual patients also had their MMP-2 level measured and plotted with time on PD (Figure 3.27). The majority of measurements for both groups were less than 40 ng/mL with only the odd sporadic sample being above this. The average MMP-2 level in the non-EPS control and EPS samples were 23 and 25 ng/mL respectively. The median (IQR) (ng/mL) for the non-EPS control was 21.9 (15.1 - 28.6) with a range (5.7 – 63.7), and for the EPS was 23.5 (19.5 – 31.1) with a range (8.2 – 43.7).



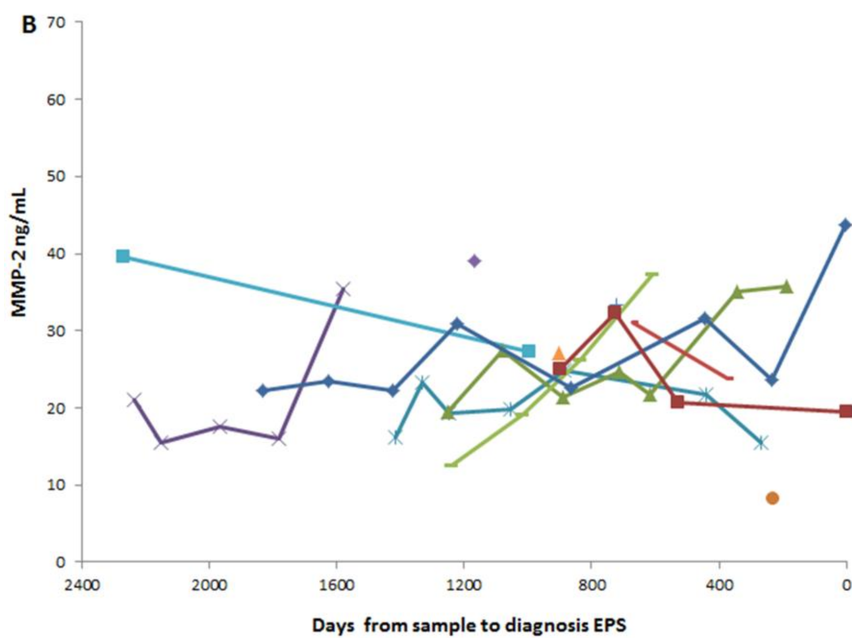


Figure 3.27 <u>Spaghetti plots of MMP-2 level in PDE samples (control and EPS)</u>

MMP-2 was assayed by commercial ELISA in the PDE samples from the GFS in control (i.e. not developing EPS) and EPS samples. (A) MMP-2 plotted against sample days till PD end in those not developing EPS and (B) MMP-2 plotted against days from sample to EPS diagnosis in the EPS group. Abbreviations: MMP: matrix metalloproteinase, PDE: peritoneal dialysis effluent, GFS: global fluid study, and EPS: encapsulating peritoneal sclerosis.

From above spaghetti plots there is no trend in MMP-2 values that suggests any value as a predictor or diagnostic for developing EPS. Mean MMP-2 were predictably flat (Figure 3.28).

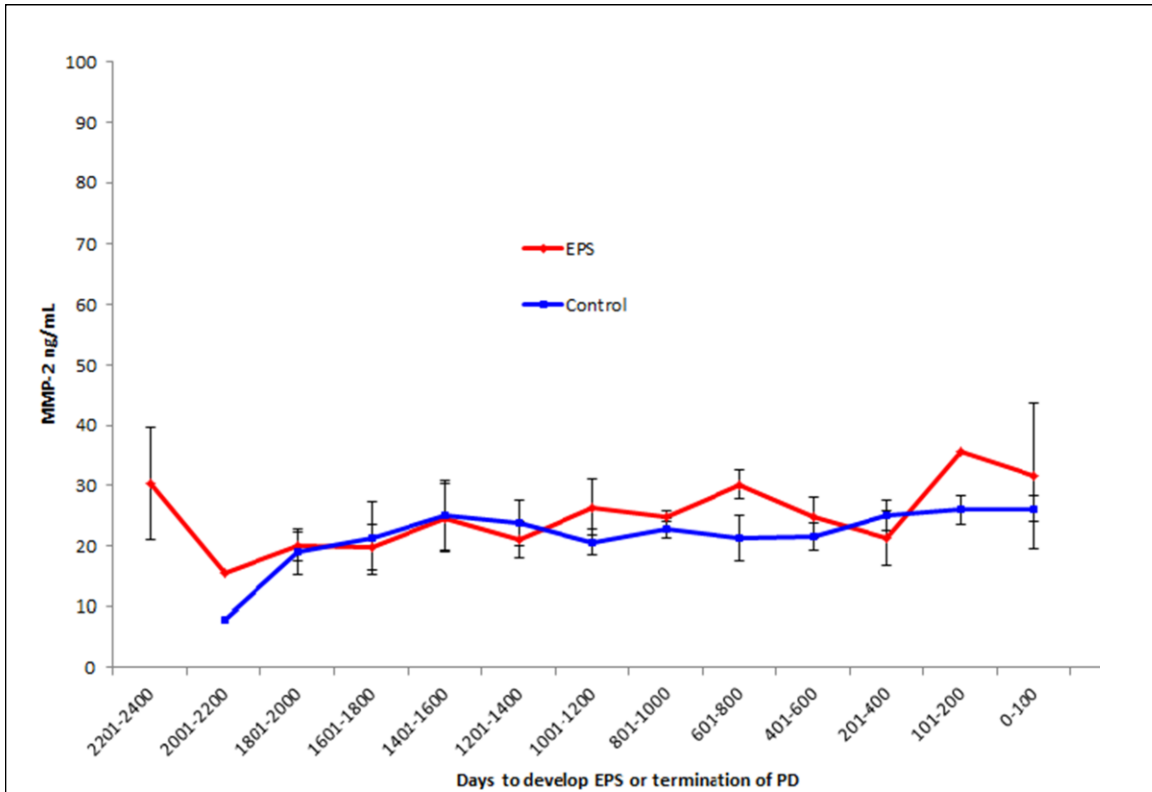


Figure 3.28 <u>Mean MMP-2 concentrations in control and EPS groups (GFS)</u>

Means MMP-2 in PDE samples were plotted for every 200 days and every 100 days in the last 200 days for EPS and control groups. MMP-2 did not show clear shift in the level of MMP-2 averages between control and EPS in last 100 days in EPS group. Data represents mean with SEM. Abbreviations: MMP: matrix metalloproteinase, PDE: peritoneal dialysis effluent, GFS: global fluid study, and EPS: encapsulating peritoneal sclerosis.

3.5.7 Statistical analysis after this study

The statistician was approached to analyse the power of the study using the program G*power 31.3. Sample size required to detect full and half effect shown in this study of TIMP-1 in SKI-2 and GFS were performed. Small studies which are significant often tend to overestimate the effect, thus half effect used as a more cautious approach. In SKI-2, sample sizes required to detect full effect of TIMP-1 correlation against D/Pcr, UF, and D/D0 glucose were 13, 21, and 28 PD patients respectively. Sample sizes required to detect half effect of TIMP-1 correlation against D/Pcr, UF, and D/D0 glucose were 68, 100, and 127 samples respectively. In Sheffield 51 samples were available for these assays.

In GFS, the statistician regarded the 2 EPS PDE samples from the GFS cohort to be acceptable to apply a statistical test on these 2 samples in order to compare them to the non EPS samples (10 samples). This was tested using an Exact Mann-Whitney as the sample size was small. TIMP-1 from the two EPS PDE samples was significantly higher than those from the control samples at 100 days (p=0.03). However, the statistician advised for more samples in any future study (16 samples EPS and 16 samples control in the last 100 days). If at 100 days the EPS and control behave as in this study with p=0.05 and power 95%, then 2 EPS patients and 2 controls are required. This sample size is too small for a study so to get reasonable sample size, then the assumption of drastic alternative behavior of the samples in the last 100 day were made. If all that is happening in the two extreme cases, then there may be expectation that EPS is averaging about double the controls, and this would give a sample size of 20 (10 controls and 10 EPS patients), p=0.05 and power=95%. However, if it is a sudden triggered, and then there will be expectation that 50% of the EPS patients behave like controls and 50% like EPS patients in the study. At that time a power calculation indicates that with p=0.05 and power=95%, then 16 EPS patients and 16 control patients are required in the last 100 days.

3.6 Discussion

The key finding from this chapter is that there is low or undetected MMP activity in the PDE samples from the start of PD suggesting that peritoneal capacity has limited ECM clearance capacity. This results from low or undetectable MMPs in PDE samples (with the exception of MMP-2 and to a lesser extent MMP-3) in compared with very high TIMPs levels, especially TIMP-1. There is a suggestion that changes in TIMP reflect membrane status with, D/Pcr being positively correlated to TIMP-1, TIMP-2, MMP-2, and MMP-3 while UF is negatively correlated to TIMP-1, TIMP-2, MMP-2 and MMP-3. TIMP-1 and TIMP-2 as well as MMP-2 and MMP-3 were higher in plasma of PD patients than in healthy volunteers. Finally looking at TIMP-1, 2 and MMP-2 in the larger GFS cohort has given some indication that TIMP-1 in PDE could have value as an EPS diagnostic tool.

Gender distribution differed between the study populations. In both SKI cohorts, male patients exceeded the number of female patients while in the GFS cohort, the reverse was seen. The majority of patients from SKI-1 cohort, and all the patients from the second Sheffield PD cohort were from a white ethnic background. Ethnicity was not recorded for the global PD cohort. These three groups of PD patients were used in this study, while none of the cohorts above were ideal in isolation, some crucial conclusions could be drawn by combining data together.

The initial assays were performed on samples from the SKI-1 cohort for proteins of interest in PDE samples. Twenty milliliters of PDE sample were made available for each patient. This amount was adequate to develop assays for PDE and establish the relative levels moving forward. One patient within this cohort was diagnosed with EPS, but the sample was taken approximately 10 months after diagnosis of EPS, although proved a useful insight into what may change.

One of the important limitations of SKI-1 cohort was unrecorded exact dwell times which can clearly affect data analysis. Furthermore, samples were not collected during a PET, thus D/P creatinine, UF and other clinical data were not available at the precise time of sample collection. Most of the clinical data was recorded from values obtained from a day close to the day PD samples were collected. Plasma samples were not collected. There was also an absence of records concerning the flushing or non-flushing of the PDE samples. This affected sample dilution. While these all impact on the accuracy of data obtainable from these samples, the cohort has large sample volumes pre stored providing material for early “first look” studies” with sufficient material for assay development.

Samples from the SKI-2 cohort were collected as the study progressed. There was no EPS patient in the SKI-2 cohort, but recorded clinical parameters were available to assess the degree of peritoneal membrane damage which were absent in the SKI-1 cohort. In the SKI-2 cohort, both PDE and blood samples were collected and samples were collected without line flushing to remove variability on sample dilution. .

The GFS has good numbers of EPS patients with sequential sampling, but limited materials were available for study, thus the good pilot data from studies in Sheffield PD cohorts gave a sound insight into how to use the GFS samples wisely and whether robust measurements could be obtained from the limited available volume of PDE samples. The target was to measure any protein of interest in 10μL or less of PDE samples. Based on the preliminary data from the Sheffield cohort of fluid samples, all three TIMPs looked to be potential markers of EPS, however only TIMPs 1 and 2 had levels sufficiently high enough to be measured in a 10 μL aliquot of PDE samples. MMP-2 and total protein were also measured in global fluid study as they also needed low quantity of PDE samples for measuring by ELISA kits.

GFS were collected from different centres. Clinical data and PD samples were available. Periodic sample collection provided the facility to study changes in the clinical data and laboratory data with time. The GFS is by far the largest and best repository of PD fluid in the world having sequential samples from over 2000 patients from its UK arm alone. Samples from 55 patients collected at 4 centres were made available for this study in Sheffield. Samples were collected at the time of PET so precise clinical data was available as well as information on dwell time. The main limitations of GFS samples were the availability of only small fluid samples (100 µl), irregular sample collection, there was also an absence of records concerning the flushing or non-flushing of the line at sample collection, and for the 12 PD patients in the EPS group, only 2 samples were collected within 100 days of EPS diagnosis.

All assays feasibility and workup studies were performed on the SKI-1 samples in which sample materials were available in good volume. From this, any changes that looked promising and could be measured on the small amount of GFS material available were subsequently investigated in the GFS samples. In the end, important assays were applied on SKI-2 samples because they weren't available in the beginning. The absence or lack of MMP activity in the PDE samples in both SKI-cohorts was predominantly due to very little MMPs being present in PDE samples which would be completely inhibited by the huge levels of TIMPs present. This lack of proteolytic decay would clearly lead to the classical thickening of the peritoneal membrane as lack of MMP activity which is normally used to balance the rate of ECM deposition to maintain a homeostatic balance in membrane thickness is missing.

The primary focus of this chapter was to look at the MMP system, but plasmin activity was also measured in PDE samples in SKI-1 and SKI-2. This is because plasmin system is closely related to MMP system. Plasmin activity is also important in activation of proMMPs to MMPs and MT-MMPs (Figure 3.1). Plasmin activity were easily detectable in PDE samples and showed a negative

correlation with time on PD i.e. longer duration on PD in years was associated with less plasmin activity in PDE. The single EPS sample was low in plasmin activity. Changes in plasmin system have already been associated with post-operative adhesions (Awonuga, Fletcher et al. 2011) and some pilot publications do relate the plasmin system to PS and EPS. The combination of loss of MMP activity early, with diminishing of plasmin activity with time could facilitate PS, with and a further drop in MMP activity due to large TIMP-1 level pushing the disease into EPS with its extensive scar tissue production.

The lack of GFS material meant performing MMP activity and plasmin activity assays on sequential GFS samples was not possible. Assaying MMP activity in GFS theoretically would have not yielded much valuable data as it was clear from the SKI-1 and SKI-2 cohorts that MMP activity is lost early and it is low or undetected in all samples. On the other hand, assaying plasmin activity in GFS may have yielded valuable data given plasmin activity with time changes in the SKI cohorts.

In the SKI-1 cohort, TIMPs, MMPs, and MMP-1/TIMP-1 complex were corrected to total protein concentration in the PD samples in order to address the problem of unknown exact dwell times in the SKI-1 PDE samples and overcome the problem of line flushing. MMP-1, MMP-9, MMP-13 and the MMP-1/TIMP-1 complex were very low or undetected in all PDE samples of SKI-1. However, TIMP-1, TIMP-2, MMP-2, and MMP-3 were detectable in PDE samples of SKI-1 and showed interesting changes in the single EPS sample in TIMP-1, TIMP-2 and MMP-2. For this reason, it was decided to measure these molecules in SKI-2 and GFS PDE samples.

MMP-1, MMP-9, and MMP-13 levels may be below the threshold of detection by ELISA in the PDE samples. MMP-1, MMP-9, and MMP-13 assays are sensitive down to 156, 31, and 6 pg/mL respectively. It is a real possibility that there are still these MMPs present in the PDE samples but they simply

cannot be measured. However, MMP-1 was also assayed by ELISA after the PDE samples were concentrated by rotary evaporation 10 fold, but MMP-1 remained low or undetected in PDE samples which suggest levels are extremely low. There are previous published papers that support these findings, for example, MMP-9 was undetected in most PDE samples in patients without infectious peritonitis (Hirahara, Inoue et al. 2007). This is match with our data as peritonitis in the two month period prior to sample was excluded.

The ELISA for MMP-1 detects total MMP-1 which was low or undetected in PDE samples. ELISA was also performed for the MMP-1/TIMP-1 complex in PDE samples. MMP-1/TIMP-1 complex were low or undetectable too. There is high TIMP-1 level in PDE samples, but low MMP-1 to bind with it. This means that there is genuinely low total MMP-1 in PDE samples and thus there is clear shut down of these proteases in PDE samples. It is thus very likely that MMP-9 and MMP-13 are also shut down in PDE samples. This is consistent with a pro-ECM and hence pro-sclerotic phenotype.

In the SKI-2 cohort, TIMP-1, TIMP-2, MMP-2 and MMP-3 in PDE samples were correlated to the degree of peritoneal membrane damage. TIMP-1 in PDE samples was best negatively correlated to UF (less UF was associated with high TIMP-1 in PDE samples). This observation on TIMP-1 correlation in PDE samples was observed in both genders. The level of TIMP-1 in PDE samples was biased by difference in gender in a study made by Hirahara et al (Hirahara, Inoue et al. 2011). The detailed comparison with this study and other similar studies are in chapter 7 (General discussion). TIMP-1, TIMP-2, MMP-2 and MMP-3 in PDE samples were positively correlated to D/Pcr and this may reflect transporter status i.e. TIMPs and MMPs may simply be markers of transport status.

In the SKI-2 cohort plasma samples were also available. Given that there is no true normal or control for peritoneal levels of proteins in PDE samples, then it

was reasoned that this may be a way to see if TIMP and MMP levels are changed in the PD peritoneum compared to healthy individuals. There was a clear elevation in TIMPs and MMPs levels in plasma from PD patients. While this is possibly due to impaired renal clearance of these molecules in PD patients, it is equally likely this is from elevated peritoneal production of these proteins. Ideally there should have been non-dialysis CKD controls to be sure whether elevated plasma levels are due to reduced clearance or increased production.

In the GFS, only TIMP-1, TIMP-2, MMP-2, protein were measured in this cohort. The data clearly shows that TIMP-1 was elevated in EPS close to diagnosis, so may have value as a late noninvasive biomarker of EPS (within 100 days of diagnosis) or in confirmation of EPS diagnosis. However, it is too early to draw that conclusion with any certainty as only 2 EPS samples were close to the time of EPS diagnosis, however a similar result was seen with TIMP-1 in PDE from the single EPS patient in cohort SKI-1. As explained previously if an arbitrary cut off of 3 x baseline is imposed as a “healthy upper limit” for TIMP-1 then measurements of TIMP-1 would be 97% accurate in predicting patients developing EPS within 100 days which is better than most clinical biomarkers used today. The changes in TIMP-2 from the SKI-1 cohort did not read through to the GFS samples, but rather showed a relatively stable level throughout dialysis in both groups in GFS. This allowed in using TIMP-2 as an internal reference point. TIMP-1 in odd samples in non-EPS patients was staying above the typical range and these samples were odd during TIMP-2 measurements too. A cutoff of 3 x of the mean TIMP-1/TIMP-2 ratio with time on PD was calculated in the control group and it was 18.3. None of the sample from the control group rose above this cutoff. In the EPS group, the 2 EPS samples in the last 100 days were higher than 18.3. Therefore, it is difficult to deliver strong conclusion about this ratio based on 2 samples. Nevertheless, it is an interesting observation that needs further validation.

Of note was that the 3% non-EPS patients that had TIMP-1 above the upper range for the bulk of the patients in the non-EPS control group was associated with high D/Pcr (0.8 or more). It could be reasoned that their high TIMP-1 level may result from systemic leakage across a compromised membrane. Calculation of TIMP-1/TIMP-2 ratio would minimise the effect of a high transport status in the control group and correct for any sample dilution due to line flushing.

TIMP-1 may have value as a biomarker/diagnostic tool, but it clearly may have a pathobiological role. TIMP-1 is a potent inhibitory effect for all the latent proMMPs and active MMPs (Ahmed 2009) and is, therefore, likely to be important in contributing to an overall reduction in matrix turnover. Previous study showed that TIMP-1 and MMP-2 are correlated to D/Pcr and they are produced locally from the peritoneum (Hirahara, Inoue et al. 2011). A study showed that MMP-2 may be useful as a marker of increased solute transport, peritoneal injury or progression to EPS (Hirahara, Inoue et al. 2007). It is likely that combinations of markers may be of far more value than single molecule in order to understand the contribution of the MMP and plasmin systems in peritoneal membrane damage, but the best predictor identified in this study is TIMP-1.

Fibrosis formation in the peritoneum like in all organs is governed in part by a decrease in degradation of ECM. The MMP2, MMP9 (gelatinases) play a role in degrading the ECM components such as fibronectin and collagen IV (Collier, Wilhelm et al. 1988). Experimental studies in EPS showed increase in MMP2 level in PDEs, and MMP2 inhibition led to less injury in the peritoneum (Hirahara, Ogawa et al. 2004, Ro, Hamada et al. 2007, Kurata, Maruyama et al. 2009). MMP-2 and MMP9 levels were investigated in a multicentre study in PDEs of PD patients with peritoneal injury and EPS patients. High MMP-9 level was observed in PD Patients with infectious peritonitis. The highest MMP-2 level was observed in PD patients with mild peritoneal injury (those had ascites less than

100 mL). The MMP-2 level was between moderate to high level in the EPS patients. Nevertheless, approximately half of PD patients (7 out of 15) with MMP-2 more than 600ng/mL ended up with EPS (Hirahara, Inoue et al. 2007). HPMC exposed to glucose in an in vitro study revealed that a decrease in the MMP-1,-8, and -13 expression as well as an increase in the TIMP expression leads to accumulation of the ECM (Kim, Li et al. 2008).

It seems that MMPs are essential, but in the right amount, duration (time frame) and place for peritoneal fibrosis. MMPs play important roles in debriding damaged ECM, re-epithelialisation, angiogenesis, remodelling of scar and wound contraction. However, there are clinical evidence which shows that high MMPs levels are also prevent wound healing, and treatments which lower MMP activities promote wound healing (Cullen, Watt et al. 2002, Veves, Sheehan et al. 2002, Lobmann, Zemlin et al. 2006, Gibson, Cullen et al. 2009).

In summary, the negligible MMP activity in PDE results from higher TIMP than MMP levels. This is consistent with a fibrotic phenotype which could underlie the development of simple PS. The elevated TIMP-1 level in PDE may have value as a diagnostic tool or late biomarker of EPS, but this requires extensive evaluation in more patients in different cohorts. Various TIMPs and MMPs in PDE showed markers of increased solute transport during peritoneal dialysis. Plasma levels of TIMP-1, TIMP-2, MMP-2 and MMP-3 are higher in PD patients in compared to healthy individuals. Plasmin activity will decline in patients who have been on PD for a long period (more than 3 years).

CHAPTER FOUR

Local and systemic production of TIMPs and MMPs

4.1 Introduction

The data from chapter three showed changes in the MMP system in patients on PD and this may play a role in the fibrotic remodeling seen in these individuals. Importantly in chapter 3, levels of TIMPs and MMPs measured in the drained dialysate were lower than in their levels in plasma hinting towards the source of theses being from the circulation and inappropriate build up in the peritoneum. Therefore the source of the TIMPs and MMPs in peritoneal dialysis effluents (PDE) could be important to understanding the pathological processes and any value as a diagnostic tool. It is known that many TIMPs and MMPs are found in the blood normally (Hirahara, Inoue et al. 2011) and because of their size could easily access the peritoneum, but it also remains possible that levels could be regulated locally in response to the presence of PD fluids which typically have high glucose which is a known trigger for changes in TIMPs (Kim, Li et al. 2008). Albumin, transferrin, β2-microglobulin (B2M), immunoglobulin G (IgG), and creatinine were used to generate a standard curve (Molecular weight versus dialysate/plasma ratio for these proteins) at 2hr dwell time and start effectively assessing the rate of transfer across the peritoneal membrane. After that, D/P ratios of TIMPs and MMPs can be plotted against their molecular weights. If the measured D/P ratios of MMPs and TIMPs are on the positive side (faster accumulation) of the standard curve, then that would suggest local production in the peritoneum. The reason why albumin, transferrin, B2M, IgG, and creatinine were selected comes from understanding their source and function.

Albumin (69 kDa) is anionic, non-glycosylated protein. It is regarded as the most abundant protein in the serum (Garcovich, Zocco et al. 2009). Albumin is produced in liver. In normal individuals, a daily amount of 10-15 g of albumin is synthesized from liver. This accounts for about 10% of total hepatic protein production. The majority of synthesized albumin will be released into the vascular compartment. A small quantity of albumin is stored in liver (<2g). About 30-40% of the synthesized albumin will be maintained in the plasma, with

remaining amount being located in tissues like skin and muscle (Redman 1969). Homeostasis of albumin is kept by balanced catabolism which take place in all tissues, mainly in liver, muscle and kidney (Beeken, Volwiler et al. 1962). One of the important functions of albumin is maintaining the colloid oncotic pressure. About 60% of intravascular protein is composed of albumin, thus maintaining 60% of plasma colloid oncotic pressure. Albumin causes water retention. It occurs through attraction of sodium ions by the negative charge on protein molecules. The remaining contribution of albumin to colloid oncotic pressure is through the Gibbs-Donnan in which there will be attraction of other active positive ions, resulting in further water retention (Nguyen and Kurtz 2006). Albumin will also participate in transporting metabolites such as bilirubin, thyroxine, fatty acids and amino acids (Garcovich, Zocco et al. 2009). Low serum albumin can be found in conditions such as liver cirrhosis and excess excretion through the kidneys in patients with nephrotic syndrome. High level of serum albumin can be found in conditions such as dehydration. Gibbs-Donnan Equilibrium is the ionic concentration difference between interstitial fluid and plasma is caused by the much higher proteins concentration in the plasma compared to the interstitial fluid. Large molecular weight substances such as proteins are not passing the capillary membrane easily. There is lack in the permeability of protein across capillary membranes and this is responsible for causing differences in the ionic concentration between the interstitial fluid and plasma and this is identified as the Gibbs-Donnan effect (Pitts 1974, Rose 1994, Nguyen and Kurtz 2004).

Transferrin (85 kDa) is an iron-binding plasma glycoprotein which is synthesized in the liver. It acts as a major transporter for iron through binding to it and transferring it to various cells in the body (Yang, Lum et al. 1984). Low transferrin may be as a result of poor transferrin production by the liver or excessive transferrin loss by the kidneys into the urine. Conditions associated with low plasma transferrin are including malignancy and infection, while iron deficiency anemia leads to abnormal high level of transferrin.

Malnutrition is associated with increase in the morbidity and mortality. There is no single effective indicator in the laboratory to identify malnutrition, but serum proteins and in particularly serum albumin are commonly used. There are some variables that could affect the levels of serum protein by decreasing their effectiveness. Serum albumin (Alb), prealbumin (PAB), transferrin, and retinol binding protein (RBP) are examples of malnutrition parameters or markers of nutritional status. Albumin has a half-life of approximately 14–20 days, which is why it has been used as a chronic nutritional status marker. PAB has a shorter half-life (2–3 days) and that is why it is expected to change rapidly with variations in the nutrient intake. Transferrin has a half-life of approximately 8–10 days, while RBP has a half-life of approximately 12 hours (Nutrition-support-team 2006).

There are several factors which cause an increase in the serum albumin level such as marasmus, dehydration, exogenous albumin and blood transfusions. In contrast, there are several factors which cause decreased serum albumin level such as kwashiorkor, over-hydration, ascites, eclampsia, infection, inflammation, metabolic, hepatic failure stress, nephrotic syndrome, burns, protein losing states, trauma, post-operative states, bed rest, cancer, collagen diseases, corticosteroid use, pregnancy, and zinc deficiency (Parrish, Krenitsky et al. 2003).

There are also situations that cause increases in the serum transferrin level such as iron deficiency, pregnancy (third trimester), dehydration, chronic kidney diseases, oral contraception (estrogens), hepatitis, chronic blood loss, and hypoxia. Decreases in the serum transferrin level can occur due to kwashiorkor, zinc deficiency, age, cancer, corticosteroids, protein, severe liver disease (hepatic congestion), iron overload (iron dextran therapy), uremia, acute catabolic states, nephrotic syndrome (permeability of glomerulus), pernicious anemia (B12 deficiency), folate deficiency anemia, anemia of chronic disease, chronic infection, and overhydration (Parrish, Krenitsky et al. 2003).

In the United States Renal Data System database (USRDS), a fall of 1 g/dL of the serum albumin level is associated with increase cardiovascular death risk factor by 39% (Fung, Sherrard et al. 2002). There is linear and incremental association between serum albumin level and mortality. It is unclear as to whether hypoalbuminemia in the CKD patients are as a result of inadequate protein intake or other disorders related to Protein-energy wasting such as comorbidity and inflammation (Friedman and Fadem 2010). Dialysis patients that tolerate the technique well show higher level of the serum albumin with better survival rates, while the reverse is true for those with an inferior dialysis performance (Lacson, Wang et al. 2009, Lacson, Wang et al. 2009). Longevity has steadily been observed in the CKD patients with a better nutritional status, including better appetite, larger muscle mass, higher intake of proteins, and fat mass (Kalantar-Zadeh, Cano et al. 2011, Jadeja and Kher 2012).

B2M (11.8 kDa) is a polypeptide and it is a subunit of the class I major histocompatibility antigens which is located on the cell surface. The B2M concentration is regulated by the kidney & it is not surprising to find B2M elevated in the plasma of patients with chronic kidney diseases (Lysaght, Pollock et al. 1989). The normal free B2M concentration in the serum is (1–2 mg/L) (Rennella, Cutuil et al. 2013). The other two molecules used to create plasma to dialysate transfer rate versus molecular weight as a standard curve were IgG and creatinine. IgG had the highest MW among the five molecules at 150 kDa, while creatinine had the smallest MW and it is 0.113 kDa. IgG molecules are created in plasma B cells and released from these cells, while serum creatinine is primarily a metabolite of creatine, which is located in mainly in the skeletal muscle (Harita, Hayashi et al. 2009)

4.2 Hypothesis

TIMPs and MMPs are produced locally in the peritoneum during PD and those local levels of production increase with the development of PS and EPS.

This local production can be demonstrated by measuring the rate of accumulation of TIMPS and MMPs in comparison to proteins of similar molecular weight known to be produced only outside the peritoneum.

4.3 Aims

To test this hypothesis, the rate of transfer of proteins of known molecular weight that are not produced in the peritoneum will be calculated. If TIMP and MMP levels in PD fluid have a potentially higher transfer rate this will indicate local production is occurring. Practically this aim will be addressed by:

1. Collecting plasma & PDE samples at set time points during PET
2. Measuring the plasma and drained dialysate levels of TIMPs, MMPs
3. Measuring the plasma and drained dialysate levels of albumin, transferrin, β2-microglobulin and IgG to calculate D/P for these proteins.
4. Obtaining D/P creatinine from clinical data
5. Constructing plasma to dialysate transfer rate standard curve by plotting of creatinine, albumin, transferrin, β2-microglobulin and IgG dialysate/plasma (D/P) ratio in a 2hr dwell vs. its molecular weight (MW).
6. Plotting TIMPs and MMPs D/P ratios against their molecular weight to calculate whether TIMPs and MMPs are produced locally or not by comparing to the plasma to dialysate transfer rate standard curve.

4.4 Methods

All ELISAs measured at optical density (OD) 450 nm in an ELISA reader immediately after adding stopping solutions except in IgG, in which an OD of 405 nm was used after suitable developing time (40 minutes) without adding stopping solution. Examples of the standard curves for the four molecules are illustrated

in the Figure 4.1. The standard curve in beta 2 microglobulin (B2M) was in pg/mL, while the standard curves of albumin, transferrin, and IgG were in ng/mL.

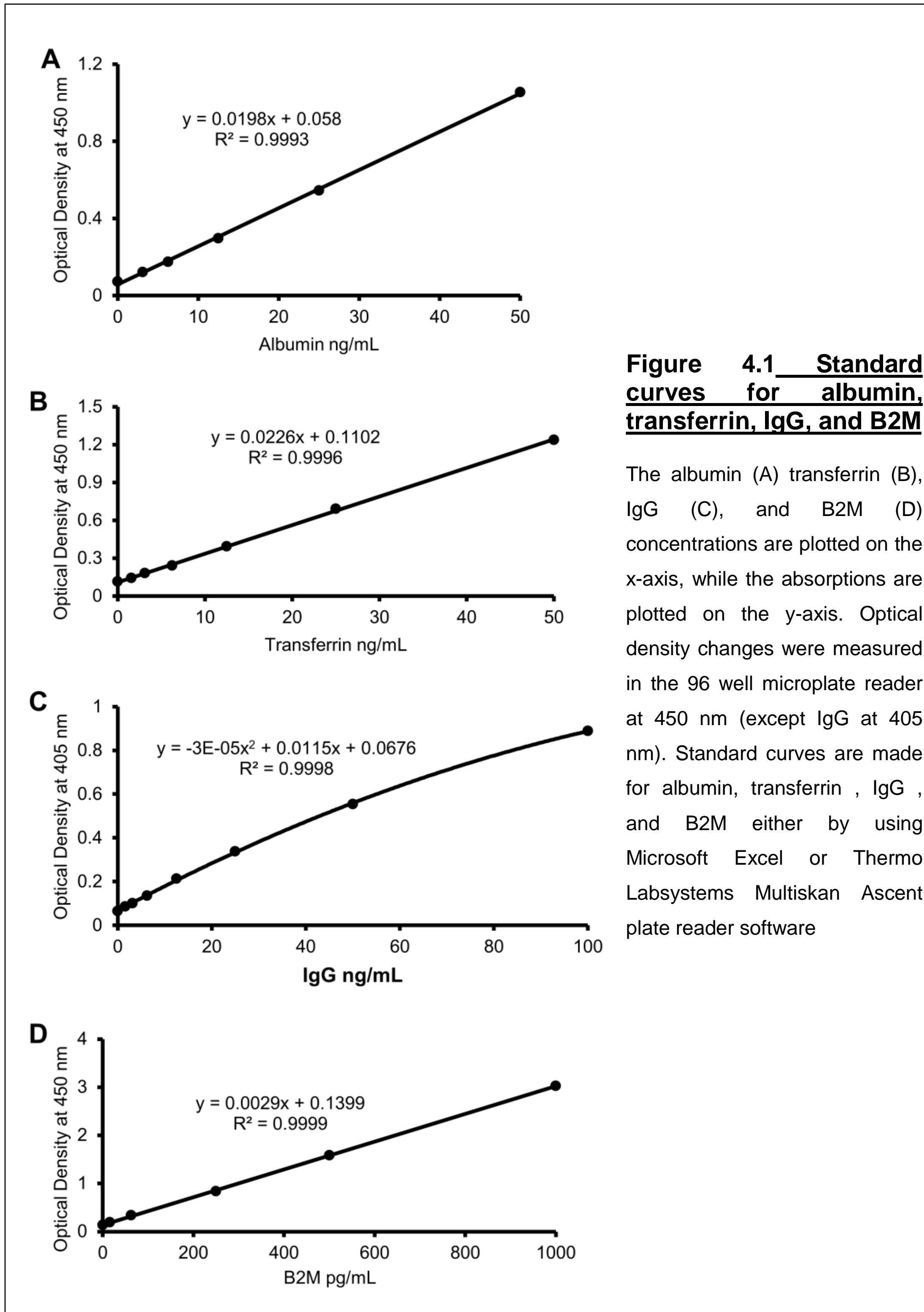


Figure 4.1 Standard curves for albumin, transferrin, IgG, and B2M

The albumin (A) transferrin (B), IgG (C), and B2M (D) concentrations are plotted on the x-axis, while the absorptions are plotted on the y-axis. Optical density changes were measured in the 96 well microplate reader at 450 nm (except IgG at 405 nm). Standard curves are made for albumin, transferrin , IgG , and B2M either by using Microsoft Excel or Thermo Labsystems Multiskan Ascent plate reader software

4.5 Results

Commercially available sandwich ELISA's for measuring albumin, transferrin, β2-microglobulin and IgG were used as described in sections 2.8.3.3, 2.8.3.4, and 2.8.3.5 to measure levels in PDE and plasma samples from SKI-cohort 2 patients undergoing PET at 0, 2 and 4 hours. Samples were diluted as listed in (Table 4.1) to ensure measurements were within the most accurate range of the ELISA.

Molecules	**Sample Dilution (plasma / PDE)**			
	0 Hour	**2 Hour**	**4 Hour**	**Plasma**
Albumin	3000	7000	13000	1500000
Transferrin	600	1750	2750	250000
IgG	1000	2500	5000	950000 (in healthy individual) 550000 (in PD patients)
Beta-2 Microglobulin	1750	5000	7250	90000 (in healthy individual) 40000 (in PD patients)

Table 4.1 Peritoneal dialysis effluent and plasma samples dilutions

The ideal approximate dilution approached for samples during ELISA, Abbreviations: PDE: peritoneal dialysis effluent, IgG: Immunoglobulin G, and ELISA: enzyme-linked immunosorbent assay.

4.5.1 Plasma level in healthy volunteers compared to PD patients in SKI-2

Albumin, transferrin, and IgG levels in plasma were higher in healthy volunteers compared to PD patients, while B2M level was lower in plasma of healthy volunteers. Albumin Median (IQR) in plasma was 44 (41–45) mg/mL in healthy but lower at 35 (30–37) mg/mL in patients on PD (Figure 4.2A). Transferrin median (IQR) was 4.7 (4.4–5.1) mg/mL in healthy individuals falling to 3.8 (3.2–4.3) mg/mL in patients on PD (Figure 4.2B). Median (IQR) was calculated for IgG level in plasma as 14.4 (11.8–15.5) mg/mL in healthy

individuals and 11.3 (9.4–14.1)mg/mL in patients on PD (Figure 4.2C). Median (IQR) was calculated for the B2M level in plasma as 1.7 (1.4–2) µg/mL in healthy volunteers and 18.3 (11.9–31.2) µg/mL in PD patients (Figure 4.2D)

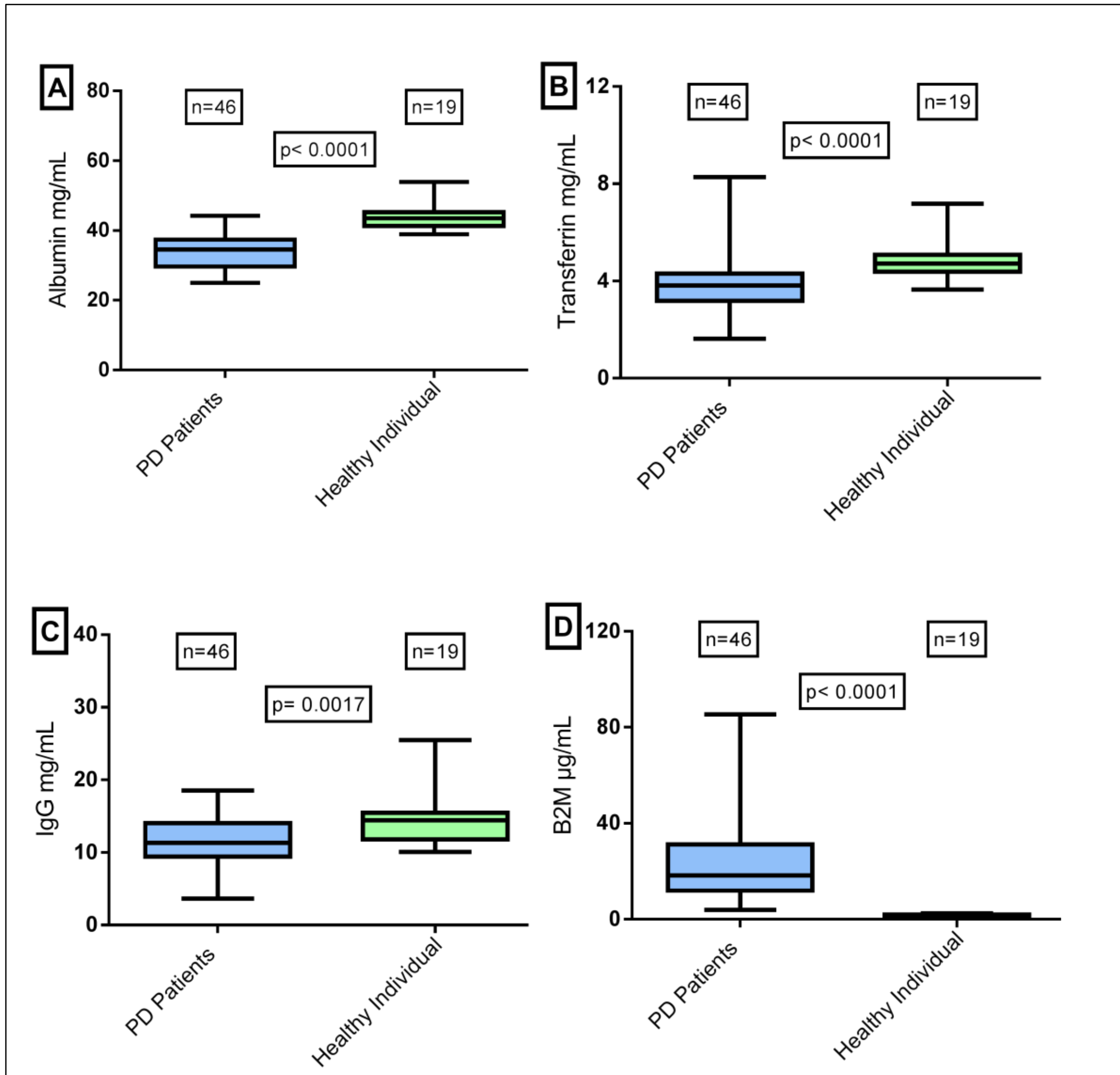


Figure 4.2 Albumin (A), Transferrin (B), IgG (C) and beta2-Microglobulin (D) in healthy volunteers and patients on PD in SKI-2

Albumin, transferrin, IgG, and beta-2 microglobulin were assayed in plasma samples by commercial ELISA. GraphPad Prism 6 used for statistical analysis (Unpaired t test in A & C and Mann Whitney test in B &D). Box and Whisker Plots show median, 25th Percentile, 75th Percentile, and the range. Abbreviations: B2M: beta-2 microglobulin, IgG: immunoglobulin, and SKI-2: Sheffield Kidney Institute PD cohort 2.

4.5.2 Effect of dwell time on Albumin, Transferrin, IgG and B2M in the PDE samples

Increases in dwell times were directly proportional to the levels of albumin, transferrin, IgG and B2M in PDE samples. Most of the 51 samples had multiple dwell time data, but 18 of them were collected prior to tube flushing in PET at different time points (0, 2hr, and 4hr) (Figure 4.3).

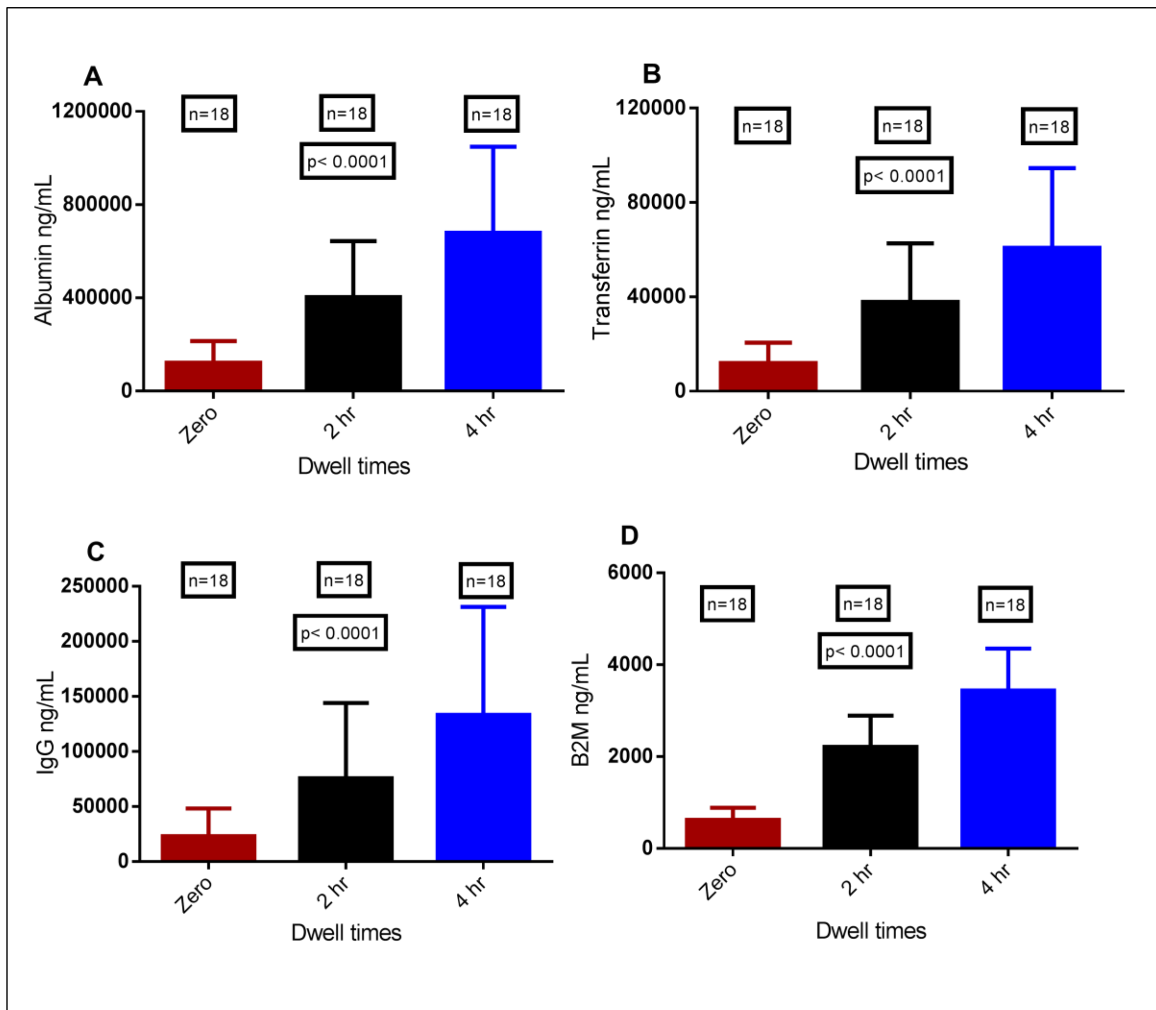


Figure 4.3 **Effect of dwell times on albumin, transferrin, IgG, and β2-microglobulin levels in PDE samples in the SKI-2 cohort**

PDE samples were collected from patients undergoing a PET test after 0, 2 and 4 hr dwell times and assayed by commercial ELISA for albumin, transferrin, IgG, and B2M. GraphPad Prism 6 was used for statistical analysis (One way ANOVA). Data represent mean with SD.

4.5.3 Rate of filtration

Patient samples were classified to three groups as less than 1 year on PD, 1-3 years on PD, and more than 3 years on PD and plots of Log10 molecular weight for creatinine, β2-microglobulin, albumin, transferrin and IgG vs the Log10 D/P creatinine, β2-microglobulin, albumin, transferrin and IgG at the 2hr dwell point to create (molecular weight) Mwt/filtration rate (standard curve). After that, Log10 molecular weight of TIMPs and MMPs were plotted against Log10 D/P TIMPs and MMPs at the 2hr dwell point for 46 patients (Figure 4.4), and at less than 1 year on PD, 1-3 years on PD, and more than 1 year on PD (Figure 4.5).

Initially standard plots of the dialysate/plasma ratio to molecular weight were performed by using albumin, transferrin, IgG, B2M (straight line). However, this had the issue that not all points were passing by the line which made them inaccurate. Post discussions with Prof Davies and Dr Lambie (Keele University), the decision was made to re-plot the data with including D/P Cr data to have wider range with performing a negative log non-linear fit (i.e. max log D/P ratio would be 0), rather than a straight line as with our initial approach.

TIMP-1, TIMP-2, MMP-2 and MMP-3 had a D/P ratio higher than would have been predicted at 2 hour from the D/P vs molecular weight plot suggesting that all had some peritoneal production. TIMP-1 and MMP-2 were much further away from the line than TIMP-2 and MMP-3 suggesting that these had greater peritoneal production. TIMP-2 was only just above the predicted level at all-time points suggesting this had low levels of peritoneal production. Therefore, there is some local MMPs and TIMPs production that exceeds the levels that would be achieved by filtration from the blood alone, but there may be increase in the MMPs and TIMPs transportation from blood to PDEs too.

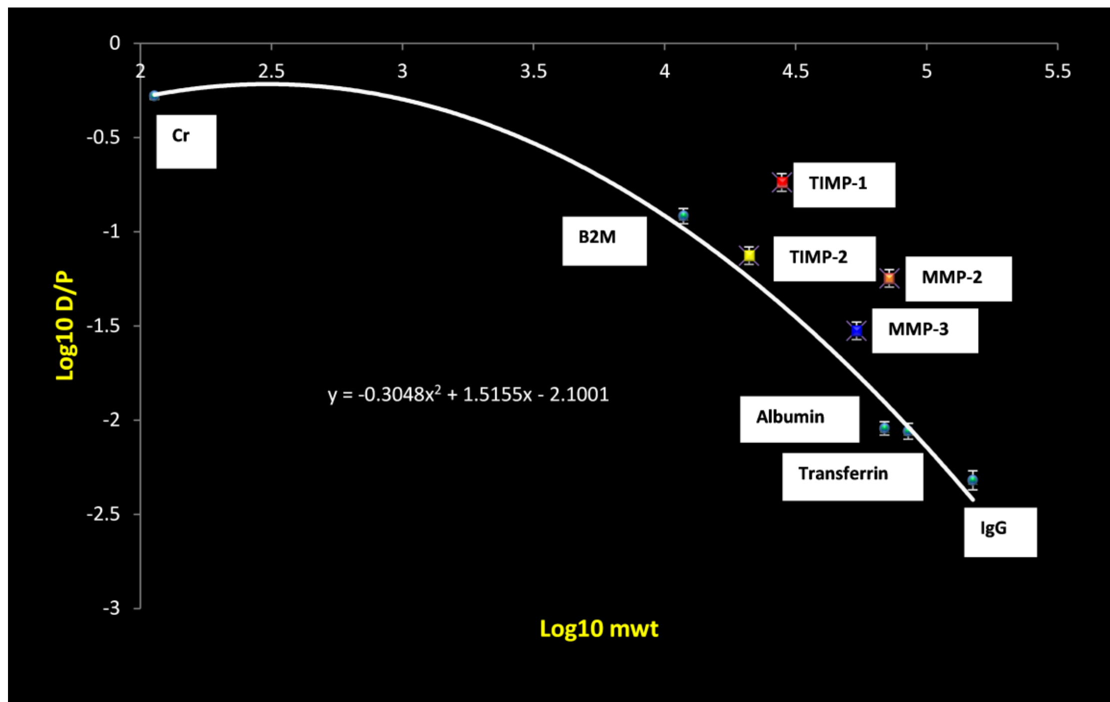
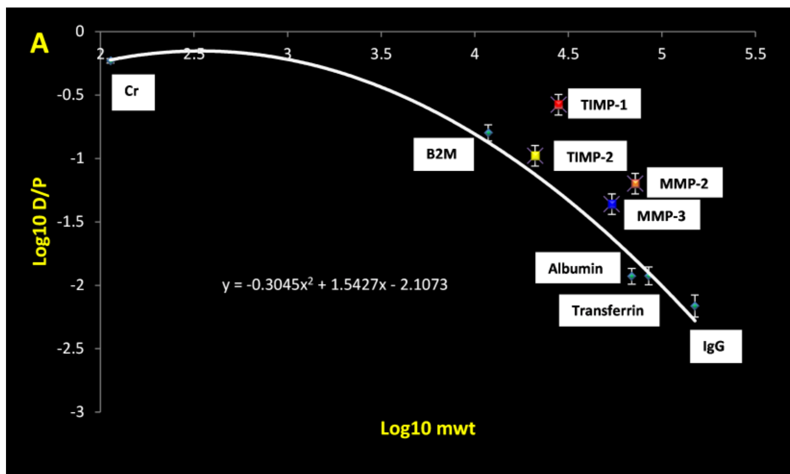
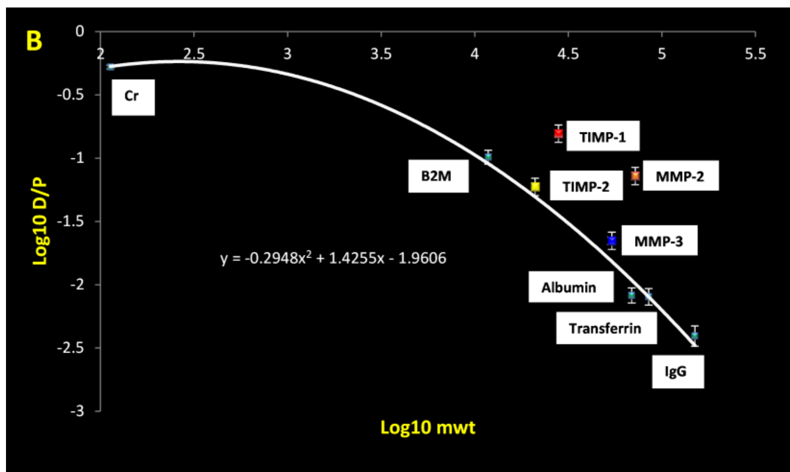


Figure 4.4 <u>TIMPs and MMPs are produced in the peritoneum (all samples)</u>

A Log10 plot of proteins in dialysate/plasma (D/P) ratio in a 2hr dwell time vs. Log10 of their molecular weight (MW) were plotted to construct a plasma to dialysate transfer rate (standard curve) by using circulating proteins with no peritoneal production i.e. creatinine (MW: 113.1 Da), β2-microglobulin (MW: 11800 Da), albumin (MW: 69000 Da), transferrin (MW: 85000 Da) and IgG (MW: 150000 Da). Log10 of TIMP and MMP in D/P ratio at 2hr dwell time for 46 patients were plotted against Log10 of their molecular weight. It demonstrated that MMP-2 (71000 Da) and TIMP-1 (28000 Da), and to lesser extent MMP-3 (54000 Da) and TIMP-2 (21000 Da) are produced in the peritoneum. Data represent Log10 of mean with SEM.





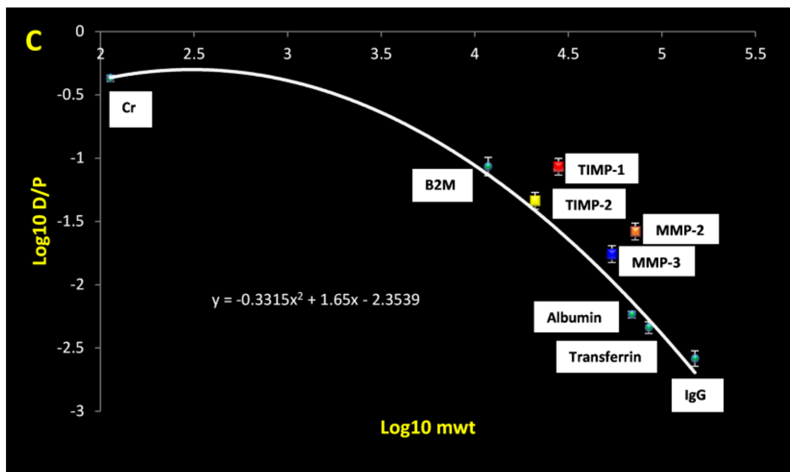


Figure 4.5 TIMPs and MMPs are produced in the peritoneum irrespective of time on PD

Patients were grouped by time on PD to less than 1 year (19 patients) (A), 1-3 years on PD (15 patients) (B), and more than 3 years on PD (12 patients) (C). A Log10 plot of proteins in dialysate/plasma (D/P) ratio in a 2hr dwell time vs. Log10 of their molecular weight (MW) were plotted to construct a plasma to dialysate transfer rate (standard curve) by using circulating proteins with no peritoneal production i.e. creatinine (MW: 113.1 Da), β2-microglobulin (MW: 11800 Da), albumin (MW: 69000 Da), transferrin (MW: 85000 Da) and IgG (MW: 150000 Da). Log10 of TIMP and MMP in D/P ratio at 2hr dwell time were plotted against Log10 of their molecular weight. It demonstrated that MMP-2 (71000 Da) and TIMP-1 (28000 Da), and to lesser extent MMP-3 (54000 Da) and TIMP-2 (21000 Da) are produced in the peritoneum in A, B, and C. Data represent Log10 of mean with SEM.

4.5.4 Correlations

4.5.4.1 Correlations of UF and D/Pcr to D/P of the albumin, transferrin, IgG, B2M, TIMPs, and MMPs

A set of correlations were run between UF and D/P of the albumin, transferrin, IgG, B2M, TIMP-1, TIMP-2, MMP-2, and MMP-3 levels at 2hr. U.F. was strongly negatively correlated to D/PTIMP-1 in both genders and similar to the correlation between UF and D/Pcr. Additional correlations are given in (Table 4.2)

Another set of correlation were run between D/Pcr and D/P of the albumin, transferrin, IgG, B2M, TIMP-1, TIMP-2, MMP-2, and MMP-3 levels at 2hr. D/Pcr was either strongly or very strongly positively correlated to D/P of albumin, transferrin, IgG, B2M, TIMP-1, TIMP-2, MMP-2, and MMP-3 levels at 2hr in both genders. Two exceptions were DPcr versus D/P B2M (female) and DPcr versus D/P MMP-2 (Male) which are both less well correlated. Additional correlations are illustrated in (Table 4.3).

This may indicate that in addition to the local production of TIMPs and MMPs, they may cross the peritoneal membrane to the peritoneal cavity. This may be because peritoneal membrane is more vascular, more inflammation with more protein crossing the peritoneal membrane.

Correlations	U.F. (Male), n=25	U.F. (Female), n=18
D/P Immunoglobulin G (IgG)	r= - 0.43 p= 0.03	r= - 0.35 p= 0.15
D/P Transferrin	r= - 0.53 p= 0.007	r= - 0.38 p= 0.12
D/P Albumin	r= - 0.59 p= 0.002	r= - 0.28 p= 0.26
D/P β2-microglobulin (B2M)	r= - 0.24 p= 0.25	r= 0.11 p= 0.67
D/P Timp-1	r= - 0.63 p= 0.0007	r= - 0.56 p= 0.016
D/P Timp-2	r= - 0.44 p= 0.03	r= - 0.32 p= 0.19
D/P MMP-2	r= - 0.39 p= 0.05	r= - 0.29 p= 0.25
D/P MMP-3	r= - 0.43 p= 0.03	r= - 0.51 p= 0.03
D/Pcr, 2 hr	r= - 0.66 p= 0.0003	r= - 0.72 p= 0.0007

Table 4.2 Correlations of Ultrafiltration (U.F.) to various D/P molecule ratio in both genders

Correlations between U.F.and dialysate/plasma (D/P) ratio for albumin, transferrin, IgG, B2M, TIMP-1, TIMP-2, MMP-2, MMP-3 and creatinine after a2hr dwell. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient). Abbreviations: tissue inhibitors of metalloproteinase (TIMP), and matrix metalloproteinase (MMP).

Correlations	D/Pcr, 2hr (Male), n=27	D/Pcr, 2hr (Female), n=19
D/P Immunoglobulin G (IgG)	r= 0.7058 $p < 0.0001$	r= 0.7216 p= 0.0005
D/P Transferrin	r= 0.7707 $p < 0.0001$	r= 0.7392 p= 0.0003
D/P Albumin	r= 0.7971 $p < 0.0001$	r= 0.6793 p= 0.0014
D/P β2-microglobulin (B2M)	r= 0.6349 p= 0.0004	r= 0.2151 p= 0.3764
D/P Timp-1	r= 0.7764 $p < 0.0001$	r= 0.7507 p= 0.0002
D/P Timp-2	r= 0.7664 $p < 0.0001$	r= 0.7772 $p < 0.0001$
D/P MMP-2	r= 0.3432 p= 0.0796	r= 0.6949 p= 0.0010
D/P MMP-3	r= 0.7791 $p < 0.0001$	r= 0.6973 p= 0.0009

Table 4.3 Correlations of D/Pcr to various D/P molecules in both genders

Correlations between D/Pcr against dialysate/ plasma (D/P) albumin, transferrin, IgG, B2M, TIMP-1, TIMP-2, MMP-2, and MMP-3 at 2hr. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient). Abbreviations: dialysate-to-plasma ratio of creatinine (D/Pcr), tissue inhibitors of metalloproteinase (TIMP), and matrix metalloproteinase (MMP).

4.5.4.2 Correlation of total protein to the albumin, transferrin, IgG, and B2M in the PDE samples

To determine if the levels of albumin, transferrin, IgG and B2M in PDE at 2hr were related to the total protein load in PDE, correlations were run between total protein in the PDE and each of the four proteins at 2hr. The correlations are illustrated in (Table 4.4) which shows much weaker correlation in B2M.

Correlations	Total protein
Albumin	r= 0.98, P < 0.0001
Transferrin	r= 0.97, P < 0.0001
Immunoglobulin G (IgG)	r= 0.92, P < 0.0001
β2-microglobulin (B2M)	r= 0.35, P = 0.01

Table 4.4 Correlations of total protein to the four molecules in peritoneal dialysis effluents (PDE)

Correlations between total protein against albumin, transferrin, IgG, and B2M in PDE at 2hr. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient).

4.5.4.3 Correlations of the albumin, transferrin, IgG, and B2M to each other in the PDE samples

A set of correlations was run between PDE levels of albumin, transferrin, IgG and B2M at 2hr to determine if there were any links between the four molecules. B2M was the least correlated protein to the other three molecules. On the other hand, the correlation between albumin and transferrin, albumin and IgG, as well as transferrin and IgG were very strongly correlated (Figure 4.6).

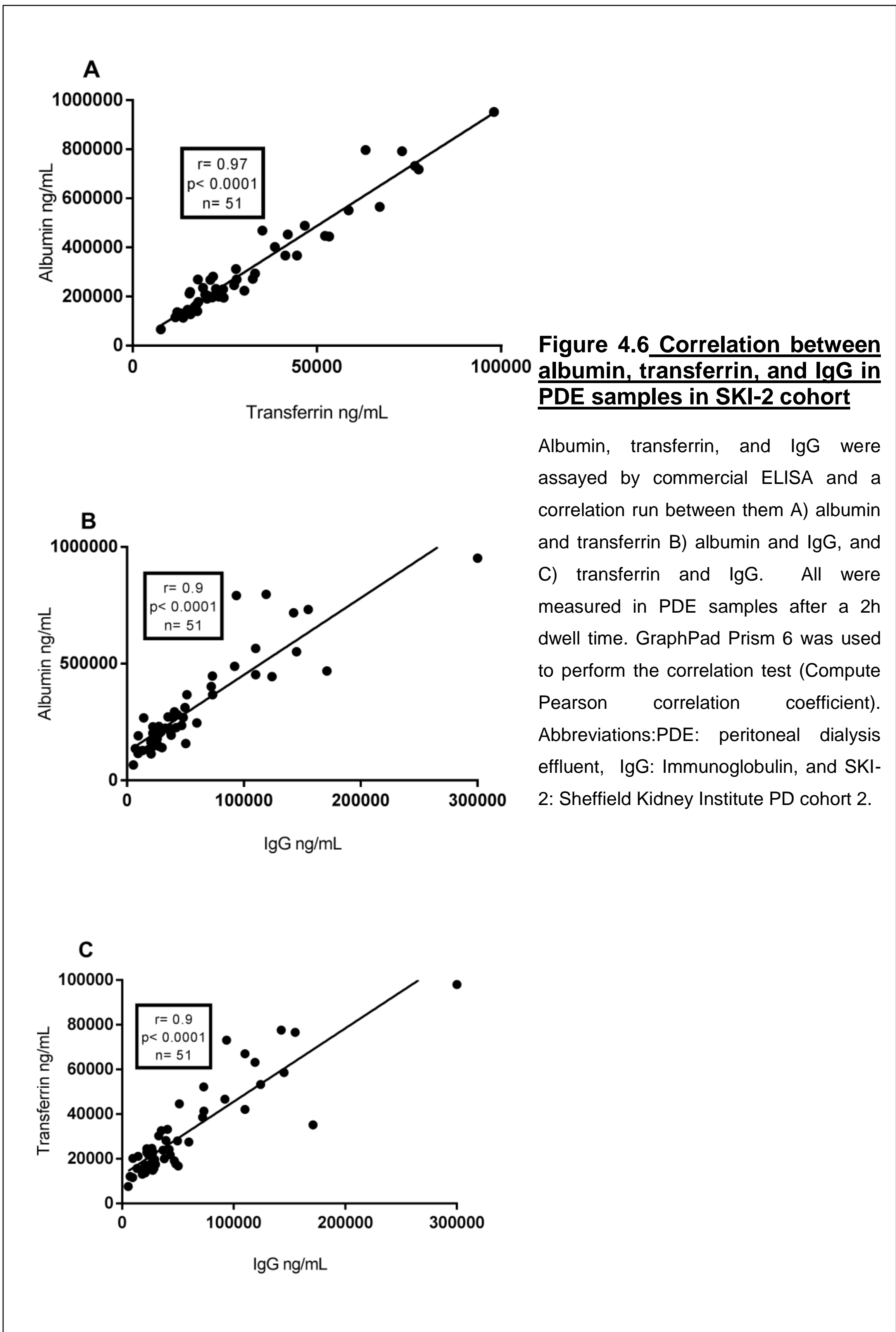


Figure 4.6 Correlation between albumin, transferrin, and IgG in PDE samples in SKI-2 cohort

Albumin, transferrin, and IgG were assayed by commercial ELISA and a correlation run between them A) albumin and transferrin B) albumin and IgG, and C) transferrin and IgG. All were measured in PDE samples after a 2h dwell time. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient). Abbreviations:PDE: peritoneal dialysis effluent, IgG: Immunoglobulin, and SKI-2: Sheffield Kidney Institute PD cohort 2.

4.6 Discussion

This chapter aimed to understand if there was local production of TIMPs and MMPs in the peritoneum during PD. Circulating proteins with no peritoneal production were chosen to construct plasma to dialysate vs molecular weight standard curve at a 2hr dwell point using samples collected accurately during routine PET. The data clearly demonstrated local production of TIMP-1, TIMP-2, MMP-2 and MMP-3 was occurring in the peritoneal cavity, in particular TIMP-1 and MMP-2 seemed to demonstrate the greatest intra-peritoneal contribution to levels in PDE. .

There were some technical challenges to overcome, the most frustrating being the effect of line flushing on PDE sample measurements as this was undertaken in different ways by those doing the PET tests. Samples after line flushing would therefore be diluted by varying amounts so all measured proteins will also be variably diluted. PDE samples at 2hr dwell time were chosen to overcome this problem as samples at 2hr were not collected with line flushing making the measurements accurate and precise.

A number of correlations were performed. They showed that albumin, transferrin, and IgG levels in PDE are strongly positively correlated to the total protein level and also to each other. These proteins were chosen as they are not produced in the peritoneum and thus need to be filtered. Only B2M was less positively correlated to these 3 proteins and total protein. Due to the fact that B2M does not correlate with total protein or other proteins measured, this does question the accuracy of this point on the molecular weight to D/p curves and may suggest the gradient of the curve is greater than it should be and thus some of the MMPs and TIMPs may have greater peritoneal production than this data predicts.

Gender has a significant effect on correlations of D/Pcr or U.F. versus D/P of all molecules tested, but U.F. was strongly negatively correlated to D/P of TIMP-1 in female and male groups, not affected by gender and thus the main finding stands cross gender. The peritoneal membrane inflammation and damage may leads to protein loss including TIMP-1.

Albumin, transferrin, and IgG levels were higher in plasma of healthy individuals in compare to patients on PD. B2M level was higher in patients on PD in compare to healthy individuals. The elevated level of B2M may be due to impaired renal clearance of this molecule.

Hirahara and collegues have previously performed similar studies to those carried out in this chapter but they were unable to detect TIMP-2 in PDE. It is likely this relates to either the assay they used as sensitivity could be an issue or the way their samples were stored, However they did show very similar findings for TIMP-1 as described here despite their lack of curve fitting in constructing plasma or serum/dialysate transfer rate standard curves. They also did not include serum or plasma creatinine which gave them a less data points for line (Hirahara, Inoue et al. 2011). The other group that did similar work was Barreto and collegues. They used four molecules curve and they only tested MMP-2 and PAI-1, and they did not perform curve fit. MMP-2 was also proved to be produced locally in this study. (Barreto, Coester et al. 2013).

The data here clearly shows local production for TIMP-1, MMP-2, and to lesser extent TIMP-2, and MMP-3 in all groups irrespective of time on PD. This is interesting because TIMP is associated with the fibrotic lesions across many organs including kidney and as such its production in response to PD could have implications for PS. What is more, this local production shows that the peritoneum has the capacity to generate TIMP-1 and thus the very high levels seen late in EPS patients in the previous chapter. Simply put, there may come a time in PD when the local TIMP-1 production is exaggerated in response to some

event and thus controlling the local production early could have benefical effects on EPS development.

In conclusion, TIMPs and MMPs specially TIMP-1 and MMP-2 are produced partly in the peritoneum. Intraperitoneal production of TIMP-1 may cause systemic elevation as measured in plasma. The process of PD may trigger this enhanced TIMP-1 and thus it can potentially be used as a biomarker of PD damage as well as a potential therapeutic target.

CHAPTER FIVE

Characterisation of three proteins identified as potential predictors of EPS by peritoneal dialysis effluent proteomics

5.1 Introduction

Recent application of 2D gel proteomics on PDE samples from the GFS carried out at the Sheffield Kidney Institute has identified changes in several proteins in patients that develop EPS compared to patients that do not. However none of these proteins have been followed up and quantified accurately by classical assays in the wider GFS cohort to validate. In this chapter, three of the more promising proteins identified by proteomics have been selected for accurate quantification in PDE samples by Enzyme-linked immunosorbent assays (ELISA). These proteins are Intelectin-1 (omentin), Dermatopontin and Collagen (alpha1) I. These 3 proteins were selected from a provisional list. The rest of the identified proteins were also measured by others (2 laboratory team workers). The reasons of choosing these 3 targets were because of the following:

1) Intelectin-1 was very clear protein spot in GFS (EPS) cohort and changed in all EPS patients put through proteomics.
2) Dermatopontin was similar to the intelectin-1. It seemed to increase with time on PD.
3) Collagen (alpha1) I was the only obvious ECM protein to come out of proteomics and thus may play as a direct marker of changes in the ECM production.

In humans, there are 2 known intelectins termed -1 and -2. Intelectin-1 (omentin) (120 kDa) is an adipocytokine. Intelectin-1 has no effect on the basal glucose uptake; however, it helps in insulin-stimulated glucose uptake in the adipocytes. It may take part in the immune defence system by having a role against microorganisms by recognising carbohydrate chains on the pathogens. Finally, it may play a role in iron metabolism (Tsuji, Uehori et al. 2001, Yang, Lee et al. 2006).

Intelectin-1 is mainly expressed in omental adipose tissue (Shibata, Ouchi et al. 2012). It is also highly expressed in the small intestine with further expression in the colon, pancreas, heart, skeletal muscle, salivary gland, thyroid, and testis. Intelectin-1 has lower expression in the prostate, uterus, thymus, lymph node and spleen (Lee, Schnee et al. 2001, Suzuki, Shin et al. 2001, Tsuji, Uehori et al. 2001, Yang, Lee et al. 2006). Goblet cells in gastrointestinal tract are the main cells secreting intelectin-1 in humans. Intelectin-1 is secreted to the intestinal lumen along with mucus (Washimi, Yokose et al. 2012). In 2012, circulating intelectin-1 level was shown to be negatively correlated against a multiplicity of metabolic risk factors. This suggested that intelectin-1 may act as a metabolic disorders biomarker (Shibata, Ouchi et al. 2012). Intelectin-2 may also have a role in defense against pathogens. Unlike intelectin-1, intelectin-2 is expressed in the small intestine only (Lee, Schnee et al. 2001)

Dermatopontin (DPT) (24 kDa) comprises a significant proportion of the non-collagenous protein in ECM. The ECM in vertebrates consists of collagens and non-collagenous proteins for example proteoglycans. The non-collagenous proteins have many functions such as maintenance of tissue architecture and integrity as well as their involvement in the cellular behavior (Yamatoji, Kasamatsu et al. 2012). Dermatopontin is a tyrosine rich acidic protein (Neame, Choi et al. 1989). Half of the tyrosine residues in dermatopontin are sulfated. Sulfated tyrosine is crucial as it helps dermatopontin to interact with other proteins in ECM. Dermatopontin interacts with numerous components in ECM, especially collagen, decorin and TGF-beta (Okamoto and Fujiwara 2006, Yamatoji, Kasamatsu et al. 2012). Dermatopontin is expressed in fibroblasts, skeletal muscle, heart, pancreas, brain, kidney, lung, placenta, and liver. It is expressed more in normal skin fibroblasts than in fibroblasts of systemic sclerosis patients and fibroblasts of patients with hypertrophic scar lesions in their skin suggesting its loss is associated with inappropriate ECM biology (Superti-Furga, Rocchi et al. 1993, Kuroda, Okamoto et al. 1999).

Collagen I represents the most abundant collagen among the collagen family. Collagen I is a heterotrimer comprising two chains of α1(I) and one chain of α2(I) encoded by theCOL1A1 and COL1A2 respectively. Collagen α1(I) is 139 kDa and Collagen α2 (I) is 129 kDa. Collagen α1(I) and collagen α2 (I) are located on chromosome 17 and 7 respectively (Dalgleish 1997). In organ fibrosis not only does collagen production go up, but there are relative increases in collagen types I & III as a percentage of the overall collagen load (Johnson, Fisher et al. 2007). Collagen I expression is increased in the other fibrosis forms such as bone marrow fibrosis, liver fibrosis, lung fibrosis, and scleroderma (Karsenty and Park 1995)

5.2 Hypothesis

Proteomics analysis of PDE samples comparing the proteome from EPS patients to that from patients not developing EPS has identified changes in several proteins. There is a possibility that some of these proteins may underlie the development of EPS or act as biomarkers. Therefore this chapter aims to test the hypothesis that is changes in intelectin-1, DPT and COL1A1 may have value as biomarkers or diagnostic markers for PS and EPS in PD patients.

5.3 Aims

The aim of this chapter is to determine if intelectin-1, dermatopontin and collagen (α1) I (COL1A1) are biomarkers of, or have diagnostic value in EPS. To do this, the aims in this chapter will be to:

1. Measure the levels of intelectin-1, dermatopontin and COL1A1 in PDE samples in the SKI-1 cohort to assess feasibility of detection.

2. Determine how these proteins change with loss of membrane function using the SKI-2 cohort.

3. Any with sufficient abundance in PDE samples will be measured in non-EPS (control) and EPS populations from the GFS to establish if they can be predictive or diagnostic.

5.4 Methods

Commercially available sandwich ELISAs for measuring Intelectin-1, dermatopontin and collagen (α1) I (COL1A1) were used as described in chapter 2 (section 2.8.3.6) using the 3 patient cohorts (SKI-1, SKI-2, and GFS). All ELISAs were ordered from USCN as following:

- E90933Hu 96 Tests, Enzyme-linked Immunosorbent Assay Kit For omentin (intelectin-1), Organism: Homo sapiens (Human), USCN
- E92432Hu 96 Tests, Enzyme-linked Immunosorbent Assay Kit For dermatopontin, Organism: Homo sapiens (Human), USCN
- E90350Hu 96 Tests, Enzyme-linked Immunosorbent Assay Kit For collagen (a1) I, Organism: Homo sapiens (Human), USCN

All ELISAs measured at optical density (OD) 450 nm in an ELISA reader immediately after adding stopping solutions. Examples of the standard curves for these three proteins are illustrated in the Figure 5.1. The standard curve in intelectin-1 was in pg/mL, while the standard curves of dermatopontin and COL1A1 were in ng/mL.

5.4.1 Assay sensitivity

The minimum detectable values are typically less than 52 pg/mL for dermatopontin, 115 pg/mL for COL1A1 and 3.1pg/mL for intelectin-1. This indicate that even low concentration of human intelectin-1 in the samples can be detected using the ELISA kit from USCN (E90933Hu).

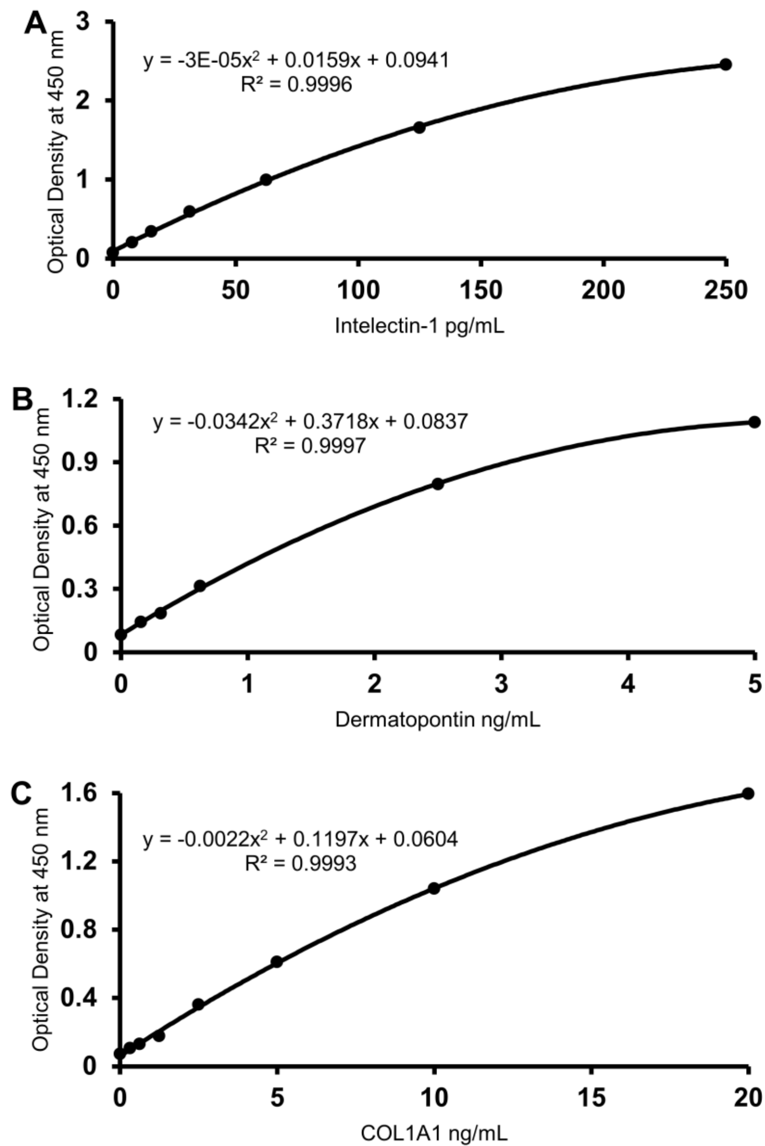


Figure 5.1 Standard curves for intelectin 1, dermatopontin and COL1A1

The intelectin-1 (A) dermatopontin (B), and COL1A1 (C) concentrations are plotted on the x-axis, while the absorptions are plotted on the y-axis. Optical density changes were measured in the 96 well microplate reader at 450 nm. Standard curves were made for intelectin-1, dermatopontin, and COL1A1 either by using Microsoft Excel or Thermo Labsystems Multiskan Ascent plate reader software

5.5 Results

PDE samples from cohort SKI-1 were used for early “first look” studies” for assay development as they had significant volumes but limited clinical data or exact dwell time information. They also had variable line flushing performed giving variable sample dilution. The SKI-2 cohort samples were collected as the study progressed during PET so had precise data available. The SKI-2 cohort contained both PDE and plasma samples. PDE samples after 2 hours dwell time were selected for use since these were collected without line flushing. The plasma samples in SKI-2 were collected at the 2 hours dwell time too. In the GFS cohort, there were only small volumes of PDE samples, but is probably the best collection of EPS samples available. Therefore anything of interest and measurable in the small samples was looked at in the GFS cohort.

SKI-1 was important cohort to practise and find the ideal dilution for the samples. Dermatopontin was assayed using neat PDE or a dilution up to 1:5. COL1A1 was assayed were between neat and 1:8 dilution while intelectin-1 was assayed between 1:50 and 1:100 dilution. Consequently all 3 proteins were measurable in the SKI-1 and 2 patient cohorts as sufficient sample was available, however only intelectin-1 was able to be measured in the GFS as intelectin-1 required less than 20 µL volumes of PDE samples to be performed.

In SKI-2, dwell times had an effect on PDE samples i.e. PDE samples (overnight) contain a higher concentration of various proteins than PDE samples after 2hr dwell time, thus need more dilution. For example, some samples required 100 times dilution to measuring intelectin-1 in PDE samples (overnight) by ELISA, while intelectin-1 measured in same samples at 2hr dwell time and it required less dilution during ELISA. To measure intelectin-1 in the PDE samples, 1:10-1:100 dilutions were used. Intelectin-1 in the plasma samples also required dilution with that from healthy individuals and patients on PD needing diluting

2500 and 5000 fold respectively. These dilutions were approximate as some individual samples required greater or lesser dilution.

5.5.1 Intelectin-1, dermatopontin and COL1A1 in PDE samples (SKI-1 cohort)

The SKI-1 cohort was used to establish the ideal dilutions for intelectin-1, dermatopontin and COL1A1 in the PDE samples, and to compare levels in PDE samples after grouping the samples according to the time on PD. SKI-1 cohort measurements were corrected to total protein concentration (mg/mL) in PDE samples to minimise the effect of variable dwell times and sample dilution caused by line flushing between PDE samples. PDE samples were divided into 4 groups based on time on PD or EPS as described previously (<1 year, 1-3 years, >3 years on PD and EPS) (Figure 5.2). Intelectin-1 expressed per mg of protein in the sample gave a higher level in the EPS patient than the other groups as would have been predicted from the proteomics. In contrast COL1A1 and dermatopontin did not show clear changes from the rest in the EPS sample. The median (range) for corrected intelectin-1, dermatopontin and COL1A1 to proteins were 4945 (1723-14754), 2531 (619-19922), and 10238 (1907-29000) pg/mg respectively.

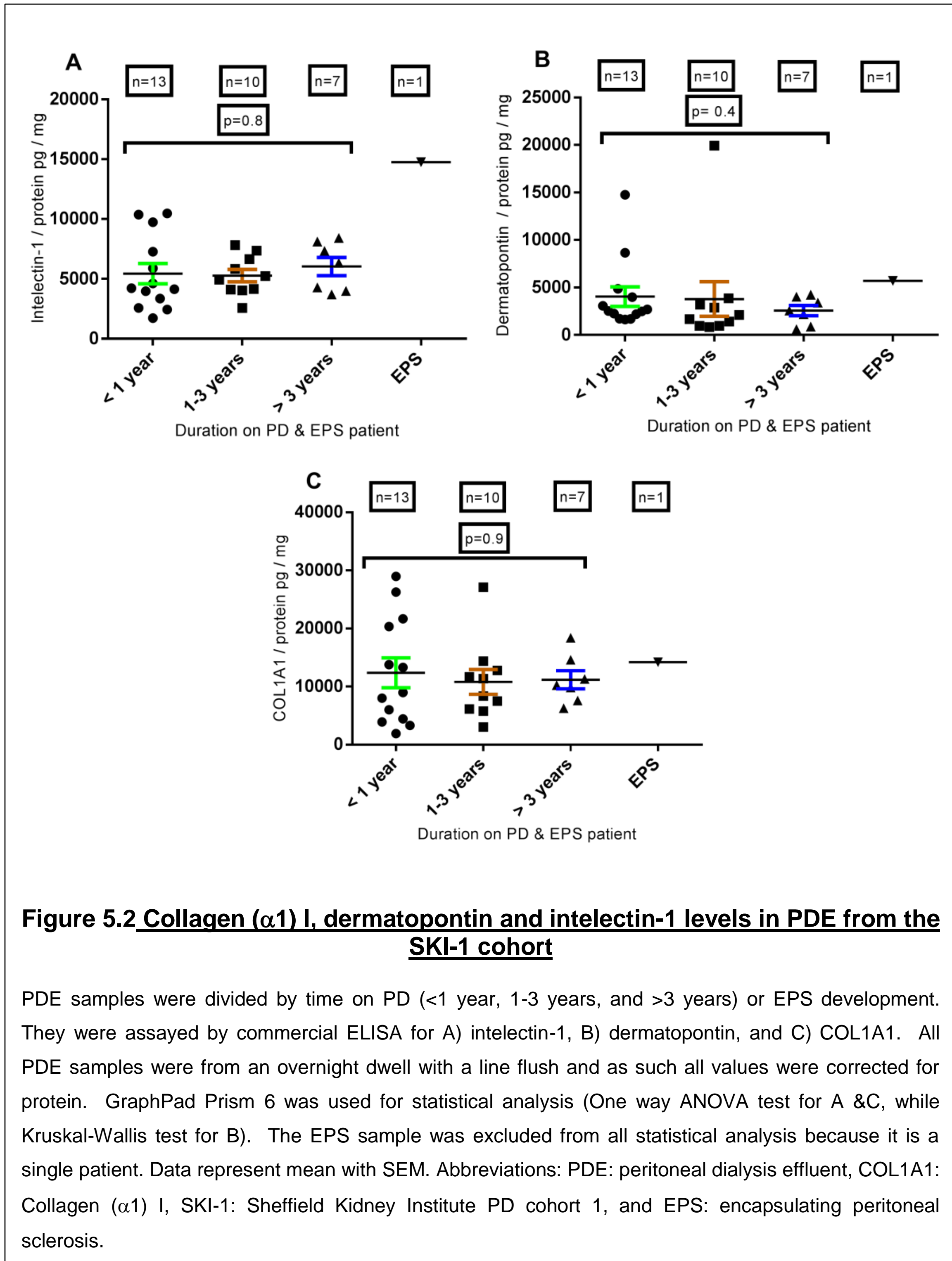


Figure 5.2 Collagen (α1) I, dermatopontin and intelectin-1 levels in PDE from the SKI-1 cohort

PDE samples were divided by time on PD (<1 year, 1-3 years, and >3 years) or EPS development. They were assayed by commercial ELISA for A) intelectin-1, B) dermatopontin, and C) COL1A1. All PDE samples were from an overnight dwell with a line flush and as such all values were corrected for protein. GraphPad Prism 6 was used for statistical analysis (One way ANOVA test for A &C, while Kruskal-Wallis test for B). The EPS sample was excluded from all statistical analysis because it is a single patient. Data represent mean with SEM. Abbreviations: PDE: peritoneal dialysis effluent, COL1A1: Collagen (α1) I, SKI-1: Sheffield Kidney Institute PD cohort 1, and EPS: encapsulating peritoneal sclerosis.

5.5.2 Intelectin-1, dermatopontin and COL1A1 in PDE samples from the SKI-2 cohort

Intelectin-1, dermatopontin and COL1A1 were detectable in the SKI-1 cohort and thus tested in the second Sheffield cohort where full dwell and clinical data was available allowing better relationship to the clinical status. Intelectin-1 was also measured in plasma of PD patients, permitting a calculation of the rate of filtration (section 5.5.2.5) to understand whether intelectin-1 is produced locally or not. The median (range) levels in PDE samples for intelectin-1, dermatopontin and COL1A1 levels median (range) in SKI-2 patient cohort were 1171 (317-10688), 801 (86-5650), and 4100 (1160-18500) pg/mL respectively.

5.5.2.1 Relationship between Intelectin-1, dermatopontin and COL1A1 with solute transport rate (D/Pcr and D/D0 glucose)

Peritoneal solute transport rate may be useful for evaluating peritoneal membrane injury. This is assessed during PET by D/Pcr and D/D0 glucose. There were positive correlations between intelectin-1, dermatopontin and COL1A1 with D/Pcr at 2hr (Figure 5.3), while the correlation was negative with D/D0 glucose (Figure 5.4). In both cases intelectin-1 and Collagen a1(I) had equivalent and better correlations than dermatopontin.

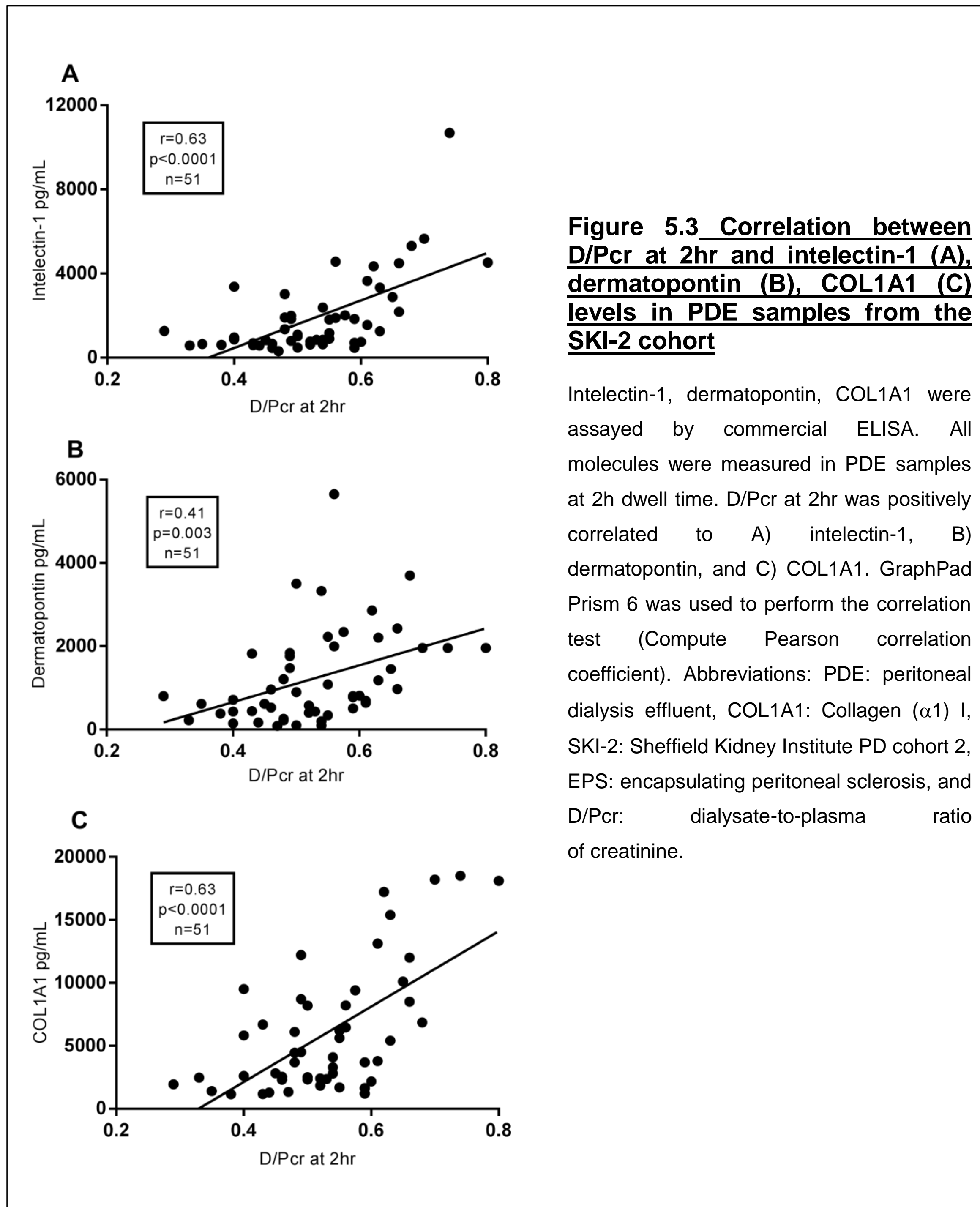


Figure 5.3 Correlation between D/Pcr at 2hr and intelectin-1 (A), dermatopontin (B), COL1A1 (C) levels in PDE samples from the SKI-2 cohort

Intelectin-1, dermatopontin, COL1A1 were assayed by commercial ELISA. All molecules were measured in PDE samples at 2h dwell time. D/Pcr at 2hr was positively correlated to A) intelectin-1, B) dermatopontin, and C) COL1A1. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient). Abbreviations: PDE: peritoneal dialysis effluent, COL1A1: Collagen (α1) I, SKI-2: Sheffield Kidney Institute PD cohort 2, EPS: encapsulating peritoneal sclerosis, and D/Pcr: dialysate-to-plasma ratio of creatinine.

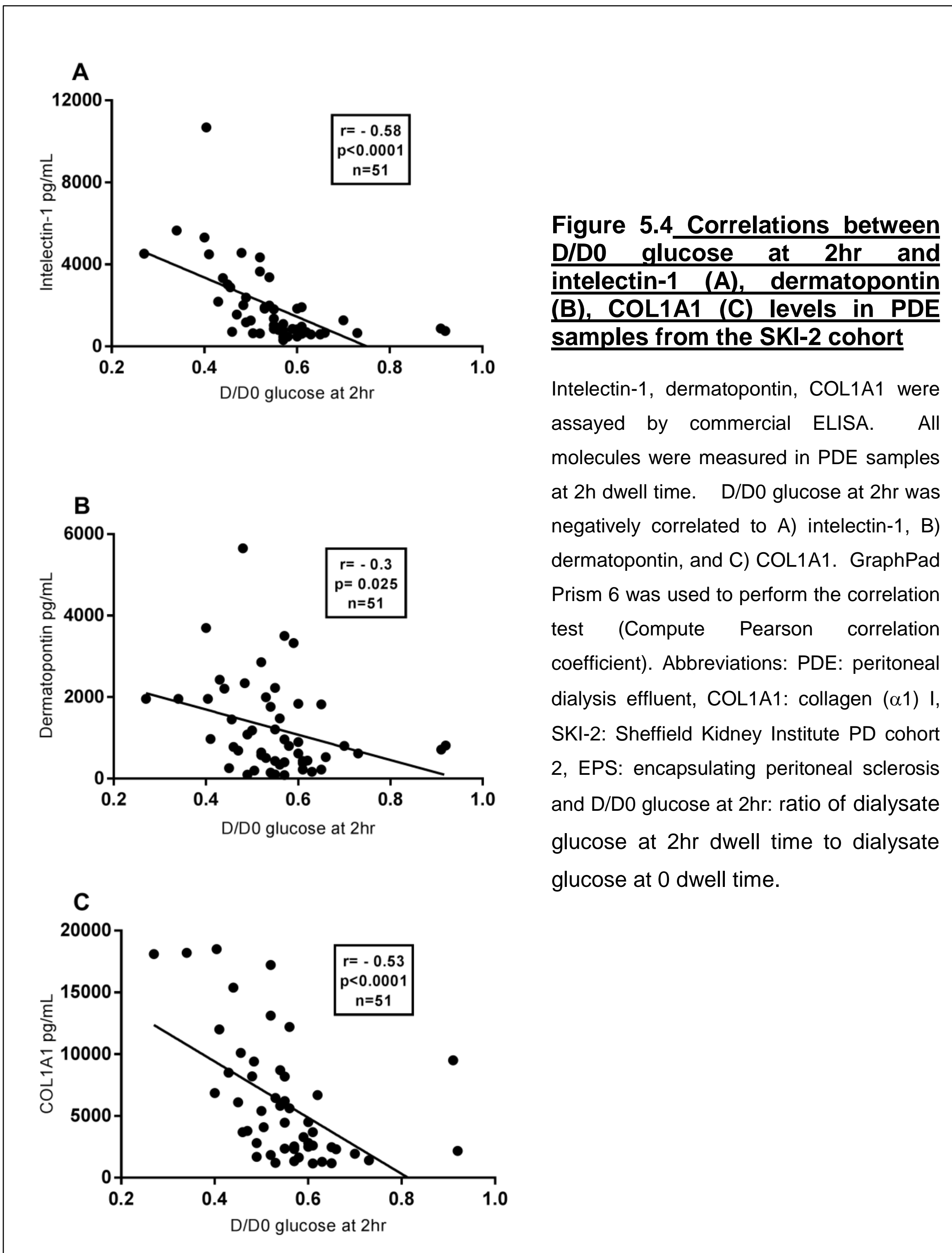


Figure 5.4 Correlations between D/D0 glucose at 2hr and intelectin-1 (A), dermatopontin (B), COL1A1 (C) levels in PDE samples from the SKI-2 cohort

Intelectin-1, dermatopontin, COL1A1 were assayed by commercial ELISA. All molecules were measured in PDE samples at 2h dwell time. D/D0 glucose at 2hr was negatively correlated to A) intelectin-1, B) dermatopontin, and C) COL1A1. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient). Abbreviations: PDE: peritoneal dialysis effluent, COL1A1: collagen (α1) I, SKI-2: Sheffield Kidney Institute PD cohort 2, EPS: encapsulating peritoneal sclerosis and D/D0 glucose at 2hr: ratio of dialysate glucose at 2hr dwell time to dialysate glucose at 0 dwell time.

5.5.2.2 Relationship between Intelectin-1, dermatopontin and COL1A1 with ultrafiltration

It is well-known that peritoneal membrane fibrosis is associated with ultrafiltration failure (Lambie, John et al. 2010, Sampimon, Coester et al. 2011). Intelectin-1, DPT and COL1A1 in the PDE samples were thus correlated against ultrafiltration rate displaying a negative correlation with UF (Figure 5.5).

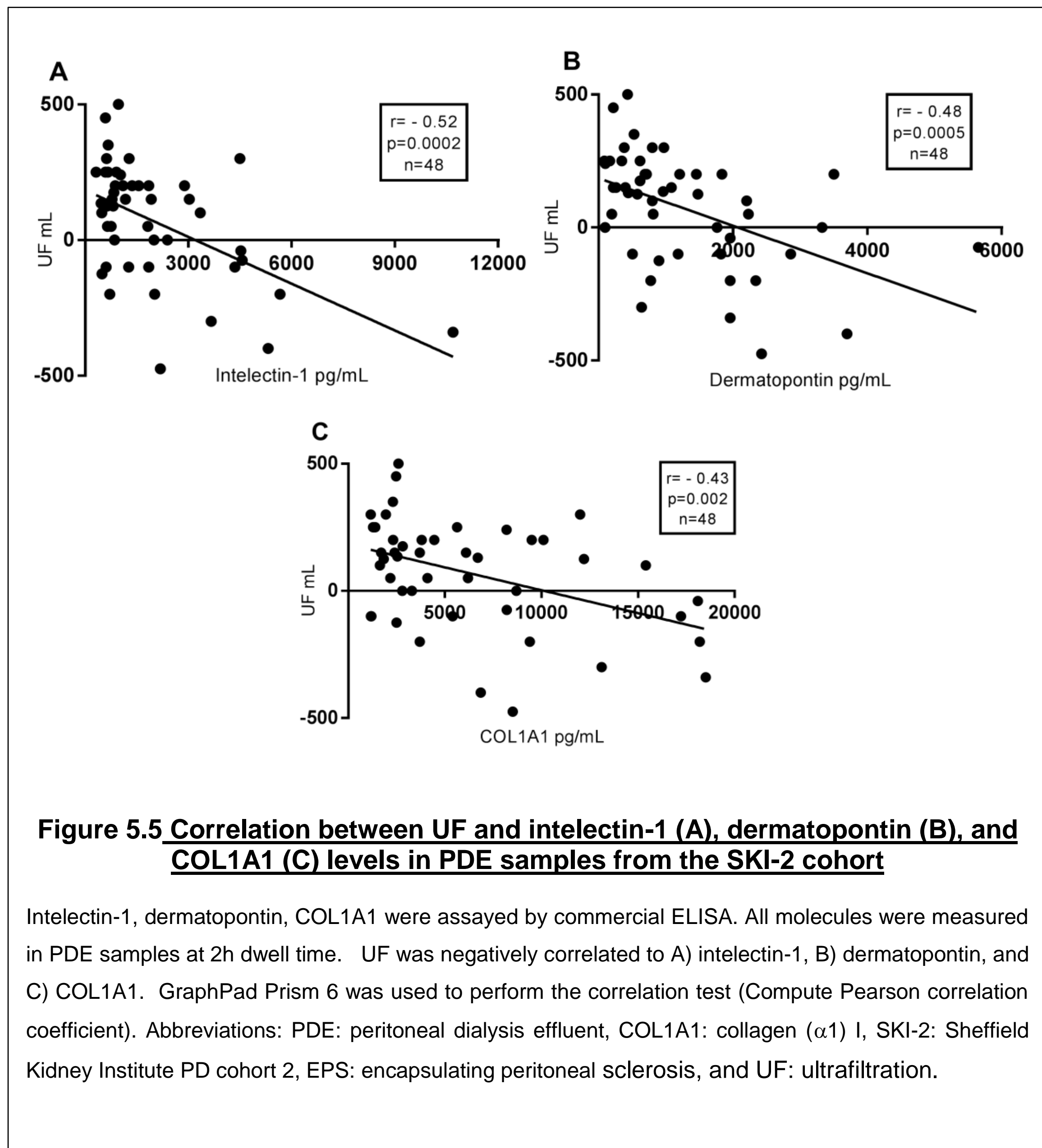


Figure 5.5 <u>Correlation between UF and intelectin-1 (A), dermatopontin (B), and COL1A1 (C) levels in PDE samples from the SKI-2 cohort</u>

Intelectin-1, dermatopontin, COL1A1 were assayed by commercial ELISA. All molecules were measured in PDE samples at 2h dwell time. UF was negatively correlated to A) intelectin-1, B) dermatopontin, and C) COL1A1. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient). Abbreviations: PDE: peritoneal dialysis effluent, COL1A1: collagen (α1) I, SKI-2: Sheffield Kidney Institute PD cohort 2, EPS: encapsulating peritoneal sclerosis, and UF: ultrafiltration.

5.5.2.3 Effect of dwell time on intelectin-1, dermatopontin and COL1A1 in the PDE samples in the SKI-2 cohort

A few samples were chosen to measure the levels of intelectin-1, dermatopontin and collagen (α1) I with increasing dwell times. Intelectin-1 and COL1A1 were similar to TIMPs and MMPs in that they progressively increased with dwell times. Dermatopontin was different as longer dwell times did not always cause an increase in the levels of dermatopontin in PDE samples. This may mean that at a certain time point there will be saturation of dermatopontin in PDE samples and therefore no further diffusion of dermatopontin from plasma to PDE samples can occur.

5.5.2.4 Plasma level in healthy volunteers compared to PD patients in SKI-2

To compare if intelectin-1 level is elevated in PD patients compared to healthy individuals, it was hypothesized that changes in intelectin-1 would find their way into the blood thus allowing a direct comparison with healthy volunteers as previously described. The healthy volunteers were lab technicians, scientists, students, lecturers with age ranging from 22 – 49 years old (7 Females and 11 males). Intelectin-1 was lower in healthy volunteers compared to those on PD ($p=0.046$). Intelectin-1 median (IQR) in plasma was 327 (272–426) ng/mL in the healthy group, compared with 508 (261–857) ng/mL in PD patients (Figure 5.6).

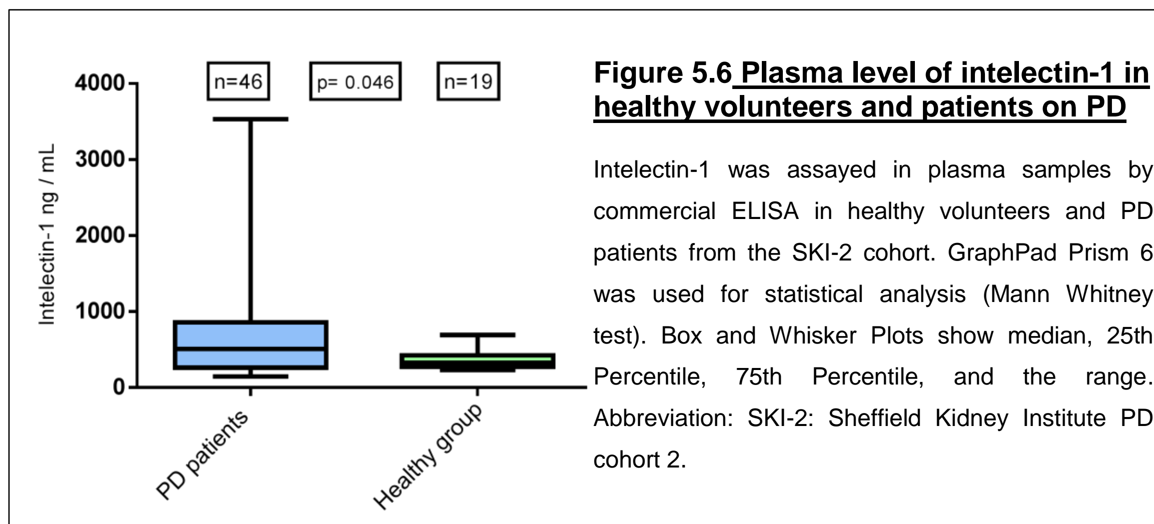


Figure 5.6 Plasma level of intelectin-1 in healthy volunteers and patients on PD

Intelectin-1 was assayed in plasma samples by commercial ELISA in healthy volunteers and PD patients from the SKI-2 cohort. GraphPad Prism 6 was used for statistical analysis (Mann Whitney test). Box and Whisker Plots show median, 25th Percentile, 75th Percentile, and the range. Abbreviation: SKI-2: Sheffield Kidney Institute PD cohort 2.

5.5.2.5 Rate of filtration

Sample volume and assay dilution indicated that Intelectin-1 was the only molecule able to be tested in GFS cohort (section 5.5.3) and as a consequence this was more fully investigated. Using the same protocol as in chapter 4 (section 4.5.3), the Log10 molecular weight of creatinine, β2-microglobulin, albumin, transferrin and IgG were plotted against their Log10 D/P creatinine, β2-microglobulin, albumin, transferrin and IgG at the 2hr dwell time to generate a Mwt/filtration rate (standard curve). After that, Log10 molecular weight of intelectin-1 was plotted against Log10 D/P intelectin-1 at the 2hr dwell point for 46 patients (Figure 5.7), and at less than 1 year on PD, 1-3 years on PD, and more than 1 year on PD (Figure 5.8). Intelectin-1 was almost on the line of the Mwt/filtration rate (standard curve) for a protein of its size. Thus the D/P vs molecular weight plot suggested that intelectin-1 did not have local production in peritoneal cavity. This did not change whether using an average of all 46 PDE samples or grouping by time on PD (less than 1 year on PD, 1-3 years on PD, and more than 1 year on PD).

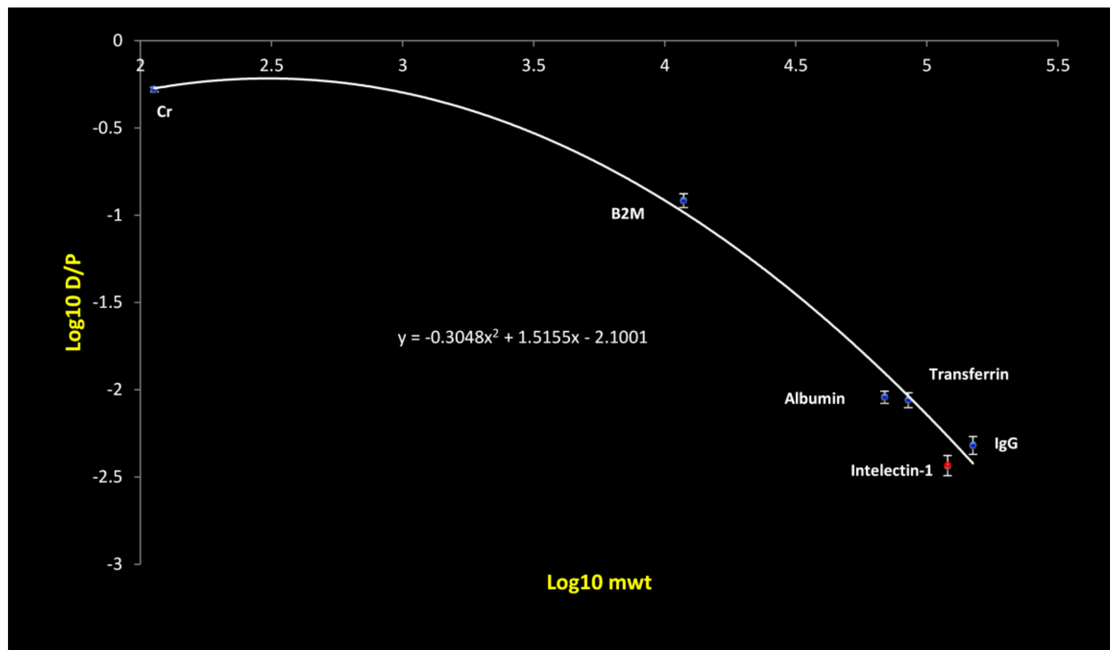
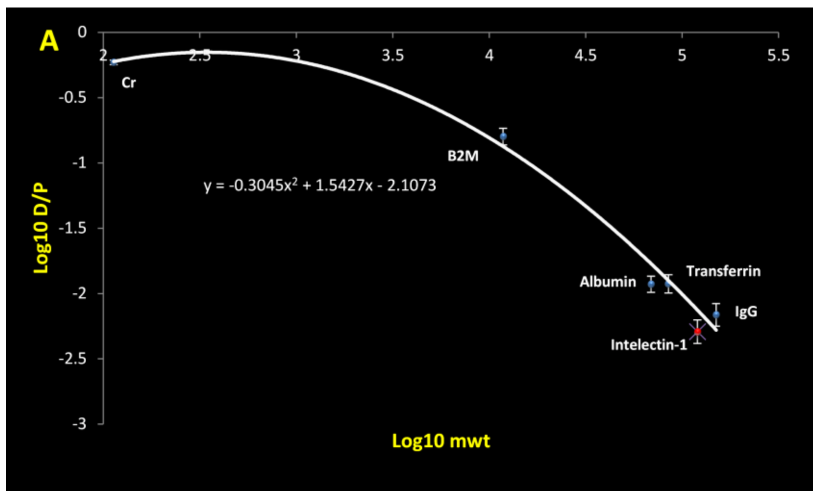
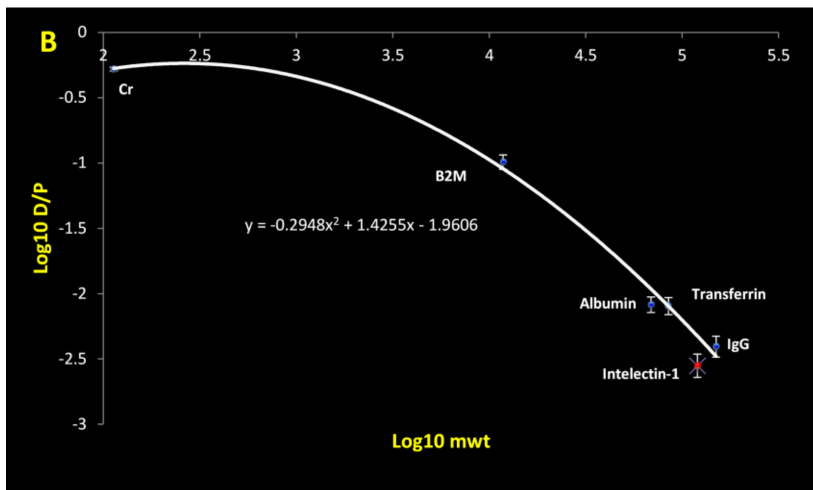


Figure 5.7 Intelectin-1 is not produced locally in the peritoneum (all samples)

A Log10 plot of proteins in dialysate/plasma (D/P) ratio in a 2hr dwell time vs. Log10 of their molecular weight (MW) were plotted to construct a plasma to dialysate transfer rate (standard curve) by using circulating proteins with no peritoneal production i.e. creatinine (MW: 113.1 Da), β2-microglobulin (MW: 11800 Da), albumin (MW: 69000 Da), transferrin (MW: 85000 Da) and IgG (MW: 150000 Da). Log10 of intelectin-1 in D/P ratio at 2hr dwell time for 46 patients were plotted against Log10 of their molecular weight. It demonstrated that intelectin-1 (120000 Da) is not produced in the peritoneum. Data represent Log10 of mean with SEM.





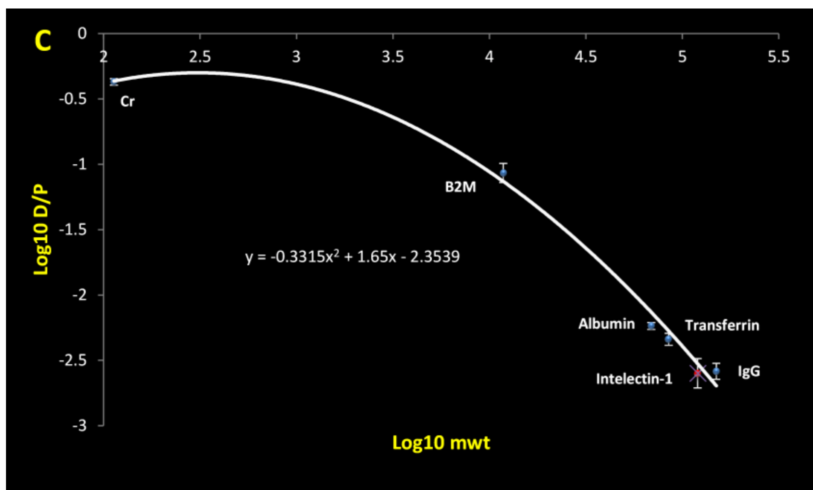


Figure 5.8 <u>Intelectin-1 is not produced in the peritoneum irrespective of time on PD</u>

Patients were grouped by time on PD to less than 1 year (19 patients) (A), 1-3 years on PD (15 patients) (B), and more than 3 years on PD (12 patients) (C). A Log10 plot of proteins in dialysate/plasma (D/P) ratio in a 2hr dwell time vs. Log10 of their molecular weight (MW) were plotted to construct a plasma to dialysate transfer rate (standard curve) by using circulating proteins with no peritoneal production i.e. creatinine (MW: 113.1 Da), β2-microglobulin (MW: 11800 Da), albumin (MW: 69000 Da), transferrin (MW: 85000 Da) and IgG (MW: 150000 Da). Log10 of intelectin-1 in D/P ratio at 2hr dwell time were plotted against Log10 of its molecular weight. It demonstrated that intelectin-1 (120000 Da) is not produced in the peritoneum in A, B, and C. Data represent Log10 of mean with SEM.

5.5.3 ELISA measurement of proteins identified as biomarkers of EPS using proteomics in the Global Fluid Study (GFS) cohort

The decision was made to only measure intelectin-1 in the GFS cohort based on that in the SKI-1 cohort intelectin-1 was the only protein elevated in EPS and that intelectin-1 measurement only requires a few microliters of PDE so it was possible to measure it in the GFS cohort where only limited volume of sample is available. In contrast, dematopontin and COL1A1 required at least 450uL to measure both molecules in PDE samples which was not realistic. Intelectin-1 also had the strongest positive correlation between intelectin-1 in PDE samples with D/Pcr and negative correlation with UF.

5.5.3.1 Intelectin-1 level in the GFS cohort

Individual patients had their intelectin-1 level plotted against time on PD (Figure 5.9). For control patients, intelectin-1 level was plotted for 158 samples taken from 42 patients. In the EPS group, intelectin-1 level was plotted for 43 samples that were taken from 12 patients. From the Spaghetti plots of the intelectin-1 there is only one sample in the EPS group which shows elevated intelectin-1 in PDE (10200 pg/mL) and this is immediately prior to diagnosis. There are a number of samples in the non-EPS control group with high level of intelectin-1 in PDE samples. The samples from control group with high intelectin-1 were associated with D/Pcr of 0.75 or more which may explain why intelectin-1 was high in these PDE samples.

Individual samples grouped by time to EPS diagnosis or stopping PD were calculated for mean and an average value plotted with time. There was no clear significant shift in the level of intelectin-1 between control and EPS diagnosed samples, although there was a late increase in intelectin-1. This late increase in intelectin-1 between control and EPS was due to a huge increase in the intelectin-1 level based on one sample only and this is not truly representative. Median (range) of intelectin-1 in PDE samples of the control and EPS groups

were 1 (0.17-12.5) and 1 (0.12-10.2) ng/mL respectively (Figure 5.10). This range is clearly showing that maximum intelectin-1 was in control group (12.5 ng/mL) and this is the highest in the control.

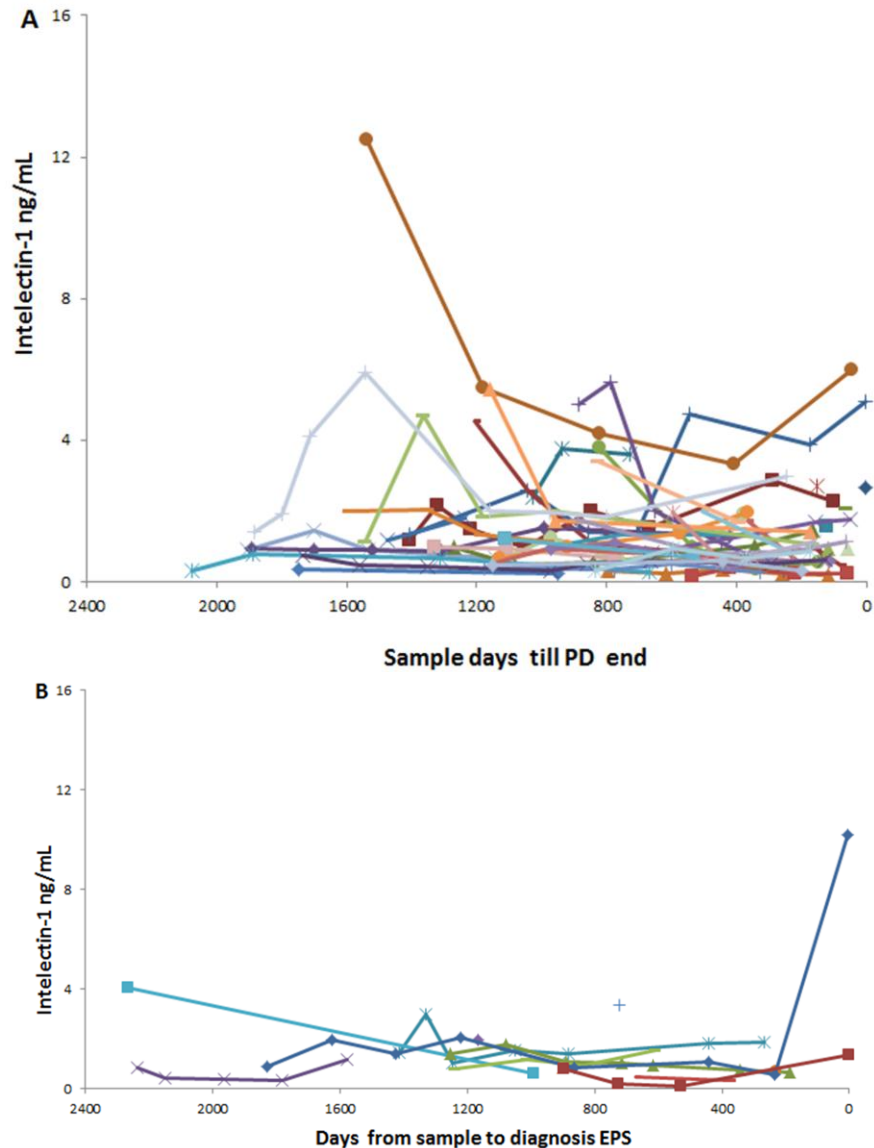


Figure 5.9 Spaghetti plots of intelectin-1 level in PDE samples (control and EPS)

Intelectin-1 was assayed by commercial ELISA in PDE samples from the GFS (control and EPS). After that, (A) Intelectin-1 was plotted against sample days till PD end without developing EPS in the control group and (B) Intelectin-1 was plotted against days from sample to EPS diagnosis in EPS group. Abbreviations: PDE: peritoneal dialysis effluent, GFS: global fluid study, and EPS: encapsulating peritoneal sclerosis.

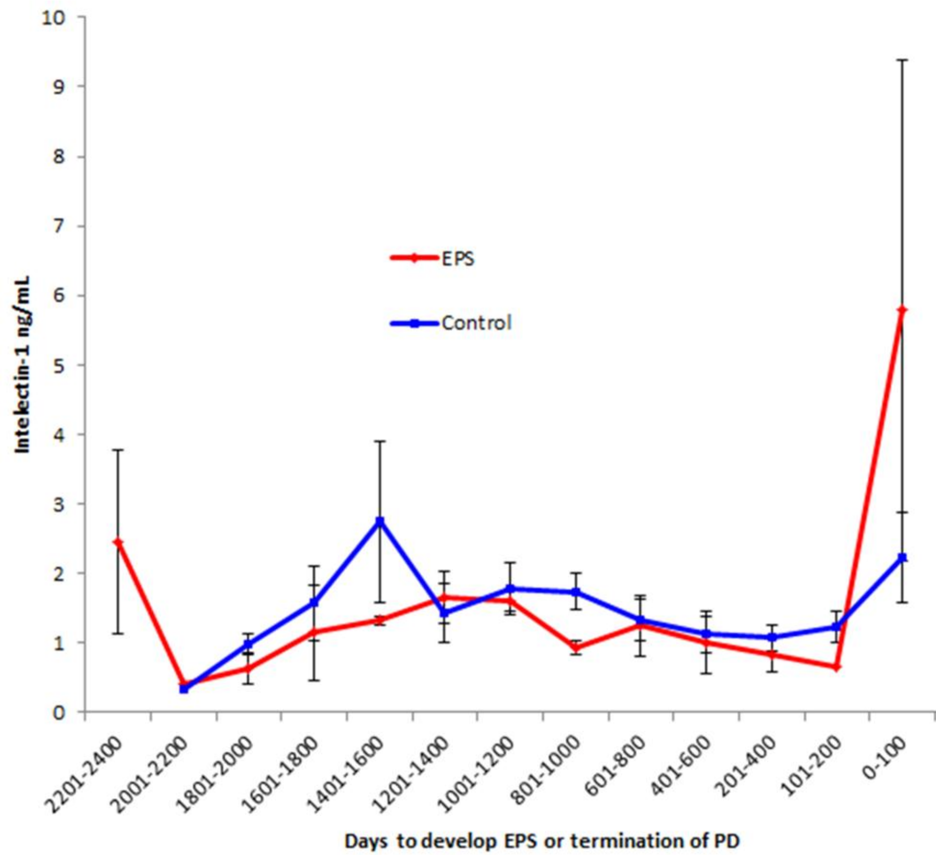


Figure 5.10 <u>Mean intelectin-1 concentrations in control and EPS groups (GFS)</u>

Means intelectin-1 in PDE samples were plotted for every 200 days and every 100 days in the last 200 days for EPS and control groups. There is huge elevation in the intelectin-1 in one sample within 100 days of the EPS diagnosis in EPS group which caused this shift in the last 100 days. Data represents mean with SEM. Abbreviations: GFS: Global Fluid Study, PD: peritoneal dialysis and EPS: encapsulating peritoneal sclerosis.

5.6 Statistical analysis after this study

5.6.1 SKI-2

Power is to allow a reasonable chance for detecting a predetermined difference (effect size) in the outcome variable, at a given level of statistical significance. Sample size is critically dependent on the purpose of the study, the proposed effect size, the outcome measure and how it is summarised, and the method of calculating the statistical tests. It is often taken as a = 0.05 and 1-b = 0.8 (i.e. 5% significance, 80% power). The statistician was approached to analysis the power of the SKI-2 cohort using the program G*power 31.3. The sample size or power required for detecting full and half effect shown in this study for intelectin-1 in SKI-2 was calculated using G*power 31.3 software. Small studies which are significant often tend to overestimate the effect, thus half effect used as a more cautious approach. In SKI-2, sample sizes required to detect full effect of intelectin-1 correlation against D/Pcr, UF, and D/D0 glucose were 22, 35, and 27 PD patients respectively. Sample sizes required to detect half effect of intelectin-1 correlation against D/Pcr, UF, and D/D0 glucose were 106, 155, and 122 samples respectively. In SKI-2, 51 samples were available for these assays.

5.7 Discussion

Previous 2D gel and ITRAQ proteomic analysis of PDE samples identified differences in the proteome between EPS and a non EPS control patients. Three of these proteins; intelectin-1, dermatopontin and COL1A1 were analysed in PDE samples by ELISA. Intelectin-1, DPT and COL1A1 were positively correlated to the D/Pcr and negatively correlated to D/D0 glucose since high transporters have a larger effective on the peritoneal surface area. Intelectin-1 may be a marker of transport status. They were also negatively correlated to the UF. Peritoneal membrane injury and fibrosis are associated with high D/Pcr and UFF (Lambie, John et al. 2010, Sampimon, Coester et al. 2011). Intelectin-1 was subsequently analysed in the GFS cohort which failed to fully validate the proteomic identification of this as a major shifting protein in the EPS proteome, although one late EPS sample did show a dramatic increase in intelectin-1 level. Of note is that while only 1 of the 2 late EPS samples showed an increase in the EPS patient PDE, the single EPS patient in the SKI-1 cohort was also higher than all the other PDE samples analysed from that cohort.

Surprisingly data also suggested that intelectin-1 was not produced locally in the peritoneum given that it is also highly expressed in the small intestine with further expression in the colon in normal individuals (Lee, Schnee et al. 2001, Suzuki, Shin et al. 2001, Tsuji, Uehori et al. 2001, Yang, Lee et al. 2006). It thus seemed logical to assume that this would be a source of intelectin-1 given the “stress” exerted on the gut during PD and especially in EPS. However, the increased levels were seen in 2 of the 3 patients with EPS. This begs the question as to why. There is also high intelectin-1 in PDE samples in some of control patients with high D/Pcr. This high intelectin-1 in PDE samples could be due to different peritoneal surface area, peritoneal membrane permeability, and pressure gradients.

Intelectin-1 was not produced locally and this mean the intelectin-1 level in the peritoneal cavity is crossing the membrane from plasma without local production. The other finding was that the plasma level of intelectin-1 was higher in PD patients than in healthy individuals and this is possibly due to impaired renal clearance of these molecules in PD patients, but it is also may be due to more production of intelectin-1 in these patients.

Intelectin-1 and COL1A1 were similar to TIMPs and MMPs described previously in that their levels increased in PDE samples with longer dwell times, but in dermatopontin, the longer dwell times were not always associated with higher dermatopontin concentrations in PDE samples. This is may be due to saturation of dermatopontin transport after certain time point.

A few samples from control group also showed sporadic high intelectin-1 in the PDE samples. The ELISA kit assays in all chapters were chosen based on the sequences the antibodies recognized or were generated against. The reason for this is because it was unclear from the proteomics if fragments (possibly post degradadtion) were being detected or the full protein. As such, the sequences identified by MS within each protein had to be within the target area of antibodies or between identified sequences. In some cases such as colα1(I) it was clear this was a fragment as the spot on the 2D gels was 50kDa compared to 139 kDa of the mature protein so a kit recognizing that fragment was essential. For intelectin-1 and dermatopontin the spots were nearer to the proteins normal molecular size but due to the resolution of 2D gels it was not conclusive that the full protein was present. In selecting ELISA a second criteria was sensitivity and specificity were always checked before purchasing the items. For example, the minimum detectable values using the commercially available ELISA kits for dermatopntin and COL1A1 were less than 52 and 115 pg/mL respectively compared to the superior 3.1pg/mL for intelectin-1. This allowed intelectin-1 to be assayed in GFS samples using a few microliters of PDE samples whereas the

other two required a larger volume of PDE samples (more than 200 μL for each) which was unavailable in the GFS cohort.

Inter and intra assay variation was established by placing the same samples on every assay plate and at different places within each plate. Values should not exceed 10% of the expected measurements and if they did the assays was repeated. Further as different PDE would have different glucose concentrations then a range of PD fluids were tested to verify high or low glucose did not alter the assay response.

In summary, changes in the intelectin-1, dermatopontin and COL1A1 in the peritoneum correlated well with higher transport rates and negatively with UF. Intelectin-1 was found to be not produced in the peritoneum. One EPS sample from SKI-1 (after diagnosis with EPS) and one out of two patients in the last 100 days before diagnosis with EPS were associated with elevated level of intelectin-1. OF note, sporadic samples in control group of GFS showed high in the intelectin-1 level in PDE samples, but all these patients had a D/Pcr of 0.75 or more.

CHAPTER SIX

Changes in the cells found in peritoneal dialysis effluent with time on dialysis

6.1 Introduction

It had been shown that long-term PD causes changes in the peritoneum morphology such as peritoneal mesothelial cell degeneration and increased fibroblasts, and these may cause defects in the peritoneal membrane function (Verger, Brunschvicg et al. 1981, Gotloib, Shostack et al. 1987). There are also changes in other peritoneal cells such as peritoneal macrophages and leukocytes as shown in (section 6.1.1) and these could influence the development of peritoneal fibrosis. Fibroblasts and Macrophages are able to secrete various ECM regulatory molecules such as TIMPs and MMPs which are able to change ECM homeostasis (Figure 3.1).

It is well-known that lymphocytes, polymorphonuclear neutrophils, macrophages and mesothelial cells are the main cell types in the peritoneal cavity, but fibroblasts and myofibroblasts are also important peritoneal cells that are highly influential on ECM levels. Proliferation of fibroblasts (Wynn 2007) or cellular differentiation such as epithelial to mesenchymal transition (EMT) are observed in CAPD patients with PS (Aroeira, Aguilera et al. 2007).

6.1.1 Leukocytes and macrophages in the peritoneal dialysis effluent

Lymphocytes and macrophages are two important elements of the host immune system, typically residing in less than 50 mL of fluid in the peritoneal cavity. Lymphocyte and macrophage levels change in PD patients. In healthy individuals, peritoneal macrophages represent about 90% of the cells, while peritoneal lymphocytes and polymorphonuclear neutrophils represent about 5% - 10%, and less than 5% respectively. The percentages of cell types found in the uninfected PDE of PD patients are: 23-86% macrophages, 0-27% polymorphonuclear neurtrophils 5-77% lymphocytes (Lewis and Holmes 1991, Brulez and Verbrugh 1995, Kazancioglu 2009). This wide range of percentages obtained from various studies reflects the large variation in published values. For

example, peritoneal macrophages account for 86% of cells in one paper (Goldstein, Bomalaski et al. 1984), while another paper showed that peritoneal macrophages represent 45% of the total cells (Cichocki, Hanicki et al. 1983). Light microscopy was used in both cases. The same variation was evident when flow cytometry was used (Brulez and Verbrugh 1995).

The macrophage is one of the most abundant cells in PDE samples, but the regular removal of cells during PD considerably weakens the capability of the peritoneal defense mechanism. In addition to that, peritoneal macrophages in CAPD patients are relatively immature cells due to rapid turnover and therefore, many macrophage functions may be impaired (Li 1999). In contrast, peritoneal lymphocytes increase in PD patients and they show signs of activation : T lymphocytes are decreased, whereas B lymphocytes are increased as compared to the peripheral blood of PD patients (Lewis, Kutvirt et al. 1993)

6.1.2 Mesothelial cells in the peritoneal dialysis effluent

Mesothelial cells (MCs) are mononuclear cells that are characterized by their polygonal shape and flattened surface. They form a continuous superficial layer of squamous epithelium that lines the peritoneal cavity. MCs have many crucial functions such as the prevention of peritoneal fibrous adhesion formation through fibrinolytic properties. They also synthesise and secrete phosphotidylcholine and phospholipids which lubricate the peritoneal surface to facilitate abdominal visceral movement (Di Paolo, Sacchi et al. 2005). In addition to that, they are involved in modulating the microcirculation of the peritoneum through the secretion of vasodilators such as nitric oxide and prostaglandin E2, as well as vasoconstrictors such as endothelin (Nagy 1996). Numerous microvilli are present on the luminal surface of MCs. These microvilli increase the surface area of peritoneal membrane and play an important role in prevention of infection through entrapment of bacteria (Yung and Chan 2012). As a consequence of time on peritoneal dialysis, mesothelial cells undergo a process called EMT

(section 6.1.4), during which peritoneal MCs lose some of their epithelial characteristics in response to certain pathological factors such as low pH, inflammatory mediators, high glucose concentrations in dialysate and exposure to glucose degradation products. The end result of EMT is that peritoneal MCs develop a fibroblast-like phenotype (Aguilera, Loureiro et al. 2013).

6.1.3 Fibroblasts in the peritoneal dialysis effluent

Fibroblasts are spindle shaped cells. They are characterised by contractility and motility. They have different origins such as regional fibroblast proliferation, fibrocytes of bone marrow and from local epithelium through the process of EMT (section 6.1.4) (Wynn 2008). Peritoneal fibroblasts are sporadically scattered within the interstitium of the submesothelial area, and have a role in inflammation, adhesion and peritoneal fibrosis (Witowski and Jörres 2006). Fibroblasts express a protein called fibroblast specific protein (FSP-1). This protein is used commonly as an indicator for identifying cells which have undergone EMT. FSP-1 is also called S100A4. It is a member of S100 family and it is a 10-kDa calcium-binding protein. FSP-1 is involved in interactions of cytoskeletal membrane, transduction of calcium signal, cell growth and differentiation (Zimmer, Cornwall et al. 1995, Barraclough 1998, Österreicher, Penz-Österreicher et al. 2011). Myofibroblasts are activated fibroblasts. They express smooth muscle actin (α-SMA). They are contractile cells with multiple functions including synthesis of cytokines, growth factors and ECM. They also participate in the inflammatory reaction (Powell, Mifflin et al. 1999).

6.1.4 Epithelial to mesenchymal transition (EMT)

EMT is the process of transition of epithelial into mesenchymal cells. The process begins with the dissociation of intercellular junctions and loss of microvilli followed by loss of apical-basal polarity of MCs. The cells acquire a more fibroblast-like phenotype including enhanced ECM protease production leading to

basement membrane degradation and thus enhanced motility (Aroeira, Aguilera et al. 2007). EMT of mesothelial cells (MCs) has been demonstrated both in vivo and ex vivo (Yanez-Mo, Lara-Pezzi et al. 2003). Cultures of confluent MCs from PD effluents demonstrate epithelioid and nonepithelioid (similar to fibroblast) morphologies. The prevalence of nonepithelioid cells is proportionally correlated with time on PD and episodes of peritonitis or hemoperitoneum (Yanez-Mo, Lara-Pezzi et al. 2003).

Immunohistochemical analysis of peritoneal membrane from PD patients taken from parietal peritoneal biopsies showed the existence of fibroblast-like cells and α- smooth muscle actin (α-SMA) expression in the fibrotic stroma (Jimenez-Heffernan, Aguilera et al. 2004). Myofibroblastic cells may arise from conversion of MC through EMT during PD (Margetts, Bonniaud et al. 2005). Transdifferentiated MC may play a crucial role in the beginning of fibrosis and subsequent decline of peritoneal function (Aroeira, Aguilera et al. 2007). Fibroblasts and peritoneal mesothelial cells play important roles in production of collagen which is often related to over expression of growth factors and cytokines (Miyazaki and Yuzawa 2005). EMT acts as trigger for peritoneal fibrosis through up-regulation of TGF-β and angiogenesis through vascular endothelial growth factor (VEGF). EMT or mesothelial to mesenchymal transition (MMT) is regulated in part by the TGF-β (Figure 6.1) (Aguilera, Loureiro et al. 2013). Recently, several authors suggested to rename the mesenchymal conversion of MCs to a more suitable term and this is mesothelial to mesenchymal transition (López-Cabrera 2014). EMT has been associated with beginning of peritoneal fibrosis. Blocking of EMT may act as a therapeutic target specially in early stages of the peritoneal fibrosis (Loureiro, Gónzalez-Mateo et al. 2013).

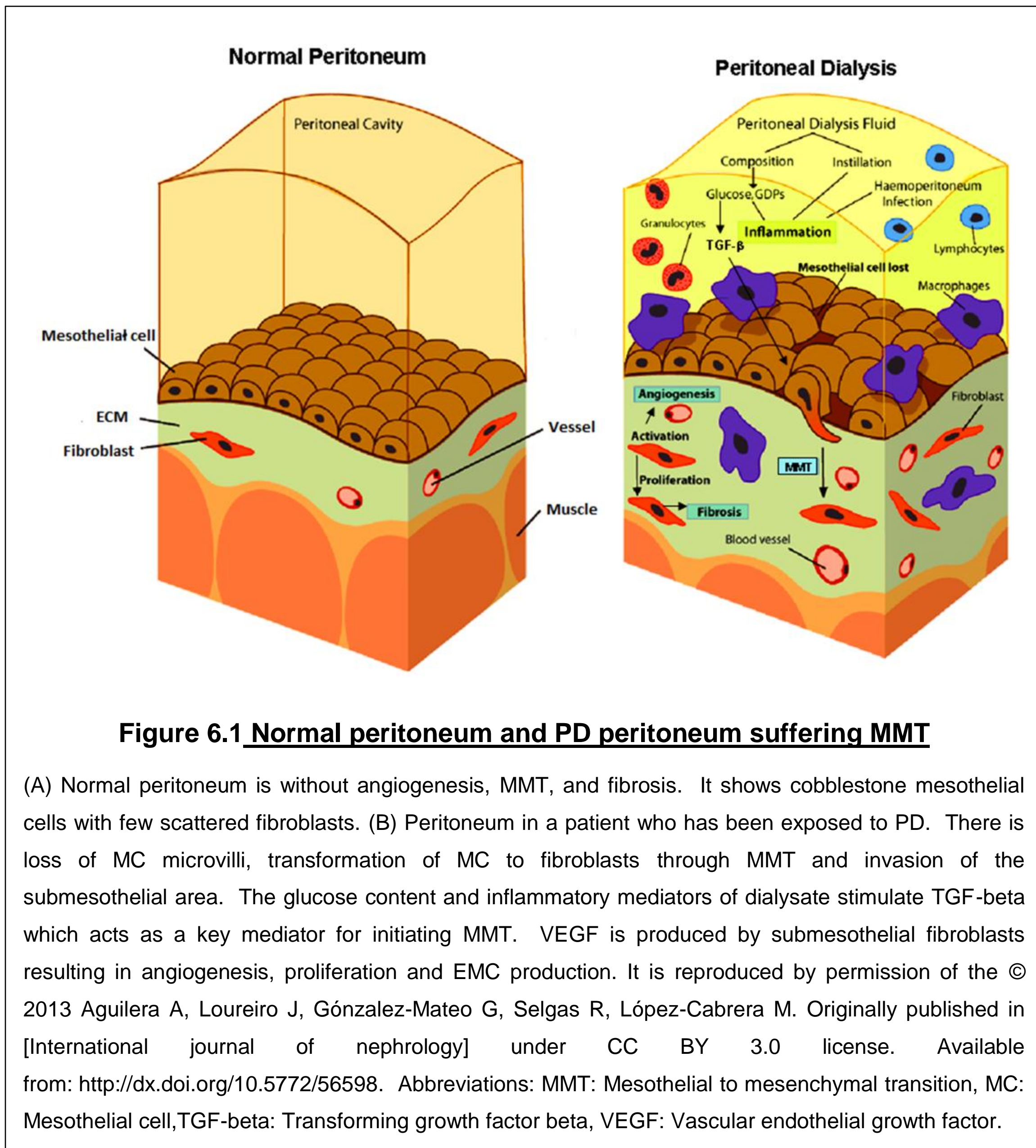


Figure 6.1 <u>Normal peritoneum and PD peritoneum suffering MMT</u>

(A) Normal peritoneum is without angiogenesis, MMT, and fibrosis. It shows cobblestone mesothelial cells with few scattered fibroblasts. (B) Peritoneum in a patient who has been exposed to PD. There is loss of MC microvilli, transformation of MC to fibroblasts through MMT and invasion of the submesothelial area. The glucose content and inflammatory mediators of dialysate stimulate TGF-beta which acts as a key mediator for initiating MMT. VEGF is produced by submesothelial fibroblasts resulting in angiogenesis, proliferation and EMC production. It is reproduced by permission of the © 2013 Aguilera A, Loureiro J, Gónzalez-Mateo G, Selgas R, López-Cabrera M. Originally published in [International journal of nephrology] under CC BY 3.0 license. Available from: http://dx.doi.org/10.5772/56598. Abbreviations: MMT: Mesothelial to mesenchymal transition, MC: Mesothelial cell,TGF-beta: Transforming growth factor beta, VEGF: Vascular endothelial growth factor.

6.2 Hypothesis

There are various cell types known to be present in peritoneal dialysis effluents (PDE) including macrophages, lymphocytes, fibroblasts, and mesothelial cells. It is likely that these will change with time on PD due to the constant stress that PD fluids place on the peritoneum. Given the varying and differing effects of each of these cell types on ECM homeostasis it is probable

that the balance of the cells found in the PDE could have a profound effect on the composition and quantity of the peritoneal membrane. This has led to the proposal of the hypothesis that the cell types found in PDE alter with time on PD. This change in cells alters ECM composition, membrane function and the development of PS or EPS.

6.3 Aims

To test this hypothesis

1) Cells will be isolated from overnight PD bag
2) Cells will be stained for cell markers of leukocytes, macrophages, fibroblasts and mesothelial cells.
3) Cell populations will be quantified by cell counting, comparing each of the peritoneal cells in 3 groups based on duration of PD (<1 year, 1-3 years, and >3 years).
4) Changes in cells will be related back to levels of ECM regulatory molecules and activities described in previous chapters

6.4 Methods

The method is illustrated in detail in chapter 2. The Processes of PD slide preparation for cell staining is described in (section 2.3). The slides were prepared from overnight bags (The dwell times should be standardized during the assessment) in PD patients. There were three groups according to exposure to PD (<1 year, 1-3 years, and >3 years) and all samples were from SKI-2 cohort. After that, cell staining was performed for leukocytes, macrophages, fibroblasts and mesothelial cells and then, cell count for the positive percentage of each peritoneal cell (section 2.8.4). Finally, these data were analysed to:

- Determine the relationship between cellular changes and time on PD.

- Determine the relationships between the dialysate glucose exposure during the year prior to sampling and cellular changes.

Slides of normal rat kidney 49F (NRK-49F) and human kidney 2 (HK-2) cells were kindly donated by Linghong Huang. Slides of human macrophage preparation from peripheral blood were kindly donated by Fiona J Wright.

6.5 Results

Macrophages, leukocytes, fibroblasts and mesothelial cells were stained for using cytospins slides obtained from overnight bags of 21 patients in the SKI-2 cohort Staining specificity was verified by replacing the primary antibody by either non-immune IgG or IgM. Positive or negative controls were generated by taking slides of normal rat kidney fibroblasts (NRK-49F), epithelial cells (HK-2) cells, and cytospins of fresh human macrophages prepared from peripheral blood.

6.5.1 Peritoneal macrophage staining

Slides of human macrophage preparations from peripheral blood were used as positive controls. These were stained with monoclonal mouse Anti-Human CD68 Clone PG-M1 as the primary antibody, showing a strong clean staining demonstrating the protocol and antibody worked (Figure 6.2.A). This protocol gave equally strong staining in some cells in a cytopsin taken from patient SKI-145 (figure 6.2 C). A cytospin of human macrophages (Figure 6.2.B) as well as a slide from patient SKI-145 (Figure 6.2.D) were used as controls for non-specific staining from the secondary antibody or endogenous peroxidase activity by replacing the primary antibody with Mouse IgG, vector laboratories inc., Burlingame, 1mg/mL (There was no staining visible in either case). To verify there was no cross reactivity with non-macrophage cell types, a slide of HK-2 cells was also stained Monoclonal Mouse Anti-Human CD68 Clone PG-M1 (Figure 6.2.E) again giving no staining.

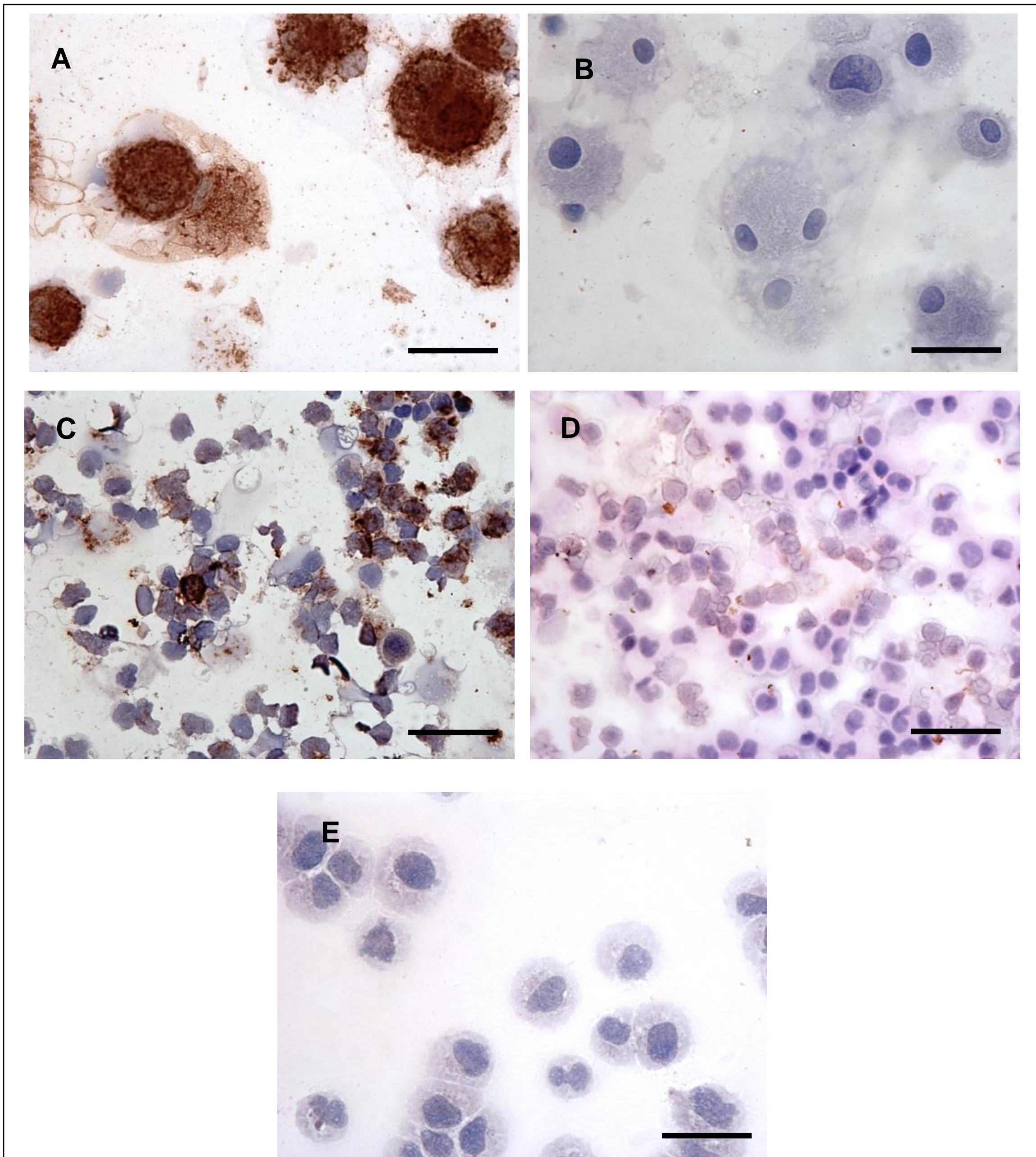


Figure 6.2 <u>Control staining for peritoneal macrophages</u>

A) Peripheral blood macrophages slide stained with Monoclonal Mouse Anti-Human CD68 Clone PG-M1 as the primary antibody (control positive). B) Peripheral blood macrophages slide stained with mouse IgG Antibody was used instead of CD68 as a control negative. C) Cells obtained from an overnight PD bag of SKI 145 were cytospun and stained with CD68 showing positive staining in some cells. D) Slide of SKI 145 was also stained with mouse IgG antibody which shows no staining. E) HK-2 epithelial cellcytospin stained with CD68 (negative control). All images were captured on an Olympus BX61 microscope with a CC-12 FW colour digital camera. Scale bar = 20μm (X400).

For analysis, patient cytospins were captured at three different magnifications (100 µm for x100, 50 µm for x200, 20µm for x400) (Figure 6.3). Cells stained positively were defined better at 400X magnification due to the high cell density on some sections and therefore this magnification was used not only for counting macrophages, but also for counting all other cells. Fifteen images were taken for each slide to calculate the percentage of macrophages on each slide of the twenty one patients. Positive peritoneal macrophages are distinguished by the brown colour in the cytoplasm. The optimal dilution for staining peritoneal macrophage was 1:100.

SKI 35 and SKI 141 are two examples of low and high macrophage levels (Figure 6.4). SKI 35 (Figure 6.4.A) was on PD for more than three years and it shows few macrophages in the slide, while SKI 141 (Figure 6.4.B) was approximately one and half year on PD, in which the percentage of macrophage was much higher

Twenty one slides from the SKI-2 patient cohort were divided into 3 groups based on time on PD as previously (<1 year, 1-3 years, and >3 years). All slides were prepared from overnight bags of PDE. There was a large range in the percentage of peritoneal macrophages counted ranging from 5%-61%, however, the percentage of macrophages showed a decline as a percentage of the total peritoneal cell count with time on PD. (Mean±SD) 37%±11% in patient on PD for less than 1 year, to, 29%±8% for those on PD for 1 to 3 years with a dramatic fall in those on PD for longer than 3 years 11%±5% (Figure 6.5).

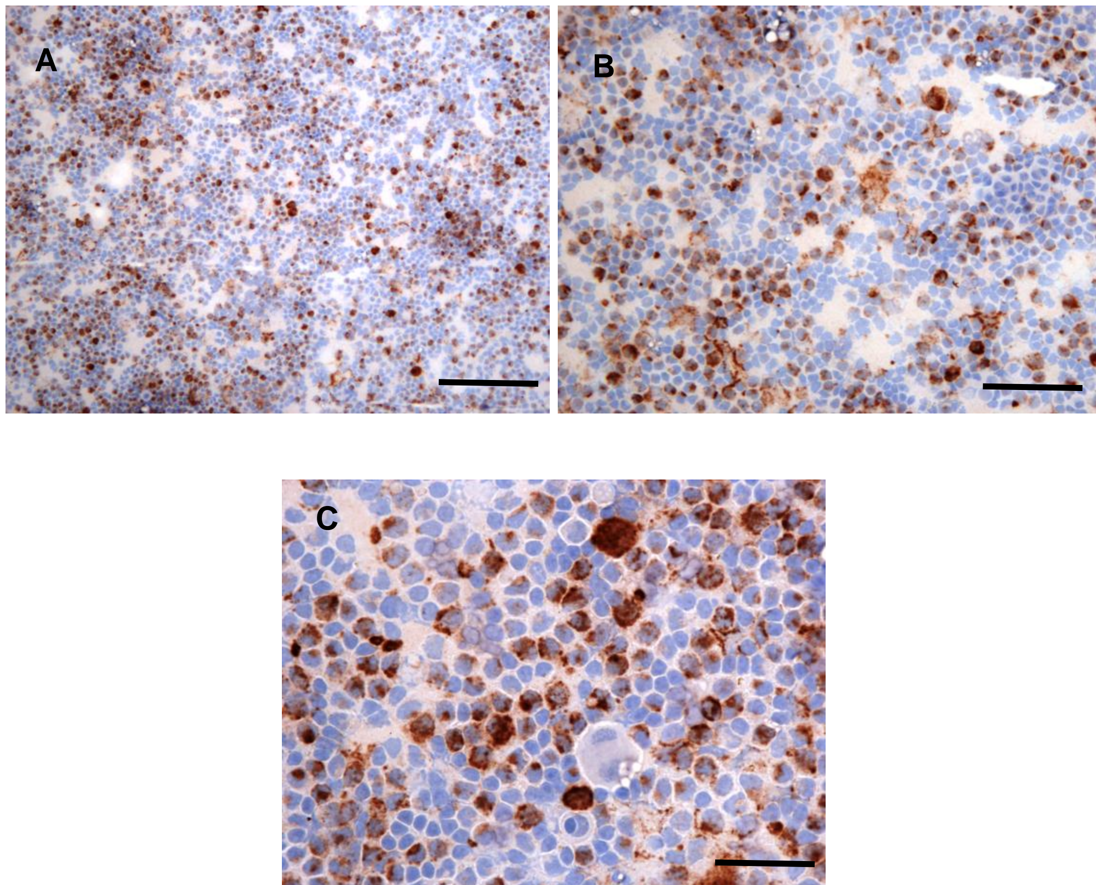


Figure 6.3 **peritoneal macrophage cell staining in three different magnifications**

8 cytospins were generated from the total cells isolated from an overnight PD bag by centrifugation. Monoclonal Mouse Anti-Human CD68 Clone PG-M1 is used as primary antibody for staining at a dilution of 1:100. Images were captured on a Olympus BX61 microscope using a CC-12 FW colour digital camera Stained peritoneal macrophage on slides generated from patient slide of SKI 123 at (A) x100, scale bar = 100µm. (B) x 200, scale bar= 50µm. and (C) x 400, scale bar= 20µm.

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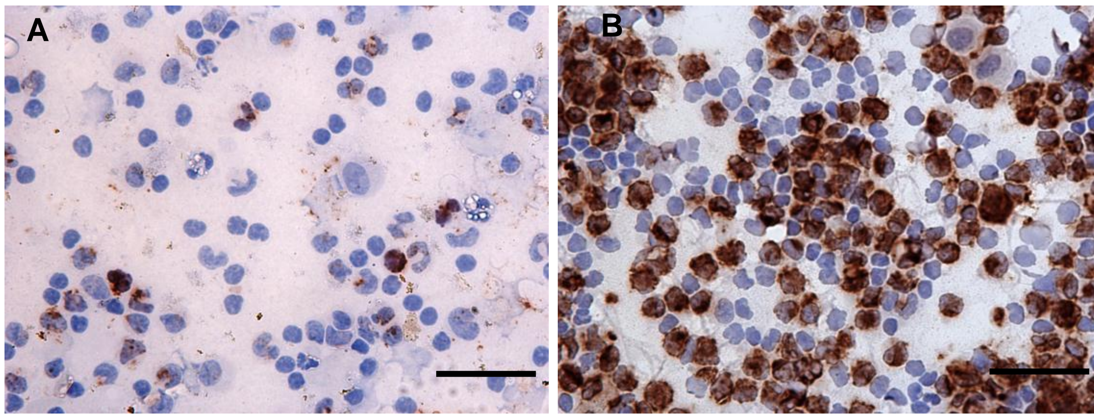

Figure 6.4 High and low levels of peritoneal macrophages in cytospins from PDE bags of PD patients

Cytospins from overnight bags collected from patients SKI-35 and SKI-141 were stained with Monoclonal Mouse Anti-Human CD68 Clone PG-M1. Images were captured on a Olympus BX61 microscope with a CC-12 FW colour digital camera. Macrophages stained brown in the cytoplasm. Magnification is x 400, scale bar = 20µm. (A) SKI 35 was on PD for more than three years. (B) SKI 141 was on PD for one and a half years.

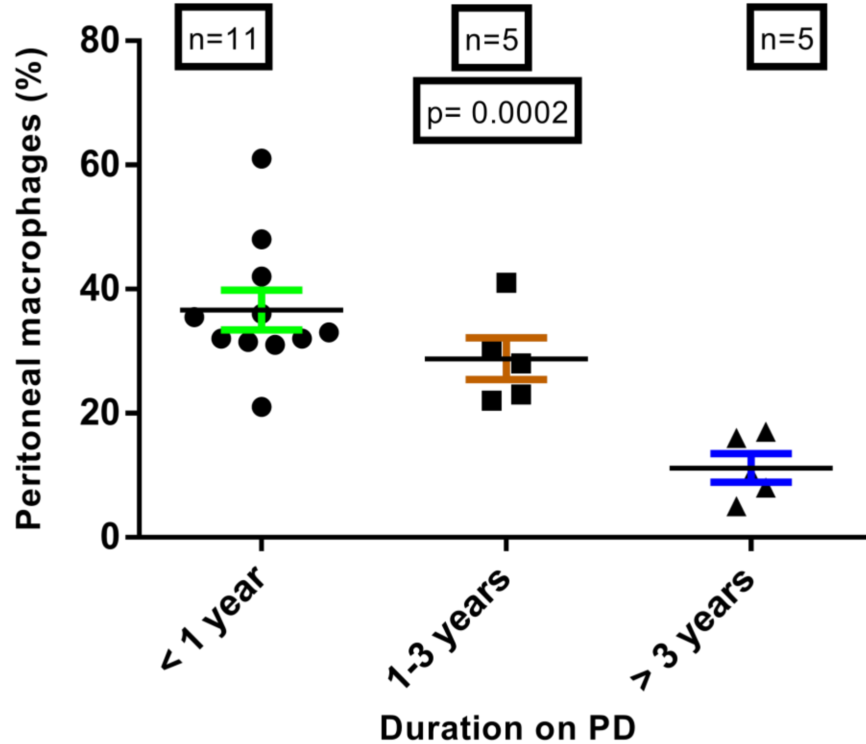

Figure 6.5 <u>Quantification of changes in peritoneal macrophages with time on peritoneal dialysis</u>

Peritoneal macrophages were quantified in 21 PDE samples in the SKI-2 patient cohort by counting the total number of cells on the cytospin and calculating the percentage of CD68 positive cells. PD patients were subsequently divided into 3 groups based on time on PD (<1 year, 1-3 years, and >3 years). The Peritoneal macrophages decreased as the duration on PD increased. Statistical analysis was done by using One-way ANOVA. Post-hoc comparisons using the Tukey's multiple comparisons test indicated that >3 years was significantly different from <1year (p=0.0002). >3 years was significantly different from 1-3 years (p=0.016).1-3 years did not differ significantly from <1year. Data represent mean with SEM. Abbreviations: PD: peritoneal dialysis. PDE: peritoneal dialysis effluent.

6.5.2 Peritoneal leukocytes staining

Fifteen images were taken in 20µm (x400) for each slide to calculate mean of leukocytes percentage in each slide of the twenty one patients. Human leukocytes are stained with Monoclonal Mouse Anti-Human CD45 Leukocyte Common Antigen (Clone 2B11 + PD7/26) as primary antibody. Positive peritoneal leukocytes are distinguished by their brown colour cell membrane. The best dilution for staining peritoneal leukocytes was 1:50.

To verify the staining protocols slides from an overnight PD bag from patient SKI 129 were used. Monoclonal Mouse Anti-Human CD45 Leukocyte Common Antigen (Clone 2B11 + PD7/26) was used to stain the cytospin giving a strong staining in a proportion of the cells and no staining in others when used at a 1:50 dilution(Figure 6.6.A). The primary antibody was then replaced by mouse IgG, vector laboratories inc., Burlingame, 1mg/mL which showed no staining at all (Figure 6.6.B) confirming no endogenous peroxidase activity of non-specific binding of the secondary antibody. Finally cytospins of normal rat kidney fibroblasts (NRK-49F) (Figure 6.6.C) and human kidney proximal tubular epithelial cells (HK-2) (Figure 6.6.D) were used as control negative to validate cell specificity of the Anti-Human CD45 Leukocyte Common Antigen (Clone 2B11 + PD7/26) antibody. Both cells slides were stained negatively.

Cytospins from patient SKI 124 and SKI 137 are examples of low and high leukocyte staining in the effluent respectively (Figure 6.7). SKI 124 (Figure 6.7.A) was on PD for approximately four months, while SKI 137 (Figure 6.7.B) was on PD for more than two and half years in which the percentage of leukocytes stained was much higher.

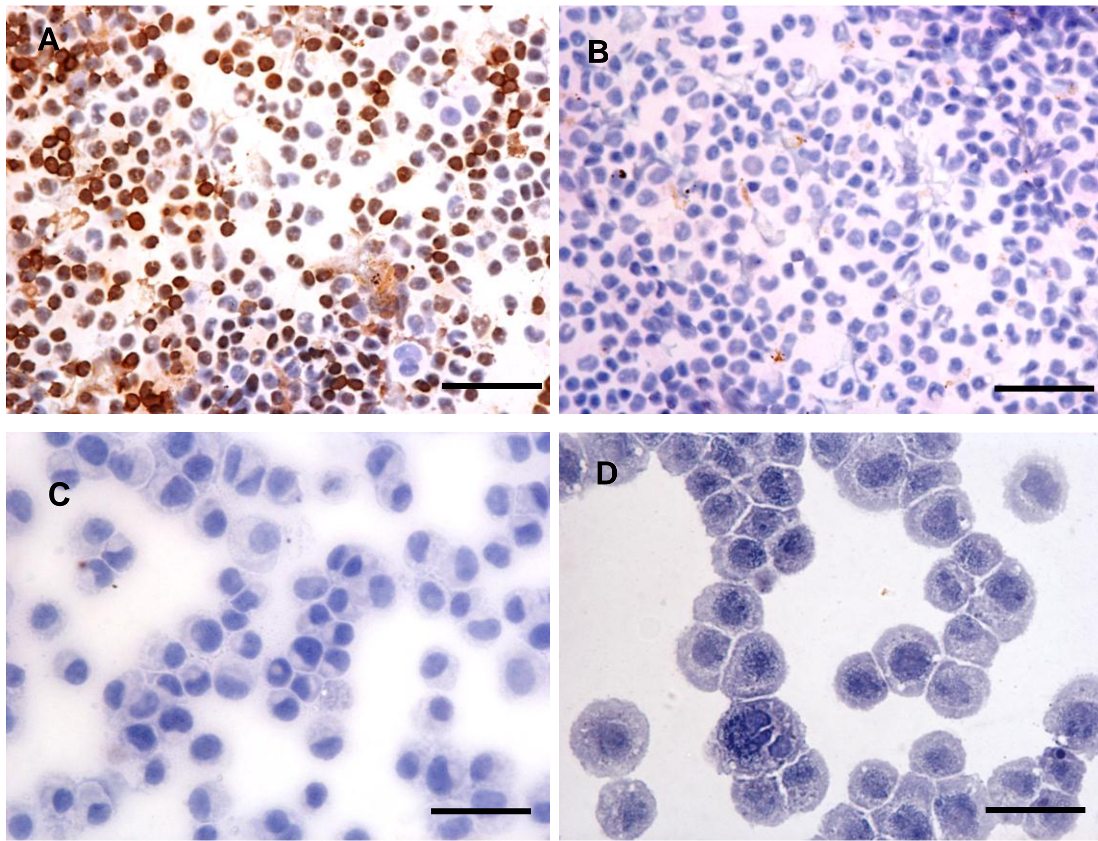

Figure 6.6 Control for peritoneal leukocytes

A) Overnight PD bag from patient SKI 129 was cytospun & slide stained for CD45 showing positive staining in a large number of cells. B) Slide of SKI 129 was also stained with mouse IgG antibody which shows no staining. C) NRK-49F fibroblasts cells and D) HK-2 proximal tubular epithelial cells were cytospun and stained with CD45 antibody and again no staining was evident demonstrating the specificity of the CD45 protocol to leukocytes. Images were captured on an Olympus BX61 microscope with a CC-12 FW colour digital camera. Scale bar = 20μm (X400).

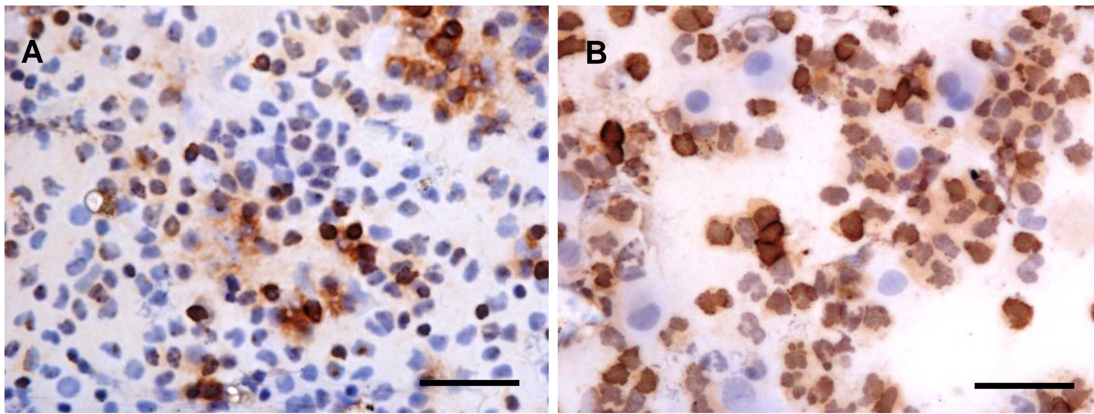

Figure 6.7 <u>High and low levels of peritoneal leukocytes in cytospins from PDE bags of PD patients</u>

Cytospins were prepared from overnight PD bags of patients SKI-124 and SKI 137 and stained with Monoclonal Mouse Anti-Human CD45 Leukocyte Common Antigen (Clone 2B11 + PD7/26) at 1:50 dilution. Images were captured by Olympus BX61 microscope with a CC-12 FW colour digital camera at x 400 magnification, scale bar = 20µm. Leukocytes appear brown colour cell membrane. A) Lower leukocyte staining was seen for SKI 124 that had been on PD for four months. Compared to B) Higher peritoneal staining in SKI 137 who had been on PD for two and half years.

Changes in leukocytes with time on PD were calculated based on time on PD as previously described (Figure 6.8). Peritoneal leukocytes were 39%±11% (mean ± SD) within the first year of starting PD, but this consistently increased with time on PD increasing to , 41%±5%, between 1 and 3 years on PD and reaching 58%±8% in those on PD for more than 3 years. Overall the range of peritoneal leukocytes were between 21% and 69%.

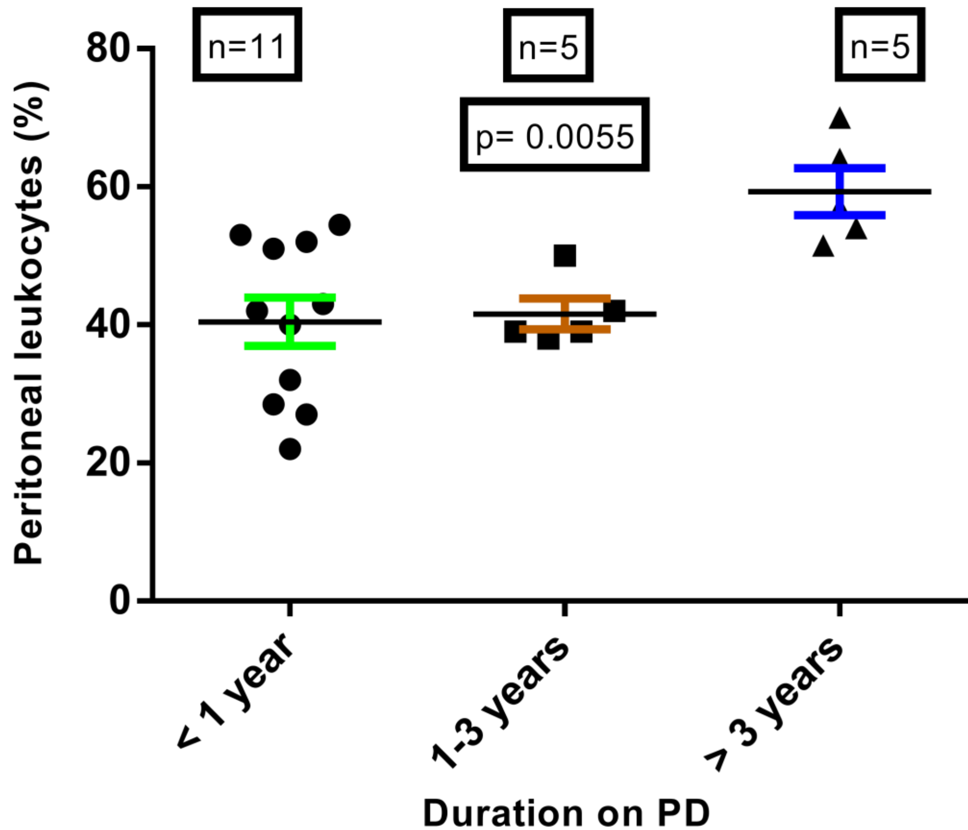

Figure 6.8 Changes in peritoneal leukocytes during peritoneal dialysis

Peritoneal leukocytes were quantified in all 21 PDE samples from the SKI-2 cohort. PD patients were divided into 3 groups based on time on PD (<1 year, 1-3 years, and >3 years) and the percentage of positive stained cells calculated. The Peritoneal leukocytes increased as the duration on PD increased. Statistical analysis was done by using One-way ANOVA. Post-hoc comparisons using the Tukey's multiple comparisons test indicated that >3 years was significantly different from <1year (p=0.005). >3 years was significantly different from 1-3 years (p=0.02).1-3 years did not differ significantly from <1year. Data represent mean with SEM. Abbreviations: PD: peritoneal dialysis. PDE: peritoneal dialysis effluent.

6.5.3 Peritoneal mesothelial cells staining

Staining for mesothelail cells was validated initially using cytospins from an overnight PD bag collected from Patient SKI 35. Using Monoclonal Mouse Anti-Human Mesothelial Cell Clone HBME-1 as the primary antibody at a dilution of 1:75, one cytospin showed strong and specific staining in discrete cells (Figure 6.9.A). Conversly when this primary antibody was replaced by an Anti-mouse IgM (μ-chain specific) Biotin Conjugate no staining was seen (Figure 6.9.B) confirming no secondary antibody non-specific binding or endogenous peroxidase activity. Normal rat kidney fibroblasts (NRK-49F) (Figure 6.9.C) and human kidney proximal tubular epithelial cells (HK-2) (Figure 6.9.D) both failed to stain with the HBME-1 antibody supporting mesothelial cell specificity

Fifteen images were taken in 20μm (at x400 magnification for each slide in the SKI-2 patient cohort to calculate mean percentage of mesothelial cells on each slide of the twenty one patients. Positive peritoneal mesothelial cells are distinguished by brown colour of cytoplasm and cell membrane.

Peritoneal mesothelial cells represented a much lower percentage of the total cell population than the other cell types examined (Figure 6.10) with counts in the region of 1-5 % of the peritoneal effluent cells. Changes in mesothelial cells with time on PD were studied in the SKI-2 cohort as described previously (Figure 6.11). Mesothelial cells showed no significant change in between the three groups being (Mean±SD) 1.8%±1.2%, 1.6%±0.5%, and 1.6%±0.5% at <1 year, 1-3 years, and >3 years respectively.

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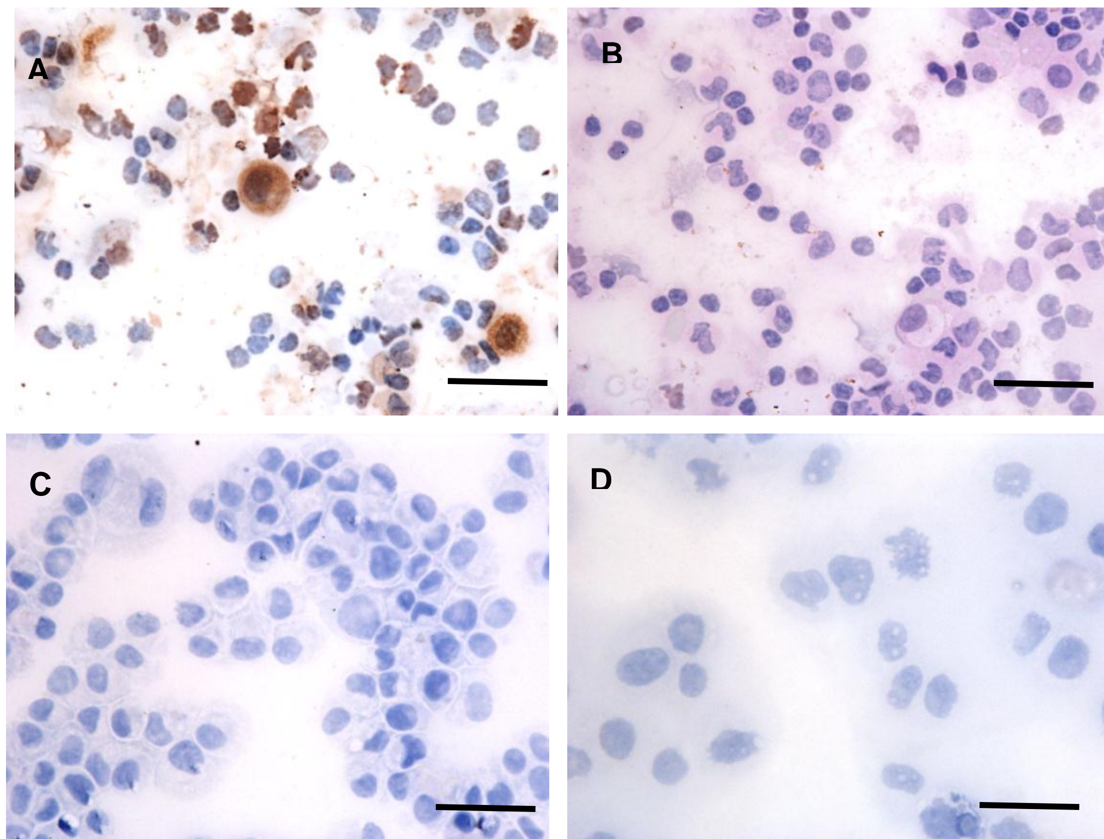

Figure 6.9 <u>Control staining for peritoneal mesothelial cells</u>

A) Overnight PD bag from patient SKI 35 was cytospun & slide stained for Monoclonal Mouse Anti-Human Mesothelial Cell Clone HBME showing positive staining cells. B) Slide of SKI 35 was also stained with Anti-mouse IgM Antibody which shows no staining. C) NRK-49F fibroblasts cells and D) HK-2 proximal tubular epithelial cells were cytospun and stained with Monoclonal Mouse Anti-Human Mesothelial Cell Clone HBME-1 and again no staining was evident demonstrating the specificity of the Monoclonal Mouse Anti-Human Mesothelial Cell Clone HBME protocol to mesothelial Cell. Images were captured on a Olympus BX61 microscope with a CC-12 FW colour digital camera at x 400 magnification with scale bar = 20µm.

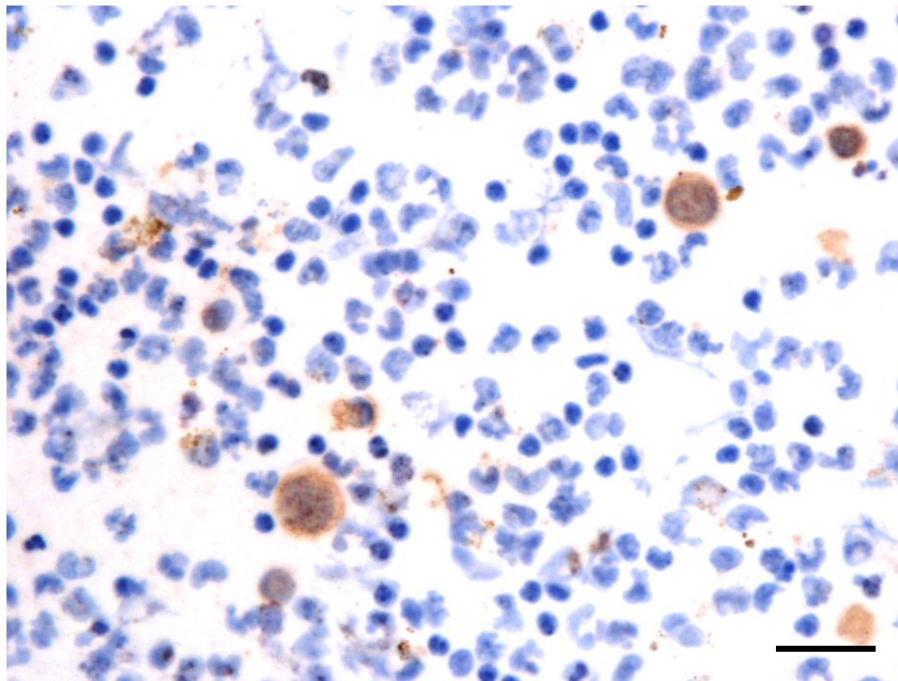

Figure 6.10 Peritoneal mesothelial cell staining

Monoclonal Mouse Anti-Human Mesothelial Cell Clone HBME-1 was used as primary antibody for staining a cytospin of cells recovered from an overnight bag from patient SKI 148. Mesothelial cells in the peritoneal dialysis effluent stained discreetly with about 1% of the total cell population. Images were captured on a Olympus BX61 microscope with a CC-12 FW colour digital camera at x400 magnification, scale bar = 20μm.

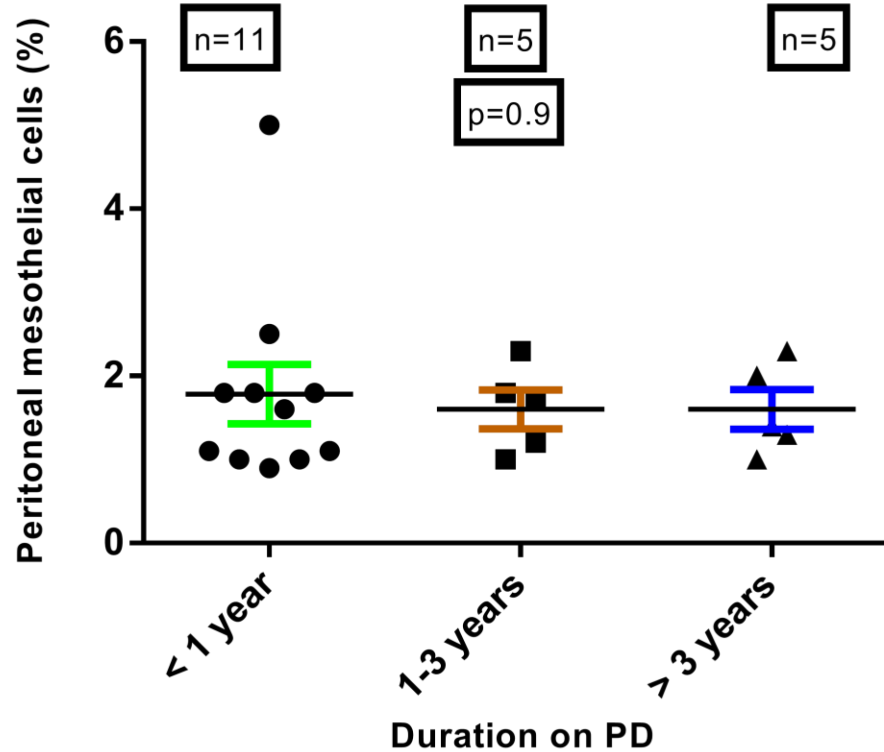

Figure 6.11 **Changes in peritoneal mesothelial cells with time on peritoneal dialysis**

Peritoneal mesothelial cells were quantified in 21 PDE samples in the SKI-2 cohort. PD patients were divided into 3 groups based on time on PD (<1 year, 1-3 years, and >3 years) and the percentage of positive stained cells calculated. Peritoneal mesothelial cells showed no significant change in between the three groups. Statistical analysis was performed using One-way ANOVA. Data represent mean with SEM. Abbreviations: PD: peritoneal dialysis. PDE: peritoneal dialysis effluent. Post-hoc comparisons using the Tukey's multiple comparisons test indicated that there are no different significant between any groups.

6.5.4 Peritoneal fibroblasts staining

FSP-1 was used as a cell marker for fibroblasts present on peritoneal cell cytospins. The NRK49F rat fibroblast cell line was used to optimise staining protocols using a 1:200 dilution of a Polyclonal Rabbit Anti-Human S100A4 as the primary antibody (Figure 6.12.A). Matching NRK49F cytospins were used to assess any endogenous peroxidase activity and nob-specific binding of the secondary antibody by replacing the primary antibody with mouse IgG, vector laboratories inc., Burlingame, 1mg/mL. No staining was seen with this antibody (Figure 6.12.B). When a cytospin from patient SKI 123 was stained with polyclonal Rabbit Anti-Human S100A4 discrete cellular staining was seen (Figure 6.12.C), which no staining was present when the S100A4 antibody was substituted for the control antibody with mouse IgG, vector laboratories inc., Burlingame, 1mg/mL (Figure 6.12.D).

It is important to refer that FSP +ve cells is not staining fibroblasts alone because there are other cells in human bodies which are staining with FSP-1. Changes in FSP +ve cells with time on PD were quantified in PD patients by calculating the mean percentage of positivity stained cells within each time frame (Figure 6.13). The range of FSP-1 +ve cells across all slides counted was 6 to 17%. FSP-1 +ve cells showed a progressive increase in the percentage of the total peritoneal cells with time on PD, increasing from 10%±2.5% (Mean±SD) of the total cell population in patients on PD for less than 12 months, rising to, 14%±1.6%,between 1 and 3 years on PD and reaching 16%±1.1% after 3 years on PD. Positive peritoneal FSP-1 +ve cells are detected by their brown colour nucleus, cytoplasm and/or cell membrane. The best dilution for staining peritoneal FSP-1 +ve cells was 1:200.

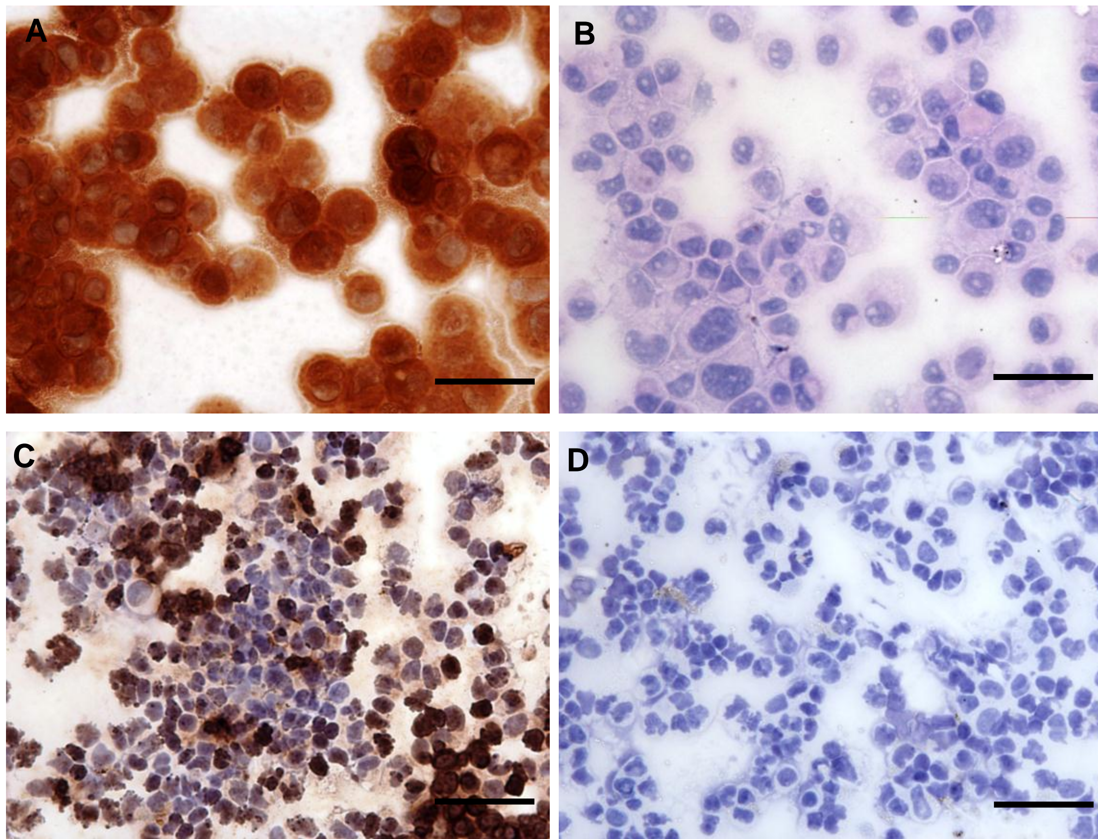

Figure 6.12 Control staining for peritoneal fibroblasts

A) cytospin of the NRK-49F renal fibroblast cell line was stained with a Polyclonal Rabbit Anti-Human S100A4 antibody to validate the staining protocol for FSP-1. The brown colour nucleus, cytoplasm and/or cell membrane can be seen. B) Parallel cytospins of NRK-49F had S100A4 replaced with mouse IgG antibody as a negative control. C) An overnight PD bag of SKI 123 had cells recovered, cytospun and the slide stained with Polyclonal Rabbit Anti-Human S100A4 showing positive discrete staining in about ¼ of the cell. D) Repeat staining of a cytospin from patientSKI 123 slide by substituting S100A4 with mouse IgG antibody which showed no staining. Images were captured on an Olympus BX61 microscope with a CC-12 FW colour digital camera at x400 magnification, scale bar = 20μm.

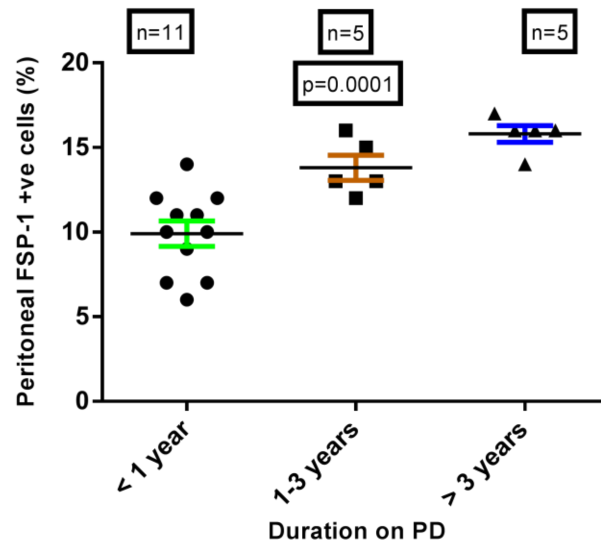

Figure 6.13 Changes in peritoneal FSP-1 +ve cells with time on peritoneal dialysis

Peritoneal FSP-1 +ve cells were quantified in 21 PDE samples in the SKI-2 cohort. PD patients were divided into 3 groups based on time on PD (<1 year, 1-3 years, and >3 years) and the percentage of positive stained cells calculated. The Peritoneal FSP-1 +ve cells increased as the duration on PD increased. Statistical analysis was done by using One-way ANOVA. Post-hoc comparisons using the Tukey's multiple comparisons test indicated that <1year was significantly different from >3 years (p=0.0001). <1year was significantly different from 1-3 years (p=0.007).1-3 years did not differ significantly from >3 years. Data represent mean with SEM.

6.5.6. Changing populations of peritoneal cells with time on PD

The four peritoneal cell populations stained in twenty one PD patients in the SKI-2 patient cohort represented the majority of total peritoneal cells. The percentages of FSP-1 +ve cells (Figure 6.14), leukocytes (CD45 +ve cells) (Figure 6.15), macrophages (CD68 +ve cells) (Figure 6.16) and mesothelial cells (Figure 6.17). All together ranged from 74%-95% of all cells counted. Cell proportions in each patient was plotted by time on PD (left to right = shortest to longest time on PD). For example SKI 71 is for a patient who is on PD for more than 9 years and it shows the highest percentage of FSP-1 +ve cells and

Leukocytes in comparison to the rest of PDE samples, while macrophage in SKI 71 is the least in comparison to the rest.

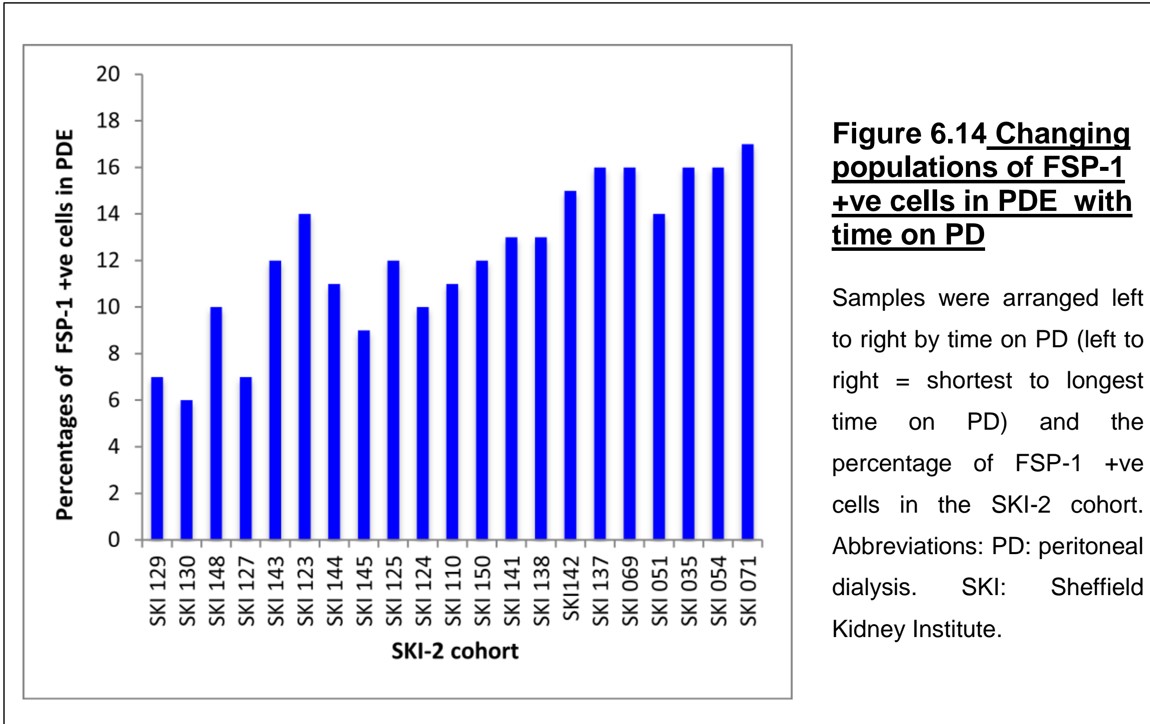

Figure 6.14 **<u>Changing populations of FSP-1 +ve cells in PDE with time on PD</u>**

Samples were arranged left to right by time on PD (left to right = shortest to longest time on PD) and the percentage of FSP-1 +ve cells in the SKI-2 cohort. Abbreviations: PD: peritoneal dialysis. SKI: Sheffield Kidney Institute.

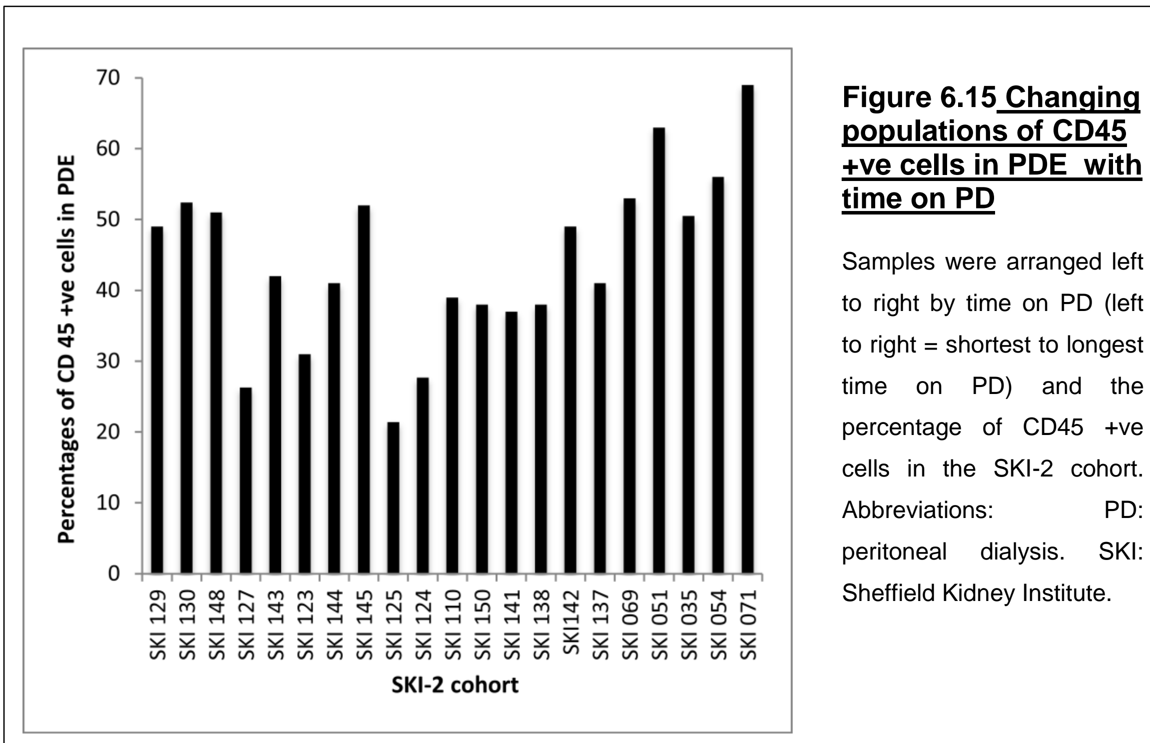

Figure 6.15 **<u>Changing populations of CD45 +ve cells in PDE with time on PD</u>**

Samples were arranged left to right by time on PD (left to right = shortest to longest time on PD) and the percentage of CD45 +ve cells in the SKI-2 cohort. Abbreviations: PD: peritoneal dialysis. SKI: Sheffield Kidney Institute.

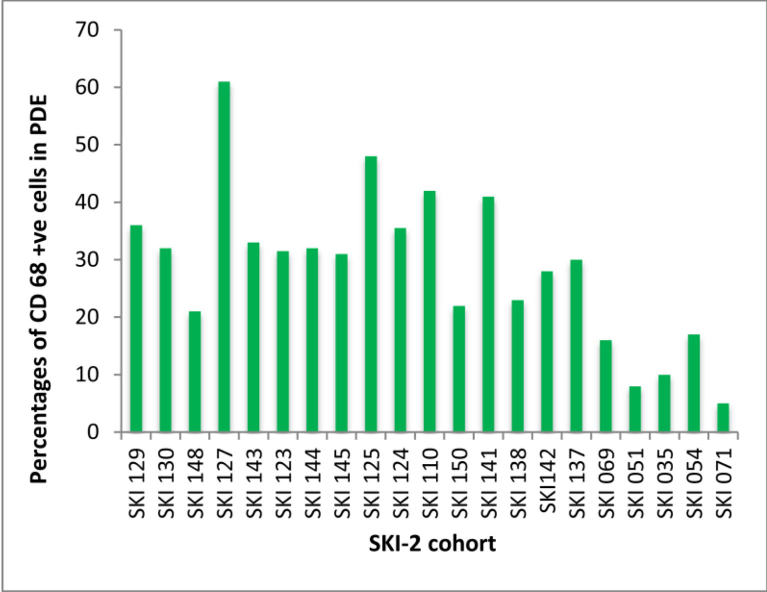

Figure 6.16 Changing populations of CD68 +ve cells in PDE with time on PD

Samples were arranged left to right by time on PD (left to right = shortest to longest time on PD) and the percentage of CD68 +ve cells in the SKI-2 cohort. Abbreviations: PD: peritoneal dialysis. SKI: Sheffield Kidney Institute.

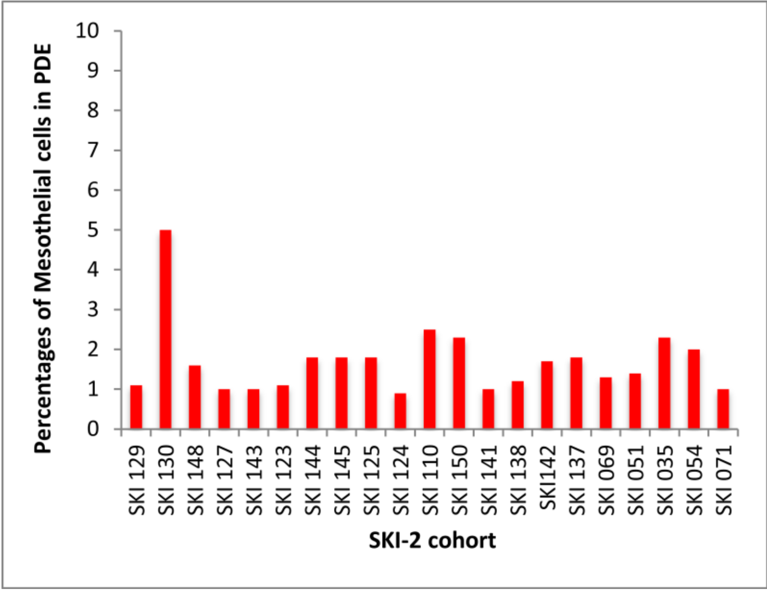

Figure 6.17 Changing populations of mesothelial cells in PDE with time on PD

Samples were arranged left to right by time on PD (left to right = shortest to longest time on PD) and the percentage of mesothelial cells in the SKI-2 cohort. Abbreviations: PD: peritoneal dialysis. SKI: Sheffield Kidney Institute.

6.5.7 Associations between peritoneal cell population and glucose exposure

Dialysate glucose exposure during the year prior to effluent sampling was calculated for each patient as described in chapter 2. Then four correlations were run between glucose exposure and percentage of each peritoneal cell population. The last year's glucose exposure to the peritoneal membrane was positively correlated to peritoneal leukocyte (r=0.45, p= 0.4) with a much stronger correlation to peritoneal FSP-1 +ve cells (r=0.7, p= 0.0004). It was also strongly negatively correlated to peritoneal macrophages (r= - 0.7, p= 0.0006) (Figure 6.18). There was no correlation to peritoneal mesothelial cells.

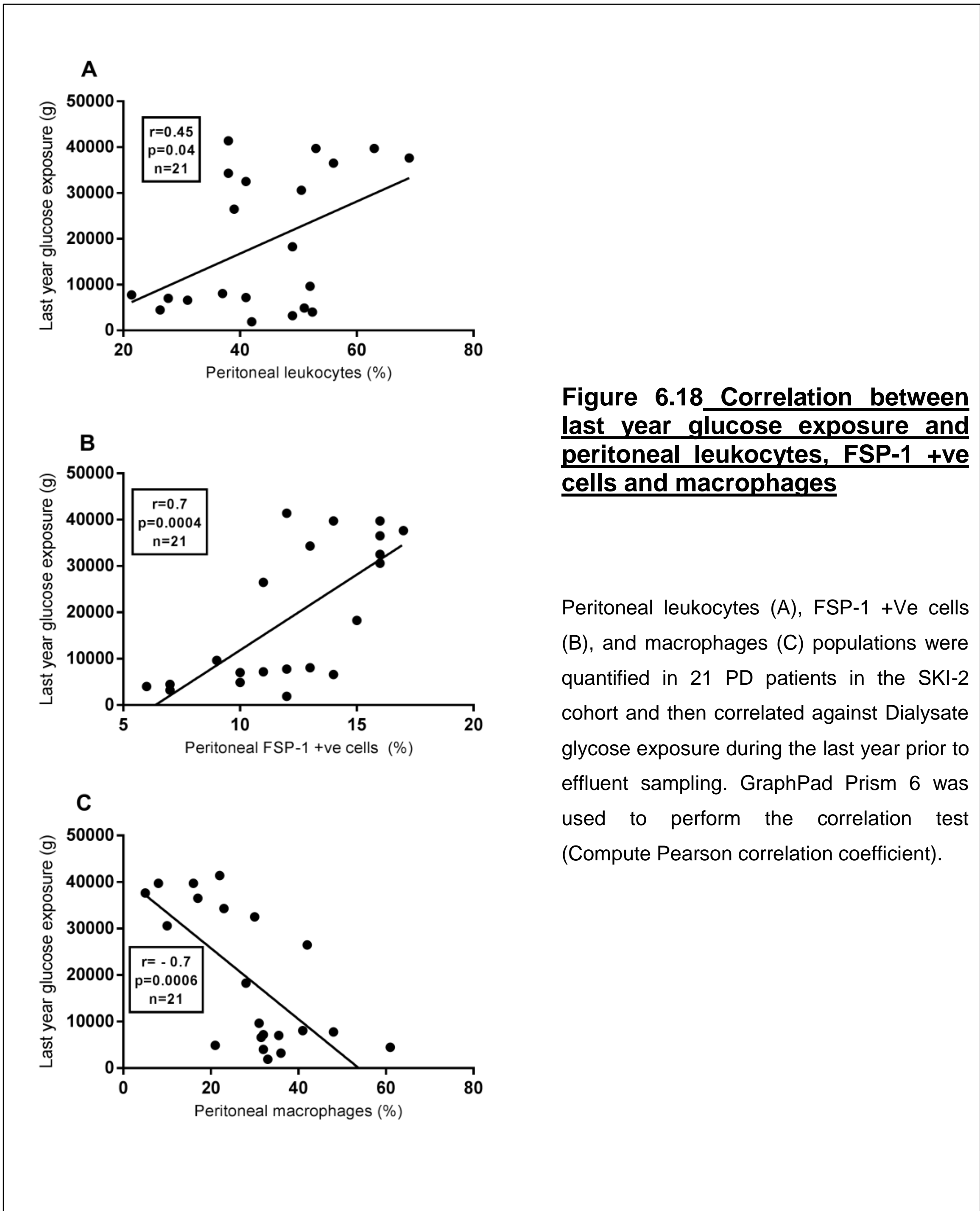

Figure 6.18 <u>Correlation between last year glucose exposure and peritoneal leukocytes, FSP-1 +ve cells and macrophages</u>

Peritoneal leukocytes (A), FSP-1 +Ve cells (B), and macrophages (C) populations were quantified in 21 PD patients in the SKI-2 cohort and then correlated against Dialysate glycose exposure during the last year prior to effluent sampling. GraphPad Prism 6 was used to perform the correlation test (Compute Pearson correlation coefficient).

6.6 Statistical analysis after this study

A statistician was approached to analyse the power of the study using the program G*power 31.3. Sample size required for detecting full and half effect shown in this study for FSP-1, leukocytes, macrophages, and mesothelial cells in SKI-2 was performed. Small studies which are significant often tend to overestimate the effect, thus half effect was used as a more cautious approach. In SKI-2, sample sizes required in detecting full effect of FSP-1, leukocytes, and macrophages were 15, 24, and 15 PD patients respectively. Sample sizes required to detect half effect were 48, 81, and 48 samples respectively. In SKI-2, 21 samples were available for these assays. Note: power calculations were performed with p=0.05 and power=95%. Mesothelial cells would need a much larger sample size as the difference detected was very small, but if the difference between the first and the second groups is D, and then between the second and the third groups is 2D and if this pattern is assumed, then with p=0.05 and power=95%, 252 samples would be required (i.e. 84 samples for each group) for mesothelial cell staining. The 3 groups based on time on PD (<1 year, 1-3 years, and >3 years).

6.7 Discussion

Cytological analysis of peritoneal washings was first performed by Keettell and Elkins in 1956. The purpose of their analysis was to assess tumour cell spreading for ovarian carcinoma (Keettel and Elkins 1956). In this chapter cell staining for four peritoneal cells was performed to analyse their changes with time on PD to see if these changes were at all linked to ECM processing changes reported in early chapters. These four cells were macrophages, leukocytes, fibroblasts and mesothelial cells.

When using cell markers to assess cell populations, there is the assumption that antibodies (primary and secondary) are bound to their targets selectively. There are several causes of weak or the complete absence of cell staining such as sub-optimal cell staining process, insufficient antigen-antibody affinity or epitope masking. Repeated cell counting for each slide is required by an operator blinded to the sample code before the average cell count can be calculated. This is a technical challenge as the cells counted can vary between individuals as a degree of subjectivity cannot be avoided. In this chapter, it had been shown that peritoneal fibroblasts and leukocytes increase with time on PD, while macrophages decrease with time. Mesothelial cells did not show significant changes. However, few samples in early stage on PD showed slight higher percentage of mesothelial cells.

In comparison with other studies, for leukocytes and macrophages various studies had measured levels of these cells in PDE samples as illustrated in section 6.1.1 Mesothelial cells undergo a process called EMT as a consequence of time on peritoneal dialysis. The end result of EMT is that peritoneal MCs develop a fibroblast-like phenotype (Aguilera, Loureiro et al. 2013) and this may explain the increase of fibroblast with time on PD. It is important to note that FSP-1, while a good marker of fibroblasts and not just myofibroblasts like Smooth Muscle actin, can be expressed by different cell types which have

mesenchymal origin (Lawson, Polosukhin et al. 2005, Österreicher, Penz-Österreicher et al. 2011) and as such it cannot be said with 100% certainty that every cell counted is a fibroblast.

Opsonins (molecules which promote phagocytosis by coating an antigen for an immune reaction) are present in the peritoneal fluids in the peritoneal cavity. They are important in the process of recognising micro-organisms that enter the peritoneal cavity. Micro-organisms coated by opsonins are recognised and phagocytised by macrophages. The regular cycling of dialysate during PD leads to removal and dilution of opsonins, resulting in a reduction in opsonin concentrations in peritoneal effluent in PD patients in compared to normal individuals (McGregor, Brock et al. 1989, de Fijter, Verbrugh et al. 1992, Brulez and Verbrugh 1995). As well as general washout, this may explain the decrease macrophage counts in peritoneal effluent with time on PD.

Opsonic activity is also affected by type of dialysate fluid in PD. There are previous studies which showed that there are different opsonic activities in cases of using different type of dialysate (Brulez, Dekker et al. 1996). It is also established in other studies that physioneal cause less death of macrophage cells in compare to dianeal. Physioneal played better role in defense cells population in the peritoneum (Pajek, Kveder et al. 2008). This is also seen with different types of PD fluid, but in this chapter the main aims were to look at the change with time on PD and to compare with changes in ECM processing systems.

The regular removal of peritoneal macrophages during PD considerably weakens the peritoneal macrophage capacity of the peritoneal defense mechanism. In CAPD patients, peritoneal macrophages may be relatively immature cells due to rapid turnover and therefore, many macrophage functions may be impaired (Li 1999). On the other hand, peritoneal lymphocytes are the main contributor to the peritoneal leukocyte population. The percentage of

peritoneal lymphocytes increased with time on PD in numerous other studies and is likely to be the cause of the increase in peritoneal leukocytes with time in this chapter. However these authors noted that effluent lymphocytes also had functional abnormalities since they demonstrated a significantly low production of IL-2 compared to peripheral lymphocytes (Lewis and Holmes 1991). This means that increase of any peritoneal cells, such as lymphocytes and fibroblasts, may not have normal functionality and as such may not perfume as required.

Peritoneal FSP-1 +ve cells and leukocytes (%) were positively correlated to glucose exposure to the peritoneal membrane during the last year prior to sampling. Peritoneal macrophage numbers declined as a percentage of the total population after a long period on PD so was negatively correlated to the glucose exposure of the peritoneal membrane in last year. It is reported in other study that high concentration of glucose can cause cell cycle stimulation of peritoneal fibroblasts (Higuchi, Sanaka et al. 1997). It is known that PD fluid will activate macrophages and mesothelial cells to produce growth factors and inflammatory cytokines and these will activate fibroblasts and this will play a role in remodeling of tissue after long-term PD (Schilte, Celie et al. 2009).

Mesothelial cells (MCs) also responsible in secreting prostaglandins, cytokines, and chemokines. MCs contribute to the leukocytes recruitment (Topley, Mackenzie et al. 1993, Lai, Tang et al. 2007). Chronic exposure of the peritoneal membrane to peritoneal dialysis fluids and peritonitis episodes will damage MCs and this will leads to partial disappearance of MCs from the peritoneal membrane (Dobbie, Anderson et al. 1994), but the remaining MCs will be activated (Mutsaers, Whitaker et al. 2000, Hekking, Zweers et al. 2005) and MCs will produce fibrotic and angiogenic factors such as VEGF, TGF-β, hyaluronic acid (HA), and FGF-2 which is a basic fibroblast growth factor (Witowski, Korybalska et al. 2000) and that is why mesothelial cells (MCs) are also important cellular components in the peritoneum. Investigators have demonstrated a reduction in PDE levels of CA 125, which is a marker of

mesothelial mass, with time on PD (Krediet 2001). However, a second study did not find a relationship between time on PD and the percentage of MCs in the cytospin of PDE (Betjes, Bos et al. 1991) as has been demonstrated here. The average of peritoneal mesothelial cells of the later study was 3.1% which this is similar to percentage of peritoneal MCs in SKI-2 cohort where levels ranged from 0.9%-5%. Betjes and colleagues demonstrated a relationship between peritonitis and decline in the peritoneal mesothelial cells (Betjes, Bos et al. 1991).

The cell staining procedure can be varied on a day to day basis for a variety of reasons including time, room temperature and unknown factors. Therefore, positive and negative controls were repeated each time cell staining was conducted so that the staining could be quality controlled to within acceptable boundaries. The majority of cells were picked up in overnight PD bags but the percentage of the cells never reached 100% so clearly some other cells were present which could not be analysed such as endothelial cells. Ideally other markers including those for better characterization would have been performed, but a lack of the slides prevented more staining as a limited number of slides were available per patient based on initial assumptions where by only 8 cytospins were done from each bag – which in hindsight was naive. Plenty of cells were recovered from each bag, & most of the cytospins were also too cell dense which complicated analysis. Thus if repeated a better approach would have been to undertake cytospins with a known cell number for giving better clarity and greater slide number. Another issue was a need for better positive and negative controls. Ideally we should have had a wide bank of cells to validate the staining against such that each antibody would be tested against fibroblasts, epithelial cells, neutrophils, B and T lymphocytes, endothelial cells, mesothelial cells etc. However, a limited number of cell lines were available as were sensible preparations of isolated blood cell and the robustness of the measurements must be taken in this context.

One of the limitations is using single markers for cell phenotype and this is a problem because there are several cell markers, but not all cells are expressing all of their markers. For example, a fibroblast may be negative for one of the markers, but it still could be a fibroblast. Another limitation is counting by manual cell counting. The limitations are cell identification errors (inter-observer variability), slide cell distribution error such as increase in the cell concentration along edges of the slides, statistical sampling error, finally manual method needs more time because there are some automated cell counters that can process 120-150 samples/hr. However, it was difficult to use automated cell counting option because some slides were crowded with cells and they were more approachable by manual cell counting. Manual cell counting was not counted only by me, but also by Dr Chra Qasm Majeed. The variability was calculated in between the manual cell counting for 2 individuals and it was below 10%.

Due to the large numbers of cells in each cytospin the decision was made to look at percentages rather than absolute counts of cells. Thus when a population is decreasing or increasing in percentage terms it could be doing so based on its absolute numbers are changing or the numbers in other populations are altering the percentage of that cell. So, for example while the data in some slides point to a reducing macrophage population in essence it could have been static and the decrease may be as a result of more fibroblasts and leukocytes. Again this needs to be recognized when interpreting the data.

Flow cytometry is a procedure through which specific characteristics of large number of cells can be measured simultaneously. Initially the cells are labeled with fluorescently conjugated monoclonal antibodies, and then in the flow cytometer the cells pass via a flow chamber and a focused laser beam to excite the flurochrome. At the excitation wavelength there will be fluorescent activation of the fluorophore. Finally the emitted fluorescence and light scattering property

for each cell is processed via a detector which can be detected (Michelson 1996). There are several advantages of using this technique such as more than one parameter can be measured for single cell by flow cytometry (Chattopadhyay and Roederer 2012), it is possible to obtain WBC differential counts (Bignardi 2015). Further dead cells can be gated out of the analysis, weakly expressed can be detected (Dunphy 2004), and the results in flow cytometry can be obtained in as short as 60-80 seconds with providing accurate quantitation (Mahieu, Vertessen et al. 2004, Butch, Wises et al. 2008, Goubard, Marzouk et al. 2011, Zur, Eichhorn et al. 2012). However, flow cytometry was not suitable for the study undertaken here. One of the major disadvantages of flow cytometer is that it requires on-site access to a flow cytometer (Herculano-Houzel, von Bartheld et al. 2015) due to cell lysis within hours of sample collection (Bignardi 2015) which made it difficult when collecting samples at SKI as there was no immediate access to a suitable flow cytometer while the availability of samples complicated this approach. However the biggest issue was the nature of the sample. The pellet from a PD effluent bag contained significant amounts of debris and fragmented cells that made a flow cytometry approach difficult with initial attempts to do this causing either blockage or a noisy signal. Immunohistchemistry approach allowed damaged cells to be counted and debris to be ignored.

In conclusion, peritoneal leukocytes and macrophages formed the majority of peritoneal cells, while the peritoneal mesothelial cells represent the smallest population. The peritoneal fibroblasts sit in between the two. Peritoneal leukocytes and fibroblasts increase with time on PD (more than 3 years), while macrophages decline with time on PD. Peritoneal fibroblasts and leukocytes show significant positive correlations with dialysate glucose exposure during the year prior to effluent sampling. As fibroblasts are a major source of TIMPs and MMPs then the increasing contribution of this cell type to the population could reflect changes in MMPs and TIMPS with time on PD.

CHAPTER SEVEN

General Discussion

7.1 General discussion

Peritoneal dialysis (PD) is one of the options for renal replacement therapy in patients with end-stage kidney failure. PD offers home dialysis permitting patients to be autonomous from the hospital. However prolonged usage of PD may be limited by scarring and fibrosis of the peritoneal membrane (Fusshoeller 2008). In some 3% of patients on PD, a rare but potentially fatal complication can develop known as encapsulating peritoneal sclerosis (EPS).

EPS is a difficult disease to study for several reasons such as it is rare disease with vague diagnostic criteria and there is no clear diagnostic test (Kawanishi, Harada et al. 2001, Korte, Sampimon et al. 2011). There is difficulty in finding suitable animal models in EPS study. The important differences in the animal models in comparison to PD in humans remain the main problem (Nikitidou, Peppa et al. 2015) for example, long incubation time is an important factor in EPS, while long incubation period is difficult in animal models. There is even disagreement as to whether EPS represents the severe end of a continuum of peritoneal sclerosis (PS) or they are entirely two different pathological entities. Aetiopathogeny of EPS has not been explained clearly and changes in peritoneal function could be due to the reflection of effects of long exposure to PD (De Sousa, del Peso-Gilsanz et al. 2012). However, those PD patients who are developing EPS have a tendency to UF failure before stopping PD (Lambie, John et al. 2010).

This thesis proposes the hypothesis that there are proteins present in the peritoneal dialysis effluent that will inform on the rate of simple peritoneal sclerosis and EPS. These proteins are likely to be those involved in tissue remodeling or fibrosis. MMPs, TIMPs, and proteins identified by proteomics are examples on these potential proteins. To address this, each chapter sought to parts of this wider hypothesis. Chapter 3 proposed that changes in the MMP system in the peritoneum would underlie PS &/or the switch to EPS and may

have value as biomarkers or diagnostics in PDE while chapter 4 tested the hypothesis that MMP family members had a peritoneal production. Chapter 6 took this information and proposed that changes in the peritoneal cell population would change with time on PD and that this underpinned changes in ECM processing systems with time on PD. Finally, using data from parallel proteomic interrogation of PDE being performed in the same laboratory, chapter 5 attempted to validate some of these data by classical ELISA in the patient cohorts available. The above chapters are important to identify markers of peritoneal injury (biomarkers) that could be monitored to provide indication of the development of peritoneal injury including PS and EPS. Biomarkers that can be measured in the PDE samples can potentially be used to monitor changes occurring in the peritoneal ECM in response to time on treatment. Therefore, the overall aims of this thesis were to identify biomarkers or diagnostic tools for PS and EPS.

This study had several key findings, perhaps the most significant findings were low ECM proteolytic activity with progressively decreasing in plasmin activity and low to undetected MMP activity in PDE samples. The lack of MMP activity seems to be due to high TIMP-1 which has a significant intra-peritoneal production and perhaps lower MMP levels than would have been predicted, especially for MMP-1. This is consistent with a fibrotic phenotype which could underlie the development of PS in PD patients where typically ECM clearance is low. Plasma levels of TIMP-1, TIMP-2, MMP-2, and MMP-3 were higher in patients on PD compared to healthy individuals. TIMP-1, 2, & MMP-2 in PDE samples were positively correlated with D/Pcr and negatively correlate with UF. The study clearly showed that TIMP-1 is rapidly elevated close to or during diagnosis of EPS and this is suggesting it may have value as a late biomarker or at least a diagnostic tool. Changes in TIMP-1 may result from a change in the cell population in the PDE with progressive increases in peritoneal fibroblasts which are powerful local TIMP producing cells. The other important finding was local production in the peritoneal cavity in particular TIMP-1 and MMP-2 which

were produced locally higher than MMP-3 and TIMP-2. MMP-3 was produced less locally and TIMP-2 was the least when compared to the other three. Plasmin activity declined in patients with long duration of PD therapy (more than 3 years).

There were limitations in the cohorts used for this study such as none of the three cohorts (SKI-1, SKI-2, and GFS) were ideal in isolation, but each contributes to the question being asked in differing ways such that each complements the others. There were deficiencies in the both SKI cohorts. In the SKI-1 cohort, there were no records for the exact dwell times and samples were not collected during a PET. There was also an absence of records concerning the occurrence or volumes used for line flushing in the collection of the PDE samples and this affected sample dilution. However these samples were already available and allowed a rapid study start being ideal for assay work up and gaining basic information. In the SKI-2 cohort, there was no EPS patients which was not ideal for either PDE biochemical analysis or cytology, instead changes with time on PD and glucose exposure to the peritoneal membrane in the last year were studied as surrogate markers of peritoneal membrane damage (Lambie, John et al. 2010). In addition, all related clinical data such as D/Pcr and UF was available which allowed good correlation analysis to be performed. The SKI-2 cohort was used for all cytology work, but in hindsight cell slides were not ideally prepared, with too many cells cytospun which made the counting difficult and time consuming as it had to be performed manually. In retrospect, rather than just loading all cells across 8 slides defined cell numbers should have been cytospun. In the GFS cohort, perhaps the primary limitation was the limited volume of the PDE samples available which restricted what assays could be performed. Irregular timing of sample collections and an absence of records concerning the line flushing at sample collection were also problematic in the GFS cohort. Finally, for the 12 PD patients in the EPS group, only 2 samples were collected within 100 days of EPS diagnosis (before diagnosis or during

diagnosis) was a significant limitation, which from the work carried out in this study appears to be a very critical period in EPS development.

This is certainly not the first study to look at the ECM proteolysis in PD. Hirahara and colleagues have previously performed similar studies in MMP system, but in their studies TIMP-2 was undetectable in their PDE samples, whileTIMP-2 was easily detectable in PDE samples of all tested cohorts (SKI-1, SKI-2, and GFS). The most likely reason for this is the detection limit of the different ELISA kits used although one cannot exclude how the samples were processed and stored. However they did show similar local production of TIMP-1 and MMP-2 as described in SKI-2 despite the lack of ideal curve fitting in their construction of a plasma or serum/dialysate transfer rate standard curve. They also did not include serum/plasma creatinine which gave them a shorter standard curve and thus inferior line fit (Hirahara, Inoue et al. 2011). Barreto and colleagues have also worked in this area, using a four point curve, but like Hirahara *et al,* they did not perform any curve fitting. This group looked at MMP-2 and PAI-1, again showing MMP-2 was produced locally in the peritoneal cavity (Barreto, Coester et al. 2013). The approach used in Sheffield was different in that five molecules were used to perform fitting curve of plasma to dialysate transfer rate standard curves. TIMP-1&-2, MMP-2&-3, and intelectin-1 were tested for locality production in peritoneal cavity of SKI-2 cohort. Finally the measured plasma levels of TIMPs, MMPs, intelectin-1, albumin, transferrin, IgG, and B2M were compared between PD patients in SKI-2 cohort against healthy individuals. A summary of approaches used for developing a dialysate transfer rate standard curve between the two published studies versus this study is illustrated in (Table 7.1).

Variable	Hirahara and colleagues	Barreto and colleagues	Present study
Tested Molecules	TIMP-1, MMP-2 and MMP-3	MMP-2 and PAI-1	TIMP-1, TIMP-2, MMP-2, MMP-3, and intelectin-1
D/P of the standard rate	Albumin, transferrin, B2M, and IgG	B2M, albumin, IgG and α2-M	Cr, albumin, transferrin, B2M, and IgG.
Molecular weight of the Standard rate (Kda)	11.8 - 150	11.8 - 820	0.1-150
TIMP and MMP levels in plasma of PD patients were compared to the healthy volunteers	No	No	Yes
Standard Curve	Linear	Linear	Polynomial excel curve fit
Produce locally	TIMP-1 and MMP-2, but not MMP-3	MMP-2 and PAI-1	TIMP-1 and MMP-2, to a lesser extent MMP-3, very low TIMP-2 and no interlectin-1 production

Table 7.1 <u>Summary of different studies to evaluate MMP family protein and intelectin-1 source in PD</u>

The comparison between published studies by Hirahara etal and Barreto etal,with the study performed in Sheffield on the SKI-2 cohort.

There are several studies showing high levels of MMPs and TIMPs in serum in various types of tissue injury (Table 7.2) (Hirahara, Kusano et al. 2011). This suggests a relationship between the pathology of tissue injury with serum or plasma levels of MMPs and TIMPs. The two studies on MMP system in PD patients [(Hirahara, Inoue et al. 2011) and (Barreto, Coester et al. 2013)] did not compare plasma or serum level of healthy individuals and PD patients, while important findings in chapter 3 and 5 of this thesis were high plasma levels of TIMP-1&-2, MMP-2&-3, and intelectin-1 in PD patients in comparison to healthy individuals. These high levels of MMPs, TIMPs and intelectin-1 in plasma may be due to high production in the body and due to impaired renal clearance. Firstly: tissue injury is associated with high MMPs and TIMPs as part of body response to the injury and the dialysate induced stress in the peritoneum. Intelectin-1 is highly expressed in the small intestine and this may be a source of intelectin-1 given the “stress” exerted on the gut during PD. Secondly, impaired

renal clearance of these molecules in PD patients may cause further high level of TIMPs, MMPs and intelectin-1 in plasma.

Increase in serum level	Tissue injury
MMP-1 and MMP-3	Rheumatoid arthritis (Green, Gough et al. 2003)
MMP-9	Chronic obstructive pulmonary disease (Brajer, Batura-Gabryel et al. 2008)
MMP-1, MMP-8, and MMP-9	Cystic fibrosis (Roderfeld, Rath et al. 2009)
MMP-9 and TIMP-1	Aortic sclerosis (Rugina, Caras et al. 2007)
MMP-2, MMP-9, and TIMP-1	Acute coronary syndrome (Tziakas, Chalikias et al. 2004)
MMP-2 and TIMP-1	Hepatic fibrosis (Kasahara, Hayashi et al. 1997)
MMP-2	Liver cirrhosis (Murawaki, Yamada et al. 1999)
MMP-2	Chronic kidney disease (Nagano, Fukami et al. 2008)

Table 7.2 Summary of studies showing changes in MMPs and TIMPs in the serum of patients with various tissue injuries

Tissue injury is associated with increased levels of various MMPs and TIMPs in plasma as these shown in various studies.

Similar to plasma levels of MMPs and TIMPs, B2M plasma levels were higher in patients on PD in compared to healthy individuals. The reverse was true for the other three molecules (albumin, transferrin, and IgG) as their plasma levels were higher in healthy individuals as compared to patients on PD. Inadequate protein intake, inflammation or protein losses from the dialysate could be responsible for the low levels of albumin and transferrin in plasma of CKD or dialysis patients (Kaysen 2003, Friedman and Fadem 2010).

In many types of fibrosis, MMPs and TIMPs are thought to play an important role (Catania, Chen et al. 2007, Hemmann, Graf et al. 2007). In this study, the hypothesis that MMPs and TIMPs may have a role in the PS & EPS was tested. The balance of MMPs and TIMPs has an important role in regulating turnover of ECM during tissue remodeling during PD. It is known that the level of MMP-2 in PDE reflects increased solute transport and peritoneal injury in PD patients (Hirahara, Inoue et al. 2007, Hirahara, Inoue et al. 2011). In a

regression analysis, TIMP-1 was one of the outstanding predictors for cell stress in PDE using a marker called HSP-27 (Musial and Zwolinska 2012)

The plasmin system also plays an important role in fibrosis alongside the MMP system. They work together to breakdown most ECM components. Both the MMP and plasmin systems have their own regulatory proteins. MMP activity is inhibited by TIMPs, while plasminogen activator inhibitors (PAIs) prevent the activation of plasminogen by blocking plasminogen activator proteins. Plasmin activity is responsible for conversion of Pro-MMPs to MMPs and MT-MMPs. There are several pro-MMPs which are activated by the plasmin system (Galis and Khatri 2002) such as MMPs-1, 3, 7, 9, 10 and 13 (Amalinei, Caruntu et al. 2007). Patients on PD for a long time (more than three years), have less plasmin activity. This will lead to decrease in MMPs-1, 3, 7, 9, 10 and 13 which means less ECM breakdown and this contributes to peritoneal fibrosis.

It would seem likely that any MMP that was present would have been completely inhibited by the huge levels of TIMPs present (Figure 7.1). Most MMPs are low or undetected in PDE samples in this study, MMP-2 level was the highest detected in PDE samples, but TIMPs levels were high in PDE samples specially TIMP-1. There were approximately 10 folds of molar excess of TIMP-1 than MMP-2 in PDE samples which would effectively block MMP activity and lead to a decrease in ECM breakdown. ECM deposition is almost certainly not down regulated to match, with the consequence being an increase in peritoneal membrane thickness. Due to the close relationship between the plasmin and the MMP systems, plasmin activity was also measured in PDE samples in the SKI-1 and SKI-2 cohorts. Plasmin activity was decreased in PDE samples from patients who were on PD for a long period (> 3 years) including the single sample from a patient diagnosed with EPS. Loss of MMP activity with diminishing plasmin activity over time could facilitate the development of PS. The large spike in TIMP-1 level in PDE samples as EPS is approached may have a role to switch the disease process into EPS with its extensive scar tissue production.

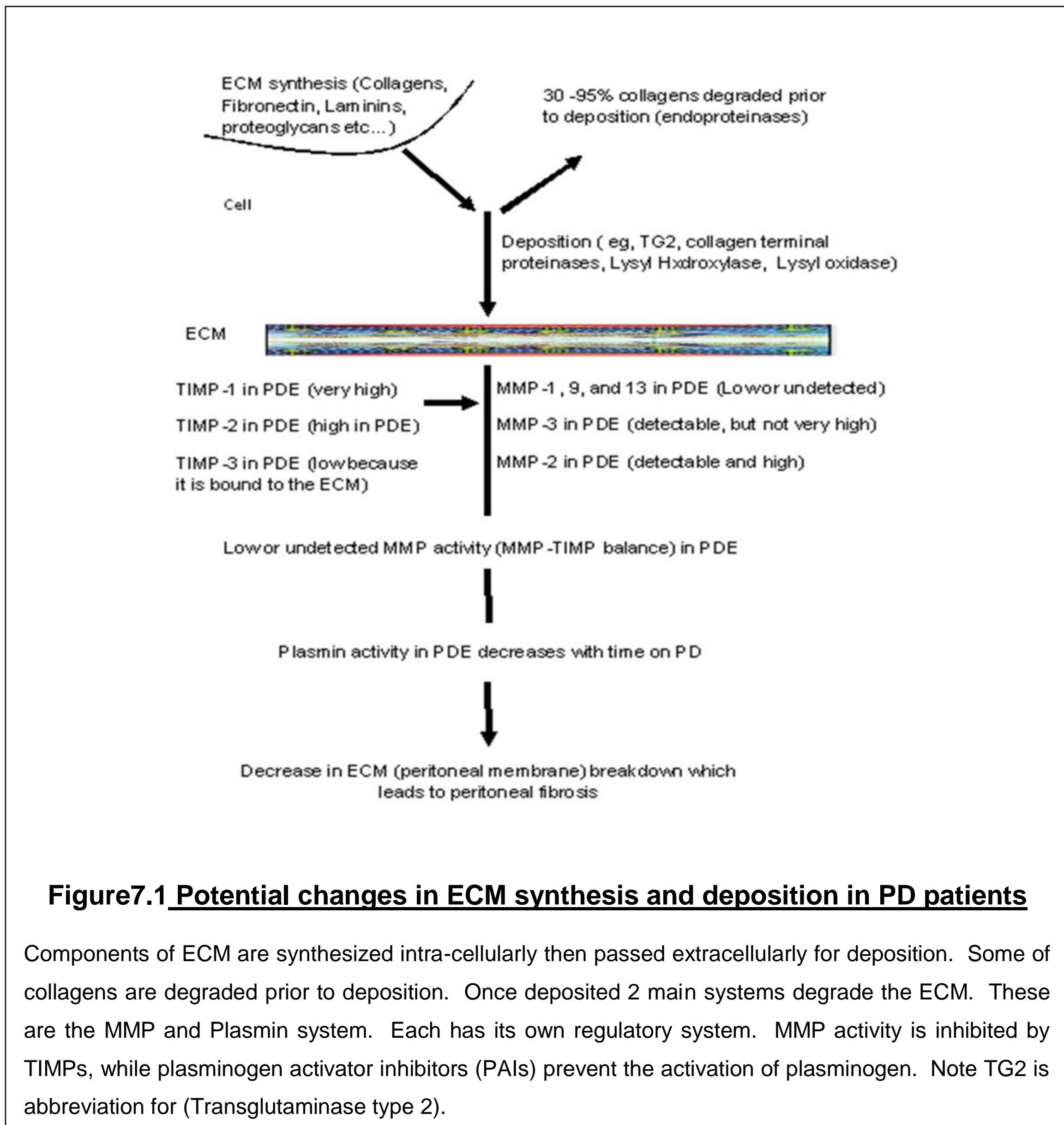

Figure7.1 Potential changes in ECM synthesis and deposition in PD patients

Components of ECM are synthesized intra-cellularly then passed extracellularly for deposition. Some of collagens are degraded prior to deposition. Once deposited 2 main systems degrade the ECM. These are the MMP and Plasmin system. Each has its own regulatory system. MMP activity is inhibited by TIMPs, while plasminogen activator inhibitors (PAIs) prevent the activation of plasminogen. Note TG2 is abbreviation for (Transglutaminase type 2).

The TIMP-1 level in PDE samples was the highest of the TIMPs measured and significantly higher than MMPs in PDE samples. TIMP-3 was low in PDE samples, although the single EPS sample in SKI-1 cohort showed the highest level of TIMP3 compared to the other samples. Low TIMP-3 in PDE samples could be due to the fact that TIMP-3 is the only TIMP which remains bound to the ECM and therefore less TIMP-3 is released as a soluble protein which would be in the PDE samples. TIMP-3 is different from the rest of TIMPs by it is property of tightly binding to the ECM (Pavloff, Staskus et al. 1992). The other cause of

low TIMP-3 may be because human TIMP-3 kit was ordered from R&D systems which is only detecting natural and recombinant TIMP-3. TIMP-3 DuoSet (DY973) does not recognize recombinant human TIMP-3 when it complexed to mature recombinant human MMP-2. TIMP-1,-2,-3 and MMP-2 were high in the EPS sample in the SKI-1 cohort, but TIMP-3 level in PDE samples was very low, therefore the decision was made to only measure TIMP-1, TIMP-2 and MMP-2 in PDE samples from the GFS due to limited sample volume from that study.

In the GFS cohort, TIMP-1 was elevated in EPS within 100 days of diagnosis, so it may have value as a late noninvasive biomarker or in confirmation of EPS diagnosis. It was also elevated in the single EPS sample compared to other samples from the SKI-1 cohort. TIMP-1 is an important molecule because it is a potent inhibitory effect for all the latent pro-MMPs and active MMPs (Ahmed 2009). Unfortunately only 2 late samples were available in the GFS cohort, but statistical advice (Dr Jean Russell, University of Sheffield medical statistics unit) was that it was appropriate to apply a statistical test to these 2 samples in order to compare them to the non EPS samples at the same time point. This was tested using an Exact Mann-Whitney as the sample size was small. TIMP-1 from the two EPS PDE samples was significantly higher than those from the control samples at 100 days ($p=0.03$). One of the key issues encountered when undertaking this study was line flushing as this was variable between clinical staff and the amounts not recorded, hence variable PDE dilution could occur. The GFS samples were taken from a 4 hours dwell time, but it was not recorded whether the “line flush” had been included or not. This could have the effected of dilution of the samples by about 10%, if a 200 mL flush in a 2 litres bag of dialysate. TIMP-2 had relatively stable level throughout dialysis in both groups in GFS and thus appeared to have the attributes of a good internal standard and thus calculation of a TIMP-1:TIMP-2 ratio was used to minimize this potential variable. Unlike TIMP-3, DuoSets for TIMP-1 and TIMP-2 are detecting natural and recombinant TIMP-1 and TIMP-2 respectively (Table 7.3). The TIMP-1:TIMP-2 ratio demonstrated an increased ratio near the time of

developing EPS. If a ratio of 3 x mean baseline was set, then no patients that did not develop EPS had "odd" samples exceeding this and it was 100% accurate in predicting patients developing EPS (given the small numbers used). MMP-2 in PDE samples of the GFS cohort did not show a clear shift in the level of MMP-2 averages between control and EPS in the last 100 days in EPS group.

ELISA kits	The detection by ELISA kits	Discussion points
TIMP-1	Natural and recombinant TIMP-1	TIMP-2 used as internal reference to TIMP-1
TIMP-2	Natural and recombinant TIMP-2	
TIMP-3	Natural and recombinant TIMP-3, but this DuoSet does not recognize recombinant human TIMP-3 when complexed to mature recombinant human MMP-2.	One of the reasons for low TIMP-3 in PDE samples is not recognizing recombinants human TIMP-3 and MMP-2 complex.
MMP-1	Pro form of recombinant human MMP-1 and the mature form of recombinant human MMP-1 by itself or when complexed to recombinant human TIMP-1 (**Total**)	Total MMP-1 was low or undetected in PDE samples. That is why MMP-1/TIMP-1 complex was low too. There is high level of TIMP-1, but very low MMP-1 in PDE samples to bind with them.
MMP-2	Active and Pro recombinant human (rh) MMP-2 but does not recognize rhMMP-2 complexed to rhTIMP-2	Recently, Total MMP-2 Quantikine ELISA Kit (MMP200), R&D systems has been announced which will be better as it detect total MMP-2.
MMP-3	Pro-, mature, and TIMP complexed forms of recombinant human MMP-3 (**Total**)	
MMP-9	This assay measures the 92 kDa Pro-MMP-9 and the 82 kDa active MMP-9. It does not measure the 65 kDa form. It recognizes human MMP-9 when complexed to Lipocalin-2/NGAL isolated from human source material.	
MMP-13	It is detecting **total** MMP-13 level.	

Table 7.3 Summary of MMPs and TIMPs ELISA kits with discussion points

The ELISA kits will or will not detect the total individual MMP or TIMP. All ELISA kits were from R&D systems except MMP-13 (abcam).

Having established TIMP-1 had potential as a diagnostic marker, and also potentially a pathogenic driver, it was important to illustrate whether TIMPs and MMPs were produced locally in the peritoneal cavity or they were just filtered from plasma to peritoneal cavity which would effectively be a filtration change. Given the absent of peritoneal biopsy samples available to us, this was carried out by calculating the rate of accumulation of TIMPs and MMPs in the PDE samples with comparison to the rate of accumulation of similar weight proteins known to only be produced else where such as creatinine, albumin, transferrin, B2M, and IgG. If the rate is consistent with similar weight proteins, then it would indicate these molecules are being purely filtered, but if the rate of accumulation in PD fluid is quicker, then it is a strong indicator of local production in peritoneal cavity.

The previous 2D gel proteomic study of PDE samples (chapter 5) facilitated the identification of several changes in proteins when comparing EPS to non-EPS developing control patients. Given this proteomic approach is semi quantitative and can compare limited samples in any run, and then it is important to validate any hits using traditional quantitative measurements in a wider population. In this study, three of these proteins were chosen to determine changes if changes identified between those developing EPS and those PD patient not. Changes in the intelectin-1, dermatopontin and collagen (α1) I in the PDE samples were associated with increased solute transport during peritoneal dialysis, most notably seen with intelectin-1 (positive correlations with D/Pcr and negative correlations D/D0 glucose and UF). The key outcome here was that the change identified by proteomics was not upheld when the GFS cohort was quantified for intelectin-1 by ELISA. Surprisingly given the known production sites of intelection-1 there was no local production in the peritoneal cavity. This suggests that the intelectin-1 level in the peritoneal cavity is due to transport of the molecule from plasma to peritoneal cavity. Proteomics incorrectly picked intelectin-1 in EPS and this may be because the “stress” exerted on the gut during PD and especially in EPS which leads to more intelectin-1. A statistical

analysis (Roc curve) was performed to compare TIMPs and MMPs (chapter 3) and intelectin-1 (chapter 5) of the control and EPS groups of GFS in the last 100 days. The analysis showed TIMP-1 and TIMP-1/TIMP-2 were the best among the measured molecules in PDE samples to predict EPS as a biomarker or diagnostic tool.

The mean level of Intelectin-1 in the plasma of PD patients was 1.6 fold higher than in the plasma of healthy individuals. The reason behind the elevated level of plasma intelectin-1 might be due to impaired renal clearance of this molecule, but it is also may be due to more intelectin-1 production. Plasma intelectin-1 level has also previously been shown to be higher in haemodialysis patients than controls (Alcelik, Tosun et al. 2011). However, this is first study to measure intelectin-1, dermatopontin and collagen (α1) I in the PDE so there are no other comparable studies. Apart from intelectin-1, dermatopontin and collagen (α1) I, a list of 9 other proteins were identified as potential predictors of EPS from proteomics analysis of GFS in Sheffield and are the subject of on-going measurement and analysis.

Given different types of cells can have hugely differing ECM processing systems, changes in the types of cells found in the PDE with time on PD could dramatically alter the ECM homeostasis. For example, fibroblasts will generate a large amount of ECM rich in fibrillary collagens, while Mesothelium derived cells will generate a basement membrane collagen rich matrix. It is known that peritoneal cells can be divided into two categories (Leung, Chan et al. 2013) as follows:

1. Residential effector cells including fibroblasts, endothelial cells, adipocytes, mesothelial, macrophages and mast cells.

2. Recruited effector cells, including macrophages, mast cells, T or B lymphocytes and polymorphonuclear cells.

In this study four types of cell were chosen for immunocytological staining and quantification to assess their changes with time on PD. These were leukocytes, macrophages, fibroblasts, and mesothelial cells (Table 7.4). These 4 types of cells were chosen because of their potential role in producing ECM proteins such as fibroblasts and macrophages. In addition to that, these chosen cells are covering the majority of peritoneal cells. In fact they represented (74%-95%) of the total cells had been found in the SKI-2 cohort. A single cell marker stain was used to see if the cell population for each cell changed with long duration of PD therapy. Cells were isolated from overnight PD bags. It would have been advantageous if cells could have also been analysed such for alpha - smooth muscle actin (α-SMA) or a marker of endothelial cells, or breaking down the leukocytes into its sub populations, but the lack of available slides prevented doing this in more detail.

Cell	**Changed with long duration of PD therapy (On PD for <1 year, 1-3 years, and >3 years)**
FSP-1 +ve cells	Increase
Leukocytes	Increase
Macrophages	Decrease
Mesothelial cells	Slight decrease, but not statistically significant

Table 7.4 Changes in the cell types found in the PDE with long duration of PD therapy

Cellular changes were observed in PDE samples for twenty one patients in SKI-2 cohort. The duration of PD were from 39 days to more than 9 years on PD. Abbreviations: PD: peritoneal dialysis and PDE: peritoneal dialysis effluent.

The Sample size for cytology study was 21 patients, and sample sizes required in detecting full effect of FSP-1, leukocytes, and macrophages were 15, 24, and 15 PD patients respectively. Post study sample sizes required to detect the half effect were 48, 81, and 48 samples respectively (power calculation with p=0.05 and power=95%). This means that more samples would be required than available to draw significant conclusions. Mesothelial cells need the largest

sample size as the difference detected was small and cell counts low, but if the difference in between first and second groups is D, and then between second and third groups is 2D and if this pattern is assumed, then with p=0.05 and power=95%, 252 samples are required (i.e. 84 samples for each group) for mesothelial cell staining. The 3 groups based on time on PD (<1 year, 1-3 years, and >3 years). It is well known that EPS is strongly associated with long duration of peritoneal dialysis therapy (Lambie, Braun et al. 2013).

It is already known that proliferation of fibroblasts (Wynn 2007) and/or differentiation to fibroblast-like cells by the process of EMT occurs in PD patients. Changes in Fibroblasts will undoubtedly change ECM homeostasis as they are a potent producer of TIMP-1. Another main function of fibroblasts is the production of fibrillary collagens and this may explain why collagen 1 was identified from the proteomics. It is thus unfortunate that the sensitivity of ELISA's for COL1A1 are insensitive and thus it was not possible to quantify in the GFS cohort. A key driver of both fibroblast proliferation and the EMT process isTGFb1 (Willis and Borok 2007). TGFb1 has the ability to induce synthesis of numerous ECM components such as Collagen I & III, but TGFb1can also block the transcription of several MMPs (Yan and Boyd 2007) & cause the expression of several TIMPS (Cotton, Herrick et al. 1998) & both PAI1 & 2 (Samarakoon, Higgins et al. 2008). Of note there is already some evidence to suggest that both FGFb & TGF beta 1 may play a role in both PS & EPS (Mlambo, Hylander et al. 1999, Honda, Nitta et al. 2003). Peritoneal leukocytes (including lymphocyte) were also increasing with time on PD. These cells also have the potential to release TGF-β which can contribute to the development of peritoneal fibrosis. T lymphocyte infiltration can occur in other organs such as lungs in animal models and patients with pulmonary fibrosis and the data here suggests similar may occur in peritoneal fibrosis. The role of lymphocytes in regulating the ECM accumulation, particularly collagen, is not completely understood (Luzina, Todd et al. 2008).

One patient among the 21 patients studied in the SKI-2 cohort had high percentage of the peritoneal mesothelial cells (up to 5%) and this patient was in the first year of PD therapy. The rest of the patients showed less mesothelial cells in all the three groups (<1 year, 1-3 years, and >3 years). Peritoneal mesothelial cells ranged from 0.9%-5% of the total population. This is close to the other published studies on peritoneal mesothelial cells. Mesothelial cells undergo the process of EMT as a consequence of time on peritoneal dialysis, resulting in peritoneal MCs developing a fibroblast-like phenotype (Aguilera, Loureiro et al. 2013). This process would likely explain in part by the increase of fibroblast with time on PD. Investigators have demonstrated a reduction in PDE CA 125, which is a marker of mesothelial mass, with time on PD (Krediet 2001). However, a second study did not find a relationship between time on PD and the percentage of MCs in the cytospin of PDE (Betjes, Bos et al. 1991). The average of peritoneal mesothelial cells of the later study was 3.1% and this is similar to the percentage of peritoneal MCs in the SKI-2 cohort. The degenerative changes of the peritoneal membrane such as loss of mesothelial cells, sub-mesothelial ECM accumulation as well as vasculopathy are associated with long-term PD (Williams, Craig et al. 2002, Fraser and Topley 2009).

It is likely that combinations of markers may be of far more value than single molecules to identify peritoneal membrane damage (figure 7.2), although in this study there was one potential protein identified, TIMP-1, but there are other adding important findings such as changes in MMP system in general and there are also changes in peritoneal cells and plasmin activity with long duration of PD therapy. TIMP-1 is the most promising marker, although it was not possible to test fully COL1A1 and dermatopontin due to detectable levels and the GFS fluid volumes available.

Based on the work here, future studies would be best aimed at expanding cell staining on other cohorts with larger sample size and inclusion of slides from EPS patients. Sequential slides are essential to monitor changes in the

peritoneal cellular in the same patient with time. Examining the impact of various types of dialysate will also be important because it affects peritoneal cells function / survival. However the most exciting finding from this work will be to examine TIMP-1 (likely as a ratio to TIMP-2) to additional cohorts and in larger numbers to fully evaluate the usefulness of it as a diagnostic tool.

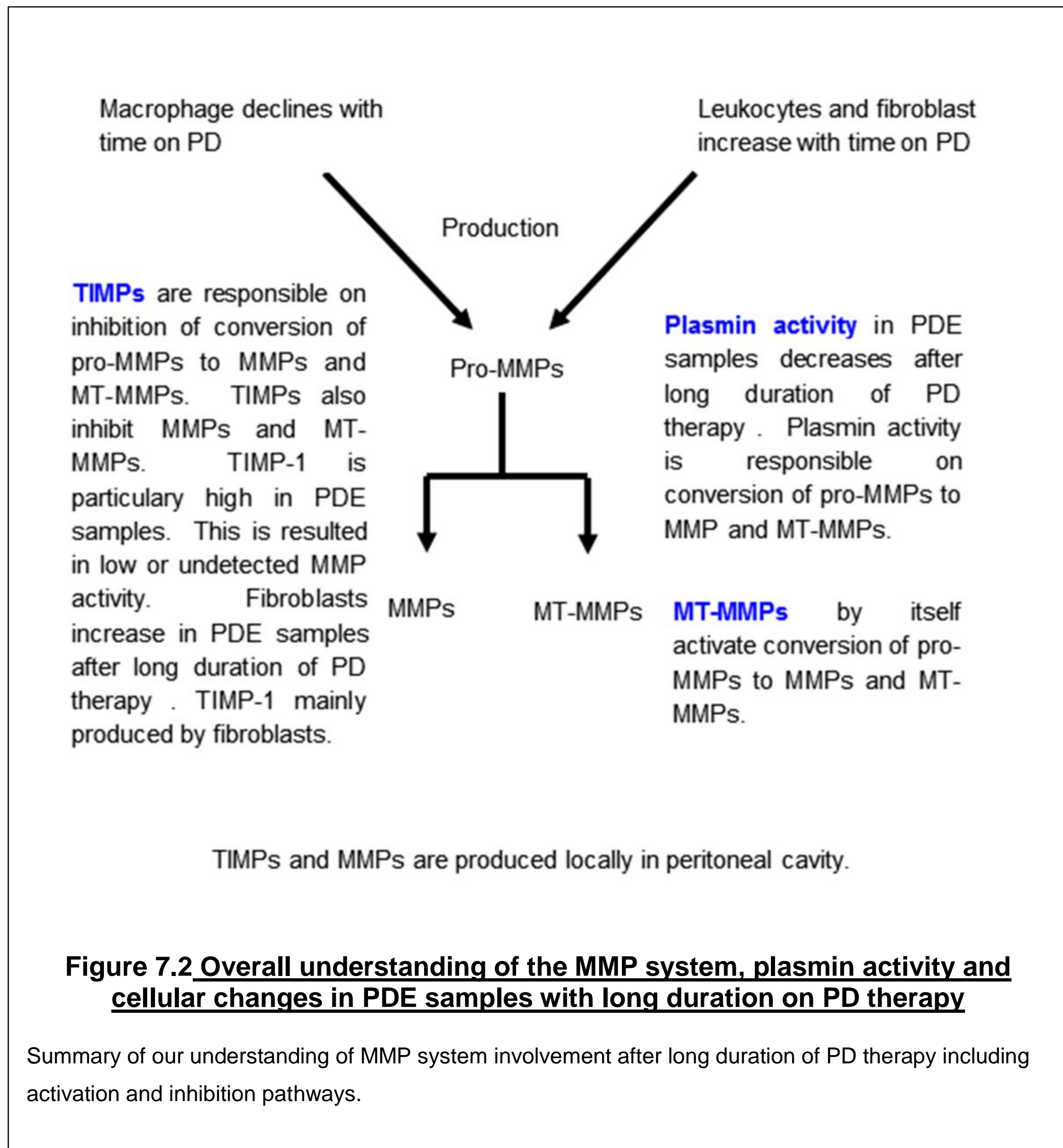

Figure 7.2 <u>Overall understanding of the MMP system, plasmin activity and cellular changes in PDE samples with long duration on PD therapy</u>

Summary of our understanding of MMP system involvement after long duration of PD therapy including activation and inhibition pathways.

In conclusion this work has identified that the TIMP-1 and TIMP-1/TIMP-2 ratio as a late predictor or early diagnostic tool of EPS onset. If this observation can be repeated and is consistent it would provide a rapid and simple test on PDE to complement or even replace existing diagnostic approaches which can be inconclusive. It may also give sufficient warning to either stop PD or possibly apply the new anti-fibrotics being introduced to clinical practice such as pirfenidone, nintedanib, Lysophosphatidic Acid receptor antagonists and LOXL2 inhibitory antibodies.

Appendices

Appendix 1: Methods of Kt/V urea and creatinine clearance calculations. It is reproduced by permission of the Baxter © Copyright 1996. Baxter Healthcare Corporation. All rights reserved.

Name patient
Date

KT/V*

1. Information required

a. Patient

Weight ______ kg
Height ______ cm

b. 24 Hour Urine

Volume ______ litres
Urea ______ mmol/l
Creatinine ______ mmol/l

c. 24 Hour Dialysate

Volume ______ litres
Urea ______ mmol/l
Creatinine $\left(\frac{\mu mol/l}{1,000}\right)$ ______ mmol/l

d. Serum

Urea ______ mmol/l
Creatinine $\left(\frac{\mu mol/l}{1,000}\right)$ ______ mmol/l

2. KT/V Calculation

a. Residual Renal KT/V

Step 1 :

$$\frac{\text{mmol/l}}{\text{mmol/l}} \times \frac{\text{litres} \times 1,000}{1,440} = \text{ml/min (Residual Renal Clearance)}$$

1,440 = min. in 24 hrs

Step 2 :

$$\frac{\text{ml/min} \times 1,440 \times 7}{\text{kg} \times (♂\ 0.6,\ ♀\ 0.55) \times 1,000} = \text{Weekly Residual KT/V}$$

b. Dialysate KT/V

$$\frac{\frac{\text{mmol/l}}{\text{mmol/l}} \times \text{litres} \times 7}{\text{kg} \times (♂\ 0.6,\ ♀\ 0.55)} = \text{Weekly Dialysate KT/V}$$

3. Total KT/V

Weekly Residual KT/V + Weekly Dialysate KT/V = Weekly Total KT/V

* K = Urea Clearance (L/WK) - T = Number of days per week the patient dialyzes - V = Volume of Urea distribution

4. Compare Patient's weekly KT/V with Target

PATIENT Weekly Total KT/V ≥ ______ TARGET Weekly KT/V of 2.0 recommended[1]

[1] Keshaviah P.R. The Peak Concentration Hypothesis : A Urea Kinetic Approach to Comparing Adequacy of CAPD and Hemodialysis. PDI - Vol. 9 - pp. 257-260, 1989

Completed by

Renal Division
Baxter

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Name patient ....

Date ....

Creatinine Clearance

1. Information required

a. Patient

Weight .... kg

Height .... cm

b. 24 Hour Urine

Volume .... litres

Urea .... mmol/l

Creatinine .... mmol/l

c. 24 Hour Dialysate

Volume .... litres

Urea .... mmol/l

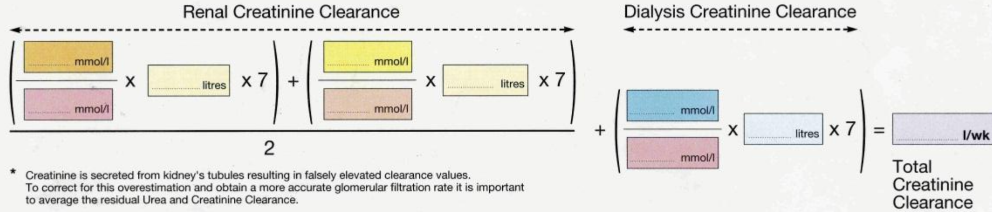
Creatinine $\left(\frac{\mu mol/l}{1{,}000}\right)$ .... mmol/l

d. Serum

Urea .... mmol/l

Creatinine $\left(\frac{\mu mol/l}{1{,}000}\right)$ .... mmol/l

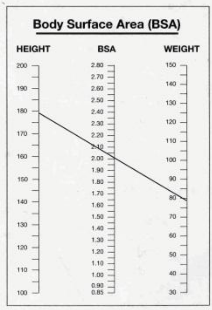
2. Creatinine Clearance Calculation*



$$\frac{\left(\frac{\text{.... mmol/l}}{\text{.... mmol/l}} \times \text{.... litres} \times 7\right) + \left(\frac{\text{.... mmol/l}}{\text{.... mmol/l}} \times \text{.... litres} \times 7\right)}{2} + \left(\frac{\text{.... mmol/l}}{\text{.... mmol/l}} \times \text{.... litres} \times 7\right) = \text{.... l/wk}$$

Total Creatinine Clearance

* Creatinine is secreted from kidney's tubules resulting in falsely elevated clearance values. To correct for this overestimation and obtain a more accurate glomerular filtration rate it is important to average the residual Urea and Creatinine Clearance.



3. Normalised Total Creatinine Clearance Calculation

$$\frac{\text{.... l/wk} \times 1.73\ m^2\ \text{BSA}}{\text{Patient BSA (refer to table)}} = \text{.... l/wk } 1.73\ m^2\ \text{BSA}$$

4. Compare Patient's Normalised Total Creatinine Clearance to target

PATIENT .... l/wk $1.73\ m^2$ BSA ≥ .... TARGET 60 l/wk normalised creatinine clearance recommended by[1]

[1] Issues and Strategies in Peritoneal Dialysis : Toward Prescription Dialysis. Highlights of a clinical conference. Part two, p. 14 : Consensus Discussion towards Prescription Dialysis

Completed by ....

Renal Division

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Appendix 2: Study protocol and related documents for Sheffield kidney institute PD cohort 1 (SKI-1)

A Study of Clinical and Genetic Risk Factors for Encapsulating Peritoneal Sclerosis

Protocol for Each Participating Centre
Stage 1
Ethical approval (2-3 months, standard application written by study co-coordinator to be adapted for regional variations)

Stage 2 (1-2 weeks)
Identification of all patients on PD by local investigator
Blood sample and Dialysis effluent from each patient
Biopsy tissue from some patients at the time of surgery.
MR scans on some patients (not all centres)

Stage 3 (Whilst collecting Blood samples)
Clinical Details entered

Stage 4 (E-mail from Study Co-coordinator every 6 months)
Regular screen for any EPS patients
Peritoneal Biopsy tissue from patients undergoing surgery for EPS or having tenckhoff catheter removal for membrane problems.

There will be regular meetings of all the local investigators and the study co-coordinator at conferences such as the renal association to maintain communication and keep all participants updated and informed. Once a critical number of samples have been collected then research proposals will be invited from the group.

Genotype Studies
Blood samples (1X 3mls EDTA) will be frozen and subsequently transported from each participating centre to Manchester. The DNA will be extracted using a standard DNA isolation kit. (Qiamp, Qiagen).
Genotyping for SNPs will be performed using Taqman allelic discrimination with the ABI PRISM 7500 sequence detection system. This method allows for high throughput genotyping.

UKEPS Protocol updated version 4 23/08/2007

Participant Information Sheet

A Study of Clinical and Genetic Risk Factors for Encapsulating Peritoneal Sclerosis

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You will be given a copy of this information sheet and a signed consent form to keep.

What is the purpose of this study?
We are investigating possible causes of Encapsulating Peritoneal Sclerosis a condition which occurs because of damage to the peritoneal membrane. The disease is rare and we think that that some people may develop this condition because of their genetic make-up. We think that variations in individual gene markers may affect the way peoples immune systems respond and hence may make some people more likely to develop EPS. If we could identify a genetic factor it may be possible to predict those people likely to develop this disease. This may allow early clinical intervention and monitoring to prevent the disease. For this study we need to recruit a large number of patients who have been on peritoneal dialysis in order to compare the genetic makeup of patients who develop EPS with those who don't.

Why have I been chosen?

1) You are on Peritoneal Dialysis
or
2) You have been identified as having Encapsulating Peritoneal Sclerosis, now or previously, and are therefore being asked to take part in this study.
or
3) You are having surgery for complications of dialysis.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?
We would ask your consent to take one 10ml (three teaspoons) blood sample. We will extract DNA from this blood for genetic studies. We would also ask you to donate 50mls of your dialysis fluid (collected after your dialysis session). We would also like permission to look at your medical notes and record details in a password protected database.
For those of you having a surgical procedure performed we ask for a small sample of your peritoneum 2-3mm to be collected by the surgeon whist you are under anaesthetic.
We may also ask you to have a MR scan of your peritoneum. This would take about 40 minutes and is a painless procedure which does not involve radiation.

What are the possible benefits of taking part?
We do not expect that there will be any benefit to you directly from this study, but neither will there be any harm. The results of this research may allow us to understand the reasons why some people develop EPS. Then we hope to identify patients at risk and develop treatments, which may prevent the development of EPs and therefore prolong the function of the peritoneum. This will help other people who receive CAPD treatment in the future.

Will my taking part in this study be kept confidential?
Your clinical information will be confidential and will not be released to anyone outside the research team. When the results of the study are reported and published, your name will not be released and it will be impossible to identify your results. It will not be possible to link the results of the genetic study to you and this data will only be seen by researchers on this study who have signed a confidentiality agreement. The genetic information collected will be anonymised and will not be released to any third party. However, it may be necessary for the ethics committee or hospital regulatory authorities to review your notes to confirm that the research has been conducted properly.

What will happen to the results of the study?
This is a long term study and it will take five years to enrol all of the patients who wish to take part. We expect to do the first analysis and publish the results two years later. We keep copies of all the research results we publish and we will give you a copy on request of any publications that come from this study. You will not be identified in any of these reports.

Who is funding the research?
The study is being funded and endorsed by the International Society of Peritoneal Dialysis.

Who has reviewed this study?
The Local Research Ethics Committee has reviewed this research study and it has agreed that it may go ahead.

Contact for further information
If you wish to know more about the study please contact:

Name of Local PI
If you wish to know more about the study please contact:
Dr Angela Summers
Dept of Renal Research
Manchester Royal Infirmary
Oxford Road,
Manchester
M13 9WL
E-mail –Angela.Summers@cmmc.nhs.uk

Participant Information Sheet
Updated Version 5 23/08/2007

Appendix 3: Study protocol and related documents for Sheffield kidney institute PD cohort 2 (SKI-2)

UK EPS Registry and DNA Bank

Protocol for Each Participating Centre
Stage 1
Ethical approval (2-3 months, standard application written by study co-coordinator to be adapted for regional variations)

Stage 2 (1-2 weeks)
Identification of all patients on PD by local investigator
Blood sample and Dialysis effluent from each patient
Biopsy tissue from some patients at the time of surgery.
MR scans on some patients (not all centres)

Stage 3 (Whilst collecting Blood samples)
Clinical Details entered

Stage 4 (E-mail from Study Co-coordinator every 6 months)
Regular screen for any EPS patients
Peritoneal Biopsy tissue from patients undergoing surgery for EPS or having tenckhoff catheter removal for membrane problems.
Extra blood samples and dialysate collected on an annual basis (not all centres).

There will be regular meetings of all the local investigators and the study co-coordinator at conferences such as the renal association to maintain communication and keep all participants updated and informed. Once a critical number of samples have been collected then research proposals will be invited from the group.

Genotype Studies
Blood samples (1X 10mls EDTA) will be frozen and subsequently transported from each participating centre to Manchester. The DNA will be extracted using a standard DNA isolation kit. (Qiamp, Qiagen).
Genotyping for SNPs will be performed using Taqman allelic discrimination with the ABI PRISM 7500 sequence detection system. This method allows for high throughput genotyping.

UKEPS Protocol version 6 02/12/2010

Participant Information Sheet

A Study of Clinical and Genetic Risk Factors for Encapsulating Peritoneal Sclerosis

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You will be given a copy of this information sheet and a signed consent form to keep.

What is the purpose of this study?
We are investigating possible causes of Encapsulating Peritoneal Sclerosis a condition which occurs because of damage to the peritoneal membrane. The disease is rare and we think that that some people may develop this condition because of their genetic make-up. We think that variations in individual gene markers may affect the way peoples immune systems respond and hence may make some people more likely to develop EPS. If we could identify a genetic factor it may be possible to predict those people likely to develop this disease. This may allow early clinical intervention and monitoring to prevent the disease. For this study we need to recruit a large number of patients who have been on peritoneal dialysis in order to compare the genetic make up of patients who develop EPS with those who don't.

Why have I been chosen?
1) You are on Peritoneal Dialysis
or
2) You have been identified as having Encapsulating Peritoneal Sclerosis, now or previously, and are therefore being asked to take part in this study.
or
3) You are having surgery for complications of dialysis.
Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.
What will happen to me if I take part?
We would ask your consent to take one 10ml (three teaspoons) blood sample. We will extract DNA from this blood for genetic studies. If you continue in the study for more than a year, we would like to take a further 10 ml blood sample each year – this blood will be stored to measure molecules that might give information about the causes of EPS. We would also ask you to donate samples of your dialysis fluid (collected after your dialysis session). We may extract cells from this fluid and perform laboratory tests on these cells.We would also like permission to look at your medical notes and record details in a password protected database.
For those of you having a surgical procedure for catheter insertion or removal we ask for a small sample of your peritoneum 2-3mm to be collected by the surgeon whist you are under anaesthetic. For those of you having a surgical procedure for EPS usually the surgeons remove the fibrous tissue we ask that this is retained for research instead of being disposed of.

We may also ask you to have a MR scan of your peritoneum. This would take about 40 minutes and is a painless procedure which does not involve radiation.
What are the possible benefits of taking part?
We do not expect that there will be any benefit to you directly from this study, but neither will there be any harm. The results of this research may allow us to understand the reasons why some people develop EPS. Then we hope to identify patients at risk and develop treatments, which may prevent the development of EPs and therefore prolong the function of the peritoneum. This will help other people who receive CAPD treatment in the future.
Will my taking part in this study be kept confidential?
Your clinical information will be confidential and will not be released to anyone outside the research team. When the results of the study are reported and published, your name will not be released and it will be impossible to identify your results. It will not be possible to link the results of the genetic study to you and this data will only be seen by researchers on this study who have signed a confidentiality agreement. The genetic information collected will be anonymised and will not be released to any third party. However, it may be necessary for the ethics committee or hospital regulatory authorities to review your notes to confirm that the research has been conducted properly.
What will happen to the results of the study?
This is a long term study and it will take five years to enrol all of the patients who wish to take part. We expect to do the first analysis and publish the results two years later. We keep copies of all the research results we publish and we will give you a copy on request of any publications that come from this study. You will not be identified in any of these reports.
Who is funding the research?
The study is being funded and endorsed by the International Society of Peritoneal Dialysis.

Who has reviewed this study?
The Local Research Ethics Committee has reviewed this research study and it has agreed that it may go ahead.

Contact for further information
If you wish to know more about the study please contact:
Dr Martin Wilkie, Sorby Renal Outpatient Dept, Northern General Hospital, S5 7AU
Martin.wilkie@sth.nhs.uk

If you wish to know more about the study please contact:
Dr Angela Summers
Dept of Renal Research
Manchester Royal Infirmary
Oxford Road,
Manchester
M13 9WL
E-mail –Angela.Summers@cmmc.nhs.uk

Participant Information Sheet
Version 6 02/12/2010

Appendix 4: Study protocol and related documents for the Global Fluid Study PD Cohort (GFS)

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, Institute of Nephrology, University of Wales College of Medicine, Heath Park, 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, 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 : 1) That the subject is free to withdraw from the study at any time 2) the subject will not benefit from participation in the study 3) the subject's GP will be contacted to inform them of their participation in the study 4) there should be some comment regarding the anonymisation of the individual's data Consent form should : 1) give permission for the subject's GP to be contacted ***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 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 :

Item	
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** ....

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)

Appendix 5: Peritoneal equilibrium test (PET). It is reproduced by permission of the Baxter © Copyright 1996. Baxter Healthcare Corporation. All rights reserved.

Peritoneal Equilibration Test (PET)

1. Collect Patient Samples

PET 1 | PET 2 | Serum | PET 3

0 Hours | 2 Hours | 2 Hours | 4 Hours — DWELL TIME

Name patient ....

Date ....

Completed by ....

2. Calculations

a. Creatinine (D/P)

D/P = dialysate concentration of corrected creatinine / serum concentration of corrected creatinine

PET 1 / Serum = A .... value (0 hrs)

PET 2 / Serum = B .... value (2 hrs)

PET 3 / Serum = C .... value (4 hrs)

b. Glucose (D/DO)

D/DO = dialysate concentration of glucose at 2/4 hrs / dialysate concentration of glucose at 0 hrs

PET 2 / PET 1 = D .... value (2 hrs)

PET 3 / PET 1 = E .... value (4 hrs)

3. Plot to Graph

D/P — CREATININE

A B C

1.1, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.0

0 h, 2 h, 4 h

1.03 HIGH 0.81 HIGH AVER 0.65 LOW AVER 0.50 LOW 0.34

D/DO — GLUCOSE

D E

1.1, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.0

0 h, 2 h, 4 h

0.61 LOW 0.49 LOW AVER 0.38 HIGH AVER 0.26 HIGH 0.12

4. Diagnosis

BASELINE PET PROGNOSTIC VALUE			
Solute Transport	Predicted Response To CAPD		Preferred Dialysis
	UF	Dialysis	
High	Poor	Adequate	CCPD dry day CAPD dry night
High Average	Poor-Medium	Adequate	Standard CAPD or APD
Low Average	Good	Adequate Inadequate	Standard CAPD High Dose PD APD
Low	Very good	Inadequate	High Dose PD Haemodialysis

Reference : Zbylut J. TWARDOWSKI, Clinical value of Standardized Equilibration Tests in CAPD Patients, *Current Concepts of CAPD Blood Purif.* 1989; 7:95-108.

Renal Division

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Appendix 6: Calculation for residual volumes

Residual volume = V injected x [D3] - [D2] [D1] - [D3]

1) Dilution of residual volume with a concentration of a molecule can be known [D1], urea and / or creatinine.
2) Volume V of fresh dialysate containing pad the molecule [D2] = 0.
3) The immediate drainage of the cavity after homogenization contains the molecule to the concentration [D3].

Appendix 7: Figure numbers and list of the permissions obtained from publishers with name of the authors and articles.

Figures	Publishers	Authors and articles
1.1	American Board of Family Medicine	Saxena, R. and C. West (2006). "Peritoneal dialysis: a primary care perspective." J Am Board Fam Med 19(4): 384.
1.2 & 1.5	Copyright © 2009, Karger	Augustine, T., P. W. Brown, S. D. Davies, A. M. Summers and M. E. Wilkie (2009). "Encapsulating peritoneal sclerosis: clinical significance and implications." Nephron Clin Pract 111(2): c149-154; discussion c154.
1.3	Peritoneal Dialysis International	Honda, K. and H. Oda (2005). "Pathology of encapsulating peritoneal sclerosis." Perit Dial Int 25 Suppl 4: S19-29.
1.4	John Wiley and Sons	Sherif, A. M., H. Yoshida, Y. Maruyama, H. Yamamoto, K. Yokoyama, T. Hosoya, M. Kawakami and M. Nakayama (2008). "Comparison between the pathology of encapsulating sclerosis and simple sclerosis of the peritoneal membrane in chronic peritoneal dialysis." Ther Apher Dial 12(1): 33-41.
1.7	John Wiley and Sons	Gerald, K. (2013). Cell and molecular biology: concepts and experiments, 7th edition, John Wiley and Sons, Hoboken, NJ.
1.8	National Academy of Sciences, U.S.A.	Buehler, M. J. (2006). "Nature designs tough collagen: explaining the nanostructure of collagen fibrils." Proc Natl Acad Sci U S A 103(33): 12285-12290.
1.10	Cambridge University Press	Lafleur, M. A., M. M. Handsley and D. R. Edwards (2003). "Metalloproteinases and their inhibitors in angiogenesis." Expert Rev Mol Med 5(23): 1-39.
1.11	BioTechniques	Snoek-van Beurden, P. A. and J. W. Von den Hoff (2005). "Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors." Biotechniques 38(1): 73-83.
2.5	UK EPS Registry & DNA Bank.	UK EPS Registry & DNA Bank, 2008.
2.10	University of Sheffield	Transfer report given by John Atkinson.
3.1	Wounds International	Gibson, D., B. Cullen, R. Legerstee, K. Harding and G. Schultz (2009). "MMP made easy." Wounds International 1(1).
6.1	Originally published in [short citation] under CC BY 3.0 license. Available from: http://dx.doi.org/10.5772/56598.	Aguilera, A., J. Loureiro, G. Gónzalez-Mateo, R. Selgas and M. López-Cabrera (2013). "The mesothelial to mesenchymal transition a pathogenic and therapeutic key for peritoneal membrane failure." The Latest in Peritoneal Dialysis. APA.
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