Microcirculatory dysfunction and glucose homeostasis in peritoneal dialysis

Submitted by Jennifer Kate Williams to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Medicine, March 2020

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University

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Abstract

Compared with hospital based therapies, peritoneal dialysis (PD) offers many advantages to patients with end stage renal disease in terms of greater autonomy and independence in addition to its health economic benefits. Cardiovascular morbidity and mortality in dialysis cohorts remains well in excess of the general population. The majority of this excess risk is attributable to a high prevalence of traditional cardiovascular risk factors and the consequences of chronic uraemia. However dialysis patients are at significant risk for systemic microvascular dysfunction which may represent an important non-traditional risk factor for cardiovascular disease in this group. In this thesis I present evidence of impairment in multiple aspects of cutaneous microvascular reactivity in PD patients compared with healthy controls and patients with similar metabolic and cardiovascular risk profiles. This impairment appears to be the result of deficiencies in multiple vasodilatory pathways and is attributable to uraemia or an aspect of PD therapy and not to co-morbidities.

During peritoneal dialysis the rate of peritoneal small solute transport is governed primarily by the peritoneal microcirculation. However in this study there was no relationship between rate of small solute transport and the degree of systemic microvascular dysfunction. Data from this study adds to the increasingly compelling evidence for intraperitoneal inflammation as the major determinant of small solute transport.

It has been suggested that the process of PD itself may also contribute to the increased cardiovascular risk, specifically the systemic effects of peritoneally absorbed glucose. In this thesis continuous glucose monitors provide novel insight into the 72 glycaemic profiles of PD patients and patients with stage 5

chronic kidney disease who are not on dialysis. In this cohort there was no significant increase in average interstitial glucose levels or the degree of glycaemic variability in PD patients compared with their non-dialysis controls. Neither was there any strong relationship between dialysate glucose exposure and interstitial glucose levels. However distinctly different patterns of glycaemia were noted in patients receiving automated peritoneal dialysis compared with those receiving continuous ambulatory peritoneal dialysis which require further investigation.

Acknowledgements

I would like to thank my supervisors Professor Angela Shore, Professor Donald Fraser, Dr Mark Gilchrist and Dr David Strain for all their support and words of wisdom.

Many thanks to my colleagues at the DVRC for supporting me to learn the techniques required to produce this thesis and for picking me up when I was down.

Thanks also go to the wonderful home care teams at the Royal Devon and Exeter Hospital and University Hospital Wales, without your help there would be many fewer participants in all the studies. Special thanks to Linda for teaching me all the practicalities of PD and being my research buddy.

This thesis is dedicated to my family – my parents who have never doubted me and Tim and Aneira who have learned to love PD and who I love very much.

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Abbreviations

3PM	Three pore model
ACh	Acetylcholine
ACR	Albumin:creatinine ratio
ADMA	Asymmetric dimethylarginine
ANOVA	Analysis of variance
APD	Automated peritoneal dialysis
AQP-1	Aquaporin-1
AU	Arbitrary units
AUC	Area under the curve
AVF	Arteriovenous fistula
BMI	Body mass index
CAPD	Continuous ambulatory peritoneal dialysis
cAMP	Cyclic adenosine monophosphate
CFR	Coronary flow reserve
CGM	Continuous glucose monitor
cGMP	Cyclic guanosine monophosphate
CKD	Chronic kidney disease
CNI	Calcineurin inhibitor
CONGA	Continuous overall net glycaemic action
COX	Cyclooxygenase
CrCl	Creatinine clearance
CRP	C reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
DCI	Davies comorbidity index
DDG	Daily dialysate glucose
DM	Diabetes mellitus
D/P_{Cr}	Dialysate to plasma ratio of creatinine
EDHF	Endothelium derived hyperpolarizing factors
eGFR	Estimated glomerular filtration rate
EPO	Erythropoetin
EPS	Encapsulating peritoneal sclerosis
ESRD	End stage renal disease
FGM	Flash glucose monitor
FMD	Flow mediated dilatation
FPG	Fasting plasma glucose
FSL	FreeStyle Libre
GV	Glycaemic variability
HA	Hyaluronic acid
HD	Haemodialysis
HOMA-IR	Homeostatic model assessment of insulin resistance
ICU	Intensive care unit
IGT	Impaired glucose tolerance
IL-6	Interleukin-6

IQR	Interquartile range
KDIGO	Kidney disease improving global outcomes
LDF	Laser Doppler flowmeter
LDPI	Laser Doppler perfusion imaging
MAGE	Mean amplitude of glycaemic excursions
MARD	Mean absolute relative difference
MFI	Microvascular flow index
MS	Metabolic syndrome
NGT	Normal glucose tolerance
NO	Nitric oxide
NOS	Nitric oxide synthase
OGTT	Oral glucose tolerance test
PBR	Perfused boundary region
PD	Peritoneal dialysis
PET	Peritoneal equilibration test
PORH	Post occlusive reactive hyperaemia
PPCl	Peritoneal protein clearance
PPV	Proportion of perfused vessels
PTH	Parathyroid hormone
PVD	Perfused vessel density
RAAS	Renin angiotensin aldosterone system
RBC	Red blood cell
RBCW	Red blood cell column width
RCT	Randomised control trial
SD	Standard deviation
SDF	Sidestream darkfield
SNP	Sodium nitroprusside
SST	Small solute transport
sVCAM-1	Soluble vascular cell adhesion molecule-1
TVD	Total vessel density
UF	Ultrafiltration
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
WHO	World health organisation
YSI	Yellow springs instrument

Chapter 1 Introduction

1.1 Peritoneal dialysis and the peritoneum as a dialysis membrane

1.1.1 Principles of peritoneal dialysis

A patient is classified as having chronic kidney disease (CKD) if they exhibit abnormalities of kidney function or structure, present for more than 3 months, which have implications for health[1]. CKD is classified on the basis of estimated glomerular filtration rate (eGFR) and level of proteinuria measured using the albumin:creatinine ratio (ACR) as detailed in Figure 1.1. Progression through the stages is associated with increased risk of adverse health outcomes[1].

Prognosis of CKD by GFR and albuminuria categories: KDIGO 2012

GFR categories (ml/min per 1.73 m²) Description and range				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30–300 mg/g 3–30 mg/mmol	>300 mg/g >30 mg/mmol
	G1	Normal or high	⩾90			
	G2	Mildly decreased	60–89			
	G3a	Mildly to moderately decreased	45–59			
	G3b	Moderately to severely decreased	30–44			
	G4	Severely decreased	15–29			
	G5	Kidney failure	<15			

Green: low risk (if no other markers of kidney disease, no CKD); yellow: moderately increased risk; orange: high risk; red, very high risk.

Figure 1.1 Classification and prognosis of chronic kidney disease using GFR and ACR categories. Reproduced with permission from Levin et al 2014 [1].

End stage renal disease (CKD G5) carries a significant burden for patients both in terms of the disease and it's treatment. Options for renal replacement therapy comprise; renal transplantation or dialysis therapy in the form of haemodialysis (HD) or peritoneal dialysis (PD). PD is classed as a home therapy as patients are trained to perform their own dialysis outside of a hospital setting. This offers several potential advantages to the patient compared with traditional hospital based haemodialysis including greater autonomy and independence and reduced routine hospital attendances [2]. However as discussed below PD is a continuous treatment, it is associated with significant emotional and physical challenges for the patient and their caregivers. It can also result in serious complications such as peritonitis. Therefore as with all forms of renal replacement therapy it has a significant impact on patients' quality of life.

PD utilises the patient's own peritoneum as a semi-permeable dialysis membrane through which solutes and fluid can be exchanged between capillary blood and the instilled dialysate (Figure 1.2).

The peritoneum is a large serous membrane whose primary purpose is to provide a smooth surface over which the abdominal viscera can move. The peritoneum consists of two anatomically distinct layers; parietal which closely adheres to the abdominal wall and visceral, adjacent to the intra-abdominal organs (Figure 1.2). The peritoneal surface area in humans varies between individuals but is usually 1-2m^2[3]. The visceral peritoneum comprises 80% of the total surface area of the peritoneum.

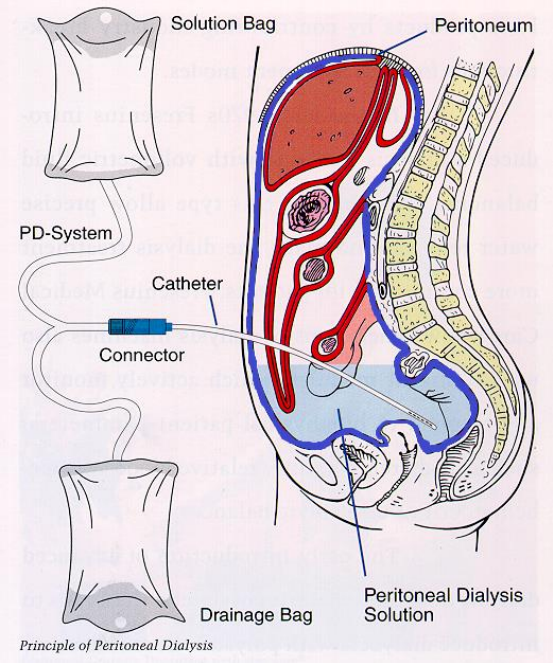

Figure 1.2 Sagittal section through the abdomen demonstrating the principles of peritoneal dialysis. Parietal peritoneum is shown in blue and visceral peritoneum in red. Dialysate is instilled into the peritoneal cavity via a catheter which has been surgically placed through the abdominal wall. The dialysate is later drained out into an empty bag.

Another natural function of the peritoneum is to convey vessels and nerves to the viscera. It therefore provides a highly vascularized and efficient system for fluid and solute delivery. Blood supply to the parietal peritoneum is derived from the abdominal wall and visceral peritoneum from the superior mesenteric artery, they drain into the inferior vena cava and the portal vein respectively[4]. Total peritoneal blood flow has been estimated at 50-150ml/min[5]. Lymphatic drainage is primarily via the sub diaphragmatic lymphatic system (70-80%).

The potential space between parietal and visceral is the peritoneal cavity which normally contains only 100mls of fluid, but can usually expand to accommodate the 2L required for standard peritoneal dialysis dwells without significant discomfort. It is here that exchange of solutes between dialysate and blood occurs during PD. Continuous ambulatory peritoneal dialysis (CAPD) is a continuous treatment. The most common regimes consist of 4 dialysis

exchanges per 24 hours with 1.5-2L of dialysate per exchange. Day time exchanges usually last 4 to 6 hours and the nocturnal exchange remains in the peritoneal cavity for around 8 hours. Automated peritoneal dialysis (APD) is a continuous treatment usually carried out at night using an automated cycling machine. Multiple shorter exchanges are performed with a longer exchange during the day.

Transport of uraemic solutes such as urea and creatinine from the plasma to the peritoneal cavity is vital for the success of this form of renal replacement therapy. The wall of the peritoneal capillaries is widely acknowledged to be the most important determinant of solute transport in PD and therefore the structure and function of the peritoneal microcirculation is of considerable interest.

1.1.2 Introduction to the microcirculation

The primary function of the microcirculation, encompassing arterioles, capillaries and venules, is to allow exchange of nutrients and gas transfer across their semi-permeable vascular wall. The combined surface area of these vessels is much greater than the larger vessels of the cardiovascular system with estimates ranging between 500 to 700 m^2.

The structure of vessels within microcirculatory beds reflects their primary function. Most exchange takes place in capillaries whose vascular wall is composed only of a single layer of endothelial cells sitting on a basement membrane with occasional pericytes embedded within it. This minimises the diffusion distance for solutes. A monolayer of endothelial cells forms the blood interface throughout the cardiovascular system. It performs multiple functions that are integral to the proper functioning of the microvascular network including

regulating vascular wall permeability, inhibiting platelet aggregation, mounting an inflammatory response against pathogens and regulating vascular tone[6]. The structure of the endothelial layer varies dependant on location and function. Continuous non-fenestrated micro-vessels have tight junctions between endothelial cells and are consequently the least permeable. These are found in skin, muscle, cardiac, respiratory and central nervous systems. Continuous fenestrated vessels contain transcellular pores (fenestrae) and therefore demonstrate greater permeability to water and small solutes. These capillaries are found in exocrine and endocrine glands, gastric and intestinal mucosa and the renal glomerulus. Discontinuous endothelium such as that found in the liver is characterised by a poorly formed basement membrane and large fenestrations, allowing for much greater permeability[7].

Resistance arteries and arterioles are the major site for regulating systemic vascular resistance as they ensure blood flow adequately meets the metabolic demands of target organ tissue by adjusting vascular tone. Consequently, their vascular wall contains a layer of smooth muscle. Vascular tone is regulated intrinsically by the myogenic response, vascular contraction as a result of increased blood pressure. The myogenic response can be modulated by the actions of locally produced vasoactive agents. Metabolically active cells produce vasodilators (e.g. adenosine, lactate) resulting in a metabolic hyperaemia. Extrinsically flow is regulated by the actions of the neuroendocrine system. Endothelial cells produce the vasoconstrictor endothelin and a variety of vasodilators including; nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin[8]. NO is a diffusible gas with a short half-life, it is synthesized from L-arginine by a family of enzymes known as nitric oxide synthases (NOS). NO is primarily produced in the endothelium by endothelial

NOS (eNOS), it then diffuses to the smooth muscle cells and causes relaxation via a cGMP-dependant mechanism[9, 10]. EDHF encompasses a number of substances that cause smooth muscle relaxation independent of NO and prostacyclins, by activating endothelial K^+ channels and causing hyperpolarisation that spreads to the underlying vascular myocytes. EDHF is thought to have a significant role in regulating vascular tone in the small resistance vessels that control tissue perfusion[10]. Prostacyclin is generated from arachidonic acid by cyclooxygenase in endothelial cells. Prostacyclin causes vascular smooth muscle relaxation via a cAMP-dependant mechanism. Collectively these pathways allow for a dynamic microcirculation, constantly changing and adapting to meet the metabolic demands of the tissue it serves.

1.1.3 The role of the peritoneal microcirculation in peritoneal dialysis

The peritoneal cavity is lined by a monolayer of mesothelial cells with microvilli on their apical surface (Figure 1.3). On top of the microvilli is a layer of glycocalyx the structure and function of which is discussed in more detail below. The mesothelial cells are supported by a basal lamina mainly composed of type IV collagen[11], however they are loosely bound to this basal lamina and even minor injury can cause cell detachment[12]. The submesothelial layer or interstitium is composed of an extracellular matrix (bundles of collagen and muco polysaccharides) and a small number of cells such as fibroblasts. The thickness of the submesothelial layer increases through infancy and childhood, reducing again in adulthood[13].

The capillaries and lymphatic vessels of the peritoneum are lined by a single layer of continuous endothelium on a basal lamina. The capillary endothelium also has a layer of glycocalyx on it’s surface[14]. These vessels appear to be

located in three distinct layers below the mesothelium and are accompanied by nerve fibers[13]. The density of lymphatic vessels is markedly lower than blood vessels[13].

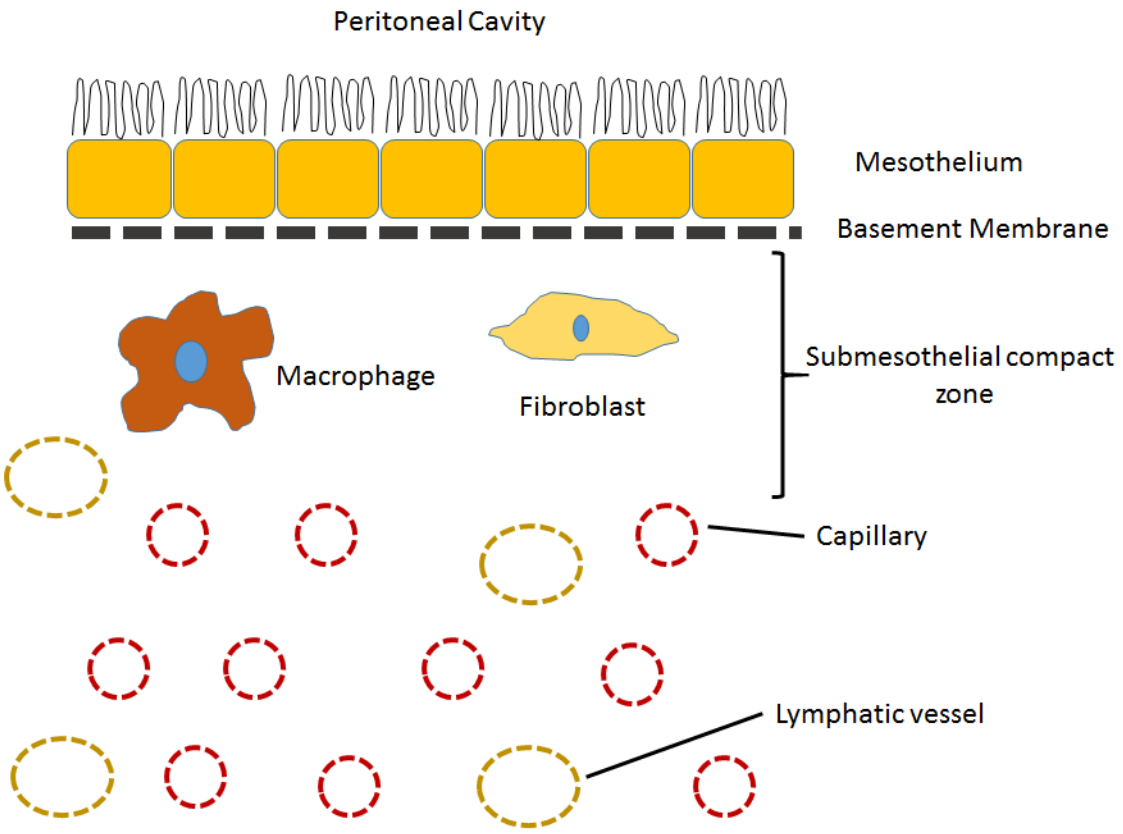

Figure 1.3 Schematic representation of the histological structure of the peritoneum

Although the visceral peritoneum has a far greater anatomical surface area than the parietal peritoneum, evisceration in animal models has demonstrated a reduction of small solute transport in the region of only 10-30%[15, 16], implying a greater contribution to small solute transport of the parietal peritoneum than it's anatomical size suggests. Histologically the visceral and parietal peritoneum have a similar structure (Figure 1.3).

As with any form of renal replacement therapy the main goals of peritoneal dialysis are the removal of solutes and excess water from the circulation. In the context of PD the peritoneum is often considered as a functional barrier rather than a precisely defined anatomical structure, the reasons for which are discussed below.

The physiological role of the mesothelium is in host defence and production of a lubricating solution to prevent local erosions and adhesions[13]. It appears that the normal mesothelium does not provide a significant barrier to solute or water movement. Except for large molecules its basement membrane appears to be freely permeable to most solutes. *In vitro* studies using an avascular section of mesentery i.e. two layers of mesothelium separated by an interstitium, demonstrate that solutes with a molecular weight up to 500kDa diffused at the rate predicted by their molecular weights[17].

The role of the interstitium is complex and becomes more important with length of time on PD as described below in the distributed model.

The three pore model

The capillary endothelium is widely considered to be the main barrier to solute transport in peritoneal dialysis. This observation forms the basis for the most widely used and best understood model of peritoneal transport – the 3 pore model(3PM)*[18]* (Figure 1.4). In the early 1990s Bengt Rippe and colleagues published their three pore model of peritoneal transport. The model was developed by combining knowledge of capillary physiology with measurements of transperitoneal solute clearances and direct volumetric assessment of drained volume as a function of dwell time in PD patients[19]. The aim was to

allow computer simulations to predict and describe peritoneal transport kinetics for a wide range of solutes. Rippe described a semi-permeable, heteroporous capillary wall that allowed for bi-directional movement of solutes and water through three different sized pores[20]. Firstly a large number of ‘small pores’, with a functional radius of 4-6nm, responsible for >90% of small solute movement. Interendothelial clefts are 15-20nm in diameter, but when studied in three dimensions there is evidence that at various points along their length they are narrowed to 4-8nm, by junctional strands[21]. Hence interendothelial clefts have been proposed as the anatomical correlate of the small pore[14].

Accompanying the ‘small pores’ were a much smaller number of ‘large pores’ (<0.1% of total pores), functionally 10-20nm in size, through which proteins and other macromolecules could be transported. The anatomical correlate of the ‘large pore’ is unclear. Evidence for a pore as opposed to transport of proteins by plasmalemmal vesicles comes from Caveolin-1-deficient mice, which lack endothelial vesicles (caveolae), and show an increased rather than decreased transport of macromolecules across microvessels[22]. The argument for highly restricted diffusion as opposed to transcytosis is supported by the observation that there is a sharp decline in clearance of small proteins as molecular radius increases[23]. Rippe proposes that the ‘large’ pores are paracellular clefts in which adjacent endothelial cells have been partly retracted such as during inflammation[24], a result of local reorganisation of the endothelial actin cytoskeleton.

Finally there were the ‘ultra-small’, transcellular pores which were selective for water only.

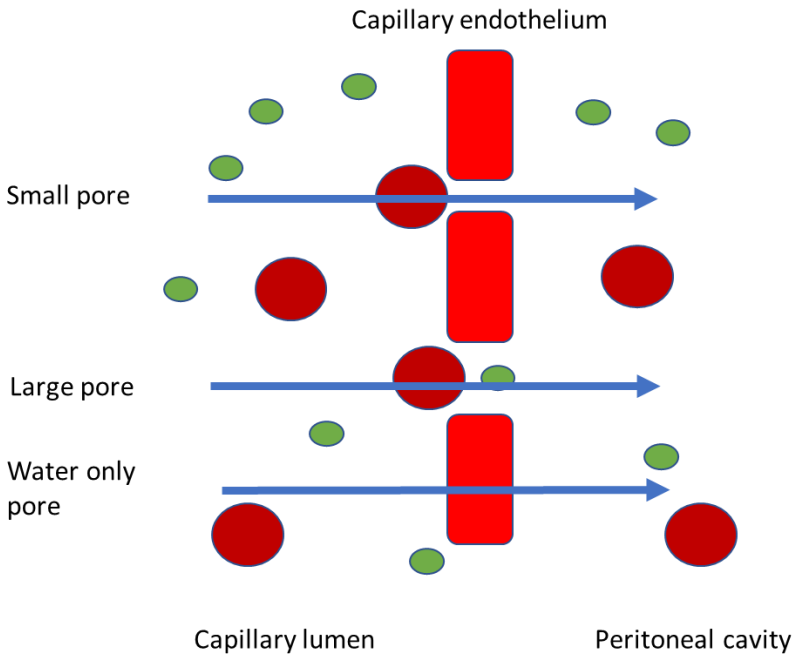

Figure 1.4 The Three Pore Model. It describes movement across the peritoneum via two paracellular pores (large pore and small pore) and one 'water-only' transcellular pore.

Compared with the histological structure the 3PM, in it's purest form, is relatively simplistic describing only the vascular compartment, the capillary wall and the peritoneal cavity. It incorporates the assumption that the capillary wall is the sole barrier to fluid and solute transport with no contribution from the interstitium. Also it does not take account of the build-up of solute gradients in the interstitial tissue. This led to criticism of the model and the development of other complementary models which will be discussed below. However the simplicity of the 3PM provides a good basis for discussion of the major influences on rate of peritoneal transport.

1.1.4 Peritoneal small solute transport

There is no evidence for active transport of solutes across the peritoneum. In cooling experiments, the transcapillary passage of albumin was reduced only to the extent that cooling reduces passive movement but not to the extent that would be expected if metabolism had been reduced[25]. Unlike the renal

glomerulus the peritoneal capillary endothelium exhibits high size selectivity but weak charge selectivity. Several studies have shown no difference in the fractional clearance of neutral and negatively charged proteins[26-28]. It is unclear why the glomerular filtration barrier senses molecular charge and the peritoneal capillaries do not. One theory is that charge phenomena are complex and may be curtailed in the long and tortuous diffusion pathway of the interendothelial cleft compared with the shorter, straighter transport pathways of the glomerulus[29].

The main forces dictating solute movement across the peritoneum are diffusion and convection.

Diffusive Flux

Diffusion is the slow but continuous, bidirectional movement of solutes through a semi-permeable membrane down a concentration gradient. This results in the net movement of solutes from an area of high concentration to an area of lower concentration. Movement continues until the concentrations are equal and net movement is zero. This is the most important force at work in solute transport in PD and is responsible for the majority of small solute (<1,000Da) movement. This diffusive flux is highest in the first hour of a dwell and decreases over time[30].

The rate of solute movement(J_S) is determined by the difference in concentration per unit of distance as demonstrated by Ficks law of diffusion

$$\mathbf{J_s = D_f/\Delta\chi * A\Delta C}$$

D_f = free diffusion coefficient

The free diffusion coefficient is determined by a number of factors. Firstly molecular weight i.e. those solutes such as urea with a relatively low molecular weight (60Da) equilibrate quickly. Conversely larger molecules such a β_2 microglobulin (11,600Da) will take much longer. Other important considerations are; temperature and the viscosity of the solvent.

$\Delta\chi$ = diffusion distance

The 3PM describe the process of peritoneal dialysis as a membrane in direct contact with the dialysate as in haemodialysis. In this case the diffusion distance is only the length of the pore. However, this is one example where the 3PM grossly simplifies the diffusion 'barrier'. The distance travelled along the long and tortuous interendothelial clefts is longer than would be predicted by measurements of the morphological thickness alone. Additionally, the thickness of the interstitial layer separating any given capillary from the peritoneal cavity differs both within and between individuals[13] and increases with time on PD. This is the basis for the distributed model*[31],* discussed below.

A = surface area

Total peritoneal surface area in humans is thought to be 1-2m^2, however a more useful concept when thinking about PD is that of the effective surface area. As the efficiency of PD relies upon transport of solutes between the blood and dialysate, the effective surface area is the number of perfused capillaries in contact with dialysate. In terms of pore theory this equates to the number of small pores in contact with dialysate. One way the effective surface area can be altered is with changes in capillary density. Normal total peritoneal microvessel density follows a U-shaped curve with age, being highest in infants (in keeping

with the rapid transport of small solutes in this age group[32]), lowest in children and higher again in adults[13]. Several studies have shown a correlation between peritoneal microvascular density measured in peritoneal biopsy specimens taken at the time of catheter insertion i.e. prior to exposure to dialysate, and subsequent baseline rate of small solute transport[33, 34]. Neoangiogenesis also occurs when the peritoneum is chronically exposed to non-physiological dialysate, this is associated with increases in small solute transport, see below.

Another route to increasing effective surface area is to increase the contact between the peritoneum and the dialysate. Fill volumes up to 3L have been shown to have a linear relationship with small solute clearance [35]. In rat models of PD, vibration significantly enhances small solute transport, this is presumed to be the result of increased contact between dialysate and visceral peritoneum[36].

Most models of peritoneal transport including the 3PM assume it is peritoneal blood volume and not blood flow that limits transport of small solutes. It has proven experimentally difficult to test this assumption. Peritoneal microvasculature is heterogeneous and only a fraction of the total blood flow through the peritoneum participates in the exchange process, the majority of vessels are too far from the peritoneal surface. Consequently it is very difficult to measure effective blood flow/volume in the human peritoneal capillary bed. Assumptions concerning the role of blood flow are based largely on animal studies. In the resting state 25% of cardiac output is directed to the splanchnic circulation[8]. In dogs subjected to circulatory shock, urea clearance from the peritoneal cavity was only reduced by 26% compared with controls[37]. In rabbit

models urea clearances only began to be affected once blood flow was reduced to 20% of normal[38]. When diffusion chambers were attached to the serosal side of the anterior abdominal wall in rats, reducing blood flow by 70% (measured using laser Doppler flowmetry) resulted in no significant difference in solute transport compared with controls[39]. In an observational study in humans, effective peritoneal blood flow estimated using diffusion of carbon dioxide, did not affect rates of small solute transport[40]. Current transport theory is therefore that under physiological conditions (blood flow >30% of normal) peritoneal blood flow does not significantly limit small solute transport.

Capillary perfusion is a dynamic process and in baseline circumstances only 25-50% of capillaries are perfused[4]. Endogenous vasoactive substances may produce a rapid and transient rise in solute transport as is seen in acute peritonitis[41] (see below). The initial instillation of dialysate fluid has also been demonstrated to cause a transient vasodilatation. Instillation of peritoneal dialysate dilated rat mesenteric arteries by more than 20%, resulting in increased capillary recruitment downstream, this was not increased by the addition of nitroprusside, indicating a maximal vasodilatation in response to the dialysate[42]. When peritoneal dialysate is co-infused with L-NAME (an inhibitor of nitric oxide synthase) arterioles remain significantly vasodilated suggesting that this dialysate induced vasodilatation is largely NO independant[43]. The exact mechanism of dialysate induced vasodilatation remains unclear. Exogenous vasoactive agents have also been shown to increase transport rates, most notably nitroprusside. Nitroprusside (an NO donor) increases clearance of small solutes in a dose dependant manner[44]. The increase in transport associated with endogenous and exogenous vasodilators is

postulated to be a result of capillary recruitment leading to increased capillary surface area[8].

ΔC = concentration gradient

In the context of the 3PM this represents the concentration difference of a given solute between dialysate and plasma. However in reality it will be the difference between the plasma concentration and the concentration in the interstitium surrounding that vessel.

Convective Flux

The second mechanism by which solute transport is achieved is convection. This is sometimes referred to as solute drag as solutes move in conjunction with a solvent. This mode of transport is especially important for the transport of macromolecules such as proteins. In the case of peritoneal dialysis the solvent, water, carries dissolved solutes (with radii less than that of the large pores) through all but the water-only pores.

The rate of solute transfer as a result of convection can be represented by the following equation;

$$J_s = J_v C(1-\sigma)$$

J_s = *solute flux*

J_v = *water flux*

C = mean solute concentration in the membrane

σ = solute reflection coefficient

Water Flux

For successful peritoneal dialysis, in addition to the removal of electrolytes and solutes we also need to enable transcapillary ultrafiltration(UF) of water from the capillaries into the peritoneal cavity. Osmosis is the movement of water from an area of low solute concentration to an area of high solute concentration across a semi-permeable membrane, i.e. the membrane must restrict the movement of solutes compared with water in order to establish an osmotic gradient. The solute reflection coefficient, referenced in the above equation, is the resistance of an osmotic substance to solute transport and consequently the maximal osmotic pressure it is able to exert across a semi-permeable membrane. In the context of the 3PM this will be different across the different pores. Values range from 0-1. Substances with a reflection coefficient of 1 are very resistant to transport across the membrane and are therefore maximally osmotically active. For solutes with a reflection coefficient of 0 the membrane offers no resistance to transport and they have minimal osmotic action. Water can move freely across all 3 pores, however the osmotic force is most pronounced across the small pores and ultra-small pores as the large pores offer little resistance to molecular movement.

Glucose is the most commonly used osmotic agent. Despite it's small molecular size and consequent net reflection coefficient of ~0.03 (this reflects a summation of the reflection coefficients across all the pores), it still has significant osmotic potential due to the presence of the ultra-small, water-only pores. In Rippe's 3PM during the early part of a dwell with hypertonic glucose-based dialysate, water passes solute free through the ultra-small pores from blood to dialysate. However, glucose is able to pass relatively freely down its diffusion gradient through both small and large pores and therefore the osmotic

gradient becomes inversely proportional to the rate of glucose transport. The presence of the ultra-small pore also explains another observed phenomenon - sodium sieving. This describes the marked fall of the dialysate-to-plasma ratio of sodium during the first hour of dialysis, when the osmotic gradient is greatest and water moves into the peritoneal cavity before any significant solute transport has taken place[45].

These ‘water-only’ pores first postulated by Rippe have subsequently been identified as Aquaporin-1 (AQP-1). AQP-1 are transcellular channels of 0.4-0.6nm in radius[46]. Having first been identified in red blood cells they are now known to be abundantly expressed in endothelial cells lining peritoneal capillaries[47]. Studies of AQP-1 knock-out mice have proven that AQP-1 is the molecular counterpart of the ultra-small pore[48]. In PD models, AQP-1 knockout mice exhibit 40% less cumulative ultrafiltration than wild-types (70% in the initial solute-free portion) and sodium sieving is abolished[49] (Figure 1.5). To date this is the only one of the three pores to be convincingly anatomically demonstrated.

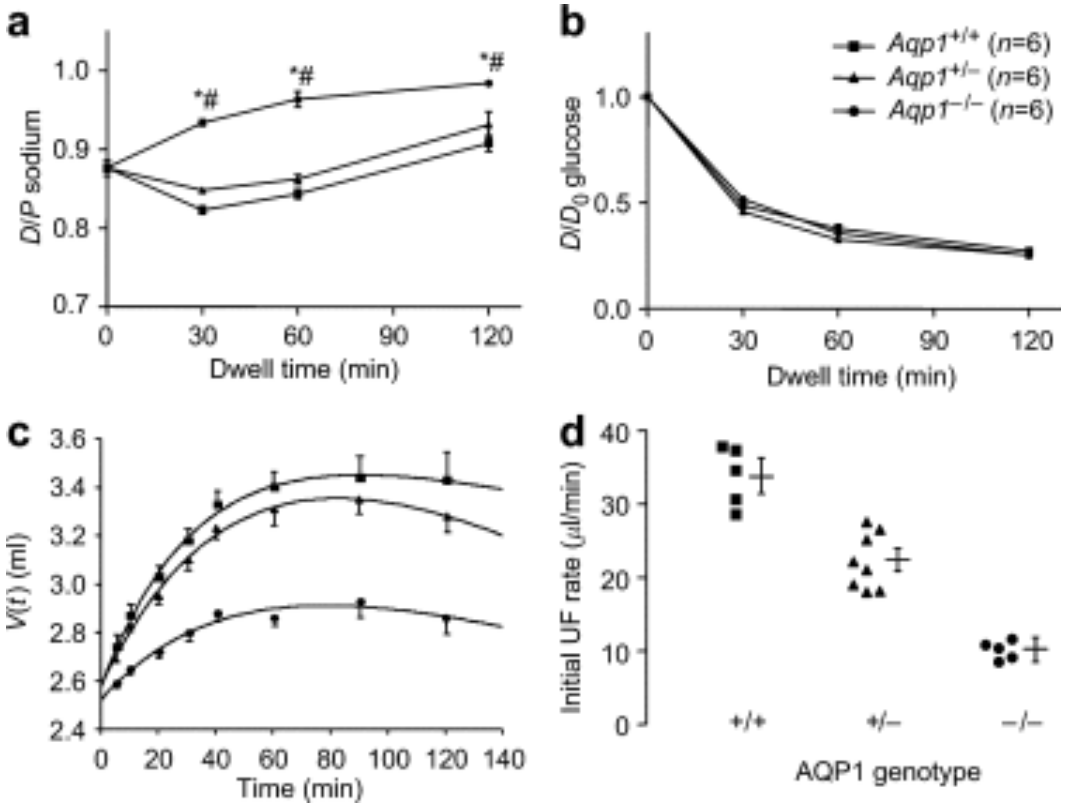

Figure 1.5 Effect of AQP1 deletion on water and solute transport parameters

a) Dialysate-to-plasma ratio of sodium, mice lacking AQP1 showed a complete loss of sodium sieving b) the progressive removal of glucose from the peritoneal cavity remained unchanged c) in volume versus time curves AQP1 knockout mice showed lower volume curves and initial UF rates d) intermediate values for initial UF were observed in AQP1+/- mice. Ni et al 2006 [49] (Reproduced with permission)

Osmosis can also be induced with colloidal agents, the most commonly used clinically is Icodextrin. Icodextrin is a mixture of starch-derived high molecular weight (1,638-45,00kDa) glucose polymers, with a structure similar to that of glycogen. Icodextrin, unlike glucose, has a net reflection coefficient approaching 1 and therefore provides an almost constant colloid osmotic pressure, able to sustain ultrafiltration for up to 16 hours even in high transporters [50]. Whereas crystalloid osmosis occurs primarily across AQP-1 colloid osmosis is primarily small pore dependant[51]. Combining data from rodent models and the above

observations in a mathematical model suggests that combining crystalloid and colloid particles may result in better UF than using them individually[52].

Net ultrafiltration achieved during PD has to take account of fluid reabsorption from the peritoneal cavity. Capillary fluid reabsorption is governed by Starling mechanisms[14], i.e. the balance between the transperitoneal hydrostatic gradient which tends to cause net ultrafiltration and the opposing transperitoneal colloid osmotic pressure. When isotonic solutions are instilled into the peritoneal cavity, fluid is reabsorbed into the plasma at a rate of approximately 1.1-1.4ml/min. It is estimated that 70% of this occurs directly into the capillaries as described above with the remaining 30% the result of lymphatic reabsorption[14]. In PD an osmotic agent (glucose) is used to prevent this reabsorption and additionally produce net ultrafiltration. However in the latter stages of long PD dwells, when crystalloid osmotic equilibrium has been reached, the Starling fluid equilibrium is shifted towards reabsorption. Increased intraperitoneal pressure reduces the transcapillary hydrostatic gradient whilst the effective peritoneal colloid osmotic pressure is increased, resulting in net peritoneal-to-capillary reabsorption.

1.1.5 Transport in the diseased state and alternative models of transport

Rippe was aware of the complexity of the “real” barrier and acknowledged that peritoneal transport needs to be viewed in the context of a more complex membrane but he argued that these additional factors were very difficult to model[19]. The simplicity of the 3PM allows for robust and simple mathematical simulation equations which have been shown to adequately describe many of

the measured properties of the peritoneum during PD including; the apparent disparity between the reflection coefficients and sieving coefficients of small solutes and the phenomenon of sodium sieving. However, due to the lack of a definite anatomical correlate for the ‘large’ and ‘small’ pores and it’s obvious simplicity other models of peritoneal transport have been proposed.

The distributed model

The distributed model describes the microvasculature in the context of its surrounding cells and the properties of the interstitium[53]. The interstitium is a highly organised structure composed of bundles of collagen in a mucopolysaccharide gel. It has been described by a two-phase model of a colloid-rich, water-poor phase from which large solutes are partially excluded, in equilibrium with a colloid-poor water-rich phase which forms a tortuous pathway for larger molecules such as proteins[54]. Tissue concentration profile studies using ethylenediaminetetraacetic acid (which has a molecular radius of 0.48nm and therefore should represent small solutes such as urea and creatinine) demonstrated the steepest portion of the concentration profile was 400-600μm from the serosa, a factor of 6 less than would be predicted based on unrestricted movement over this diffusion distance (3mm)[55]. This also demonstrates that the role of any given capillary in solute and water exchange is influenced by its position in the interstitum, not only with regards the diffusion distance for small solutes but also which portion of the solute concentration gradient set up in the interstitum it is exposed to.

A draw back of the distributed model is that it assumes a homogenous interstitium with evenly distributed capillaries. In reality the structure of the interstitium and capillary density are both heterogeneous[13]. The 3PM and the

distributed model have recently been combined in an ‘extended 3PM’, describing the heteroporous capillary wall coupled in series to a homogenous interstitium[23].

Pore matrix theory and the role of the glycocalyx

A third model of solute transport is the pore matrix theory. The glycocalyx is a layer of negatively charged glycoproteins found on the surface of many cells including the luminal surface of vascular endothelial cells. It was first visualised in the 1960s using ruthenium red staining[56] and later with electron microscopy[57]. Vink and Duling were able to demonstrate an ‘exclusion zone’ adjacent to endothelial cells in hamster skeletal muscle capillaries, that excluded not only red blood cells but also macromolecular dyes[58]. This barrier was demonstrated to limit permeation of dextrans in a molecular size and charge dependant manner. Destruction of the glycocalyx, by insults such as inflammation and hyperglycaemia, leading to increased permeability has been demonstrated in other tissues [59, 60]. New vessel formation secondary to electrical stimulation in rat striated muscle was associated with significant reductions in glycocalyx in these new vessels [61]. It was postulated that this reduction in glycocalyx would not only increase the permeability of the vessels but also allow the release of circulating growth factors and other proteins that themselves may promote angiogenesis[62], thereby increasing vascular surface area.

The above observations led to the hypothesis that there is only one intercellular pore with greater or lesser degrees of glycocalyx density which defines their size[53]. With regards this model of peritoneal transport, acute inflammation would result in destruction of elements of the glycocalyx and consequent

increases in solute transport as seen in acute peritonitis, similarly chronic exposure to hyperglycaemic dialysate would damage glycocalyx and lead to neoangiogeneis and the increases in small solute transport seen over time. Flessner proposed that the pore-matrix model be used in conjunction with the distributed model as the best representation of the three dimensional 'peritoneal barrier'[62]. Several elements of this theory have been critiqued[24]. The density of the glycocalyx appears to be too low to present a sieving barrier to small solutes such as urea and creatinine as observed in peritoneal transport. Two barriers in series i.e. the glycocalyx and the junctional strands of the interendothelial cleft would result in a 'protected' area beneath the glycocalyx and above the junctional strands. This would mean that the Starling equilibrium in this region would be virtually unaffected by interstitial colloid osmotic pressure, it is difficult to reconcile this with the action of colloidal osmotic agents such as icodextrin. Direct testing of this theory has been impeded by a number of experimental considerations. Histological processing of tissue in such a way that the fragile glycocalyx is not destroyed is very difficult, consequently no-one has been able to directly measure the glycocalyx in human peritoneal vessels and relate it's structure to small solute transport. Although measuring glycocalyx components such as hyaluronic acid(HA) in peritoneal dialysate has been successfully achieved, it has been suggested that the majority of HA measured in dialysate is derived from the peritoneal mesothelial cells[63], making this a poor marker for vascular endothelial properties. Until accurate assessment of endothelial glycocalyx properties either *in vivo* or in histological samples is possible, its role in small solute transport will continue to be debated.

There are several scenarios in which small solute transport is significantly increased, most notably inflammation and chronic exposure to dialysate. The histological and physiological changes associated with these scenarios aid our understanding of the factors that are most influential in solute transport.

Inflammation

Clinically during episodes of peritonitis there is an increase in small solute transport and consequent reduction in UF due to more rapid loss of the osmotic gradient [41]. NO, released in response to acute inflammation, resulting in vasodilation is thought to play a significant role in this[64]. Peritonitis models in mice lacking specific NOS isoforms have been used to demonstrate the role of NO. Deletion of endothelial NOS had no effect on baseline transport but during an episode of peritonitis small solute transport was significantly reduced and UF improved compared with wild-type[65]. Deleting neuronal NOS and inducible NOS had no such effect on small solute transport. Increased expression and activity of eNOS and iNOS has been found in a rat model of acute peritonitis[66].

Even in the absence of acute peritonitis there is accumulating evidence for an association between local inflammation and rate of small solute transport. Dialysate levels of interleukin-6 have repeatedly been associated with small solute transport rate[67-69]. A recent study reported that in CAPD patients dialysate levels of interleukin-6 correlated more strongly with solute transport rate than any other measured variable[70]. Even in a peritoneum that has never been exposed to dialysate the levels of IL-6 are correlated with subsequent small solute transport rate, when PD is commenced[33, 34].

Membrane changes over time

Chronic exposure to non-physiological dialysate results in structural changes in the peritoneum. These include; submesothelial fibrosis, neoangiogenesis and hyalinising vasculopathy[71] (Figure 1.6). These changes are accompanied by; increased small solute transport[72] and eventual UF failure, necessitating a change of dialysis modality.

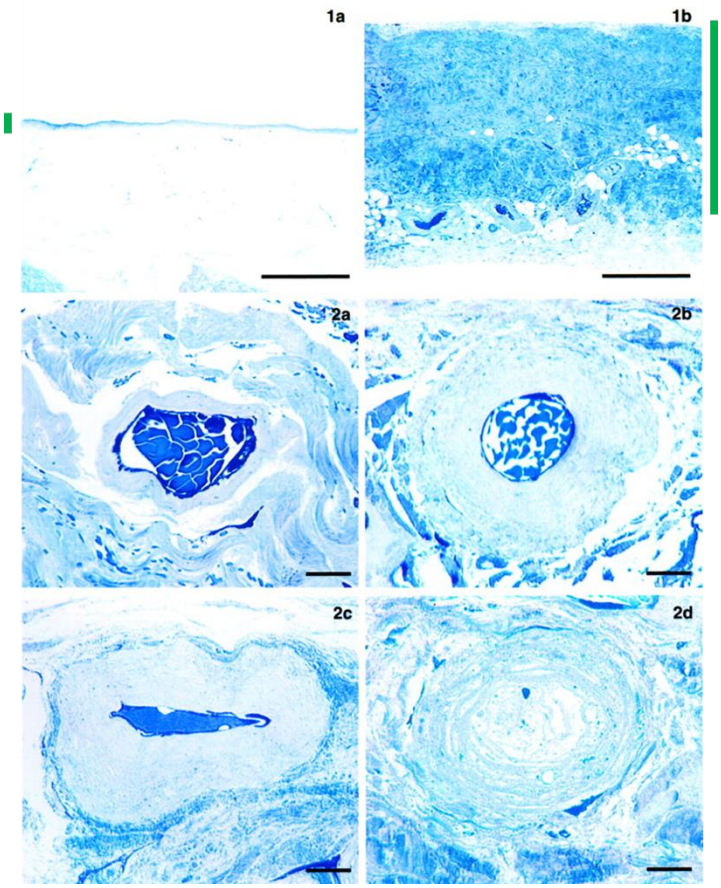

Figure 1.6 Morphologic features of the parietal peritoneum (Toluidine blue)[71]

1a) parietal peritoneum biopsy from normal individual (scale bar 500µm), 1b) a patient who has undergone PD for 9 years, the compact zone is markedly thickened (green line). 2a-d) transverse sections of small peritoneal blood vessels showing progressing grades of hyalinising vasculopathy, ending with complete occlusion of the vascular lumen (scale bar 10µm) Williams et al 2002 (Reproduced with permission).

The exact mechanisms linking the histological and clinical findings are still much debated. Increased small solute transport implies increased small pore area. Chronic PD is associated with neoangiogenesis in the peritoneum[73, 74]. The role of these newly formed vessels in small solute transport will necessarily depend on both their proximity to the peritoneal cavity and the degree to which they are perfused. In theory neoangiogenesis near the interface would increase the effective surface area (i.e. number of small pores available to participate in solute transport). It has also been suggested in the pore-matrix model that these newly formed vessels may have increased permeability as seen in other tissues.

Long term PD is also associated with increased production of intraperitoneal pro-inflammatory and angiogenic mediators such as eNOS[74] and cytokines[70] which acutely result in vasodilatation. The increased levels of eNOS may be a result of angiogenesis and increased endothelial area but it has also been suggested that NO may contribute to angiogenesis within the peritoneum[74]. NO is necessary for the biological activity of VEGF, augmented VEGF expression has been associated with peritoneal angiogenesis [74]. Although there are many factors involved in new blood vessel formation it is conceivable that enhanced VEGF expression plus augmented NO release play a role. In other tissues VEGF has been shown to increase vascular permeability although the molecular mechanisms remain unclear[75].

Thus it is proposed that vascular proliferation and vasodilatation of pre-existing vessels may represent the structural basis for the increase in effective peritoneal surface area observed in chronic PD. The failure of osmotic fluid removal that accompanies prolonged PD therapy is due in large part to this

increase in small solute transport and consequent rapid loss of osmotic gradient. However, it has also been suggested that in some patients it is the result of functional defects in the aquaporin-1 channel. The nature of this defect is currently unclear however studies in rat models of PD and a single, small observational study in humans have shown improvements in AQP-1 function associated with steroid use[76, 77].

A biopsy study from patients with encapsulating peritoneal sclerosis (EPS), a devastating complication of PD characterised by a thickened peritoneal interstitium with sclerotic changes, showed that reduced UF and sodium sieving were directly related to the degree of fibrosis and collagen density[78]. This supports predictions by the combined 3PM and distributed models that fibrosis reduces osmotic conductance either by forming a mechanical barrier outside the capillary or by reducing the penetration of the osmotic agent into the interstitium and thereby limiting the osmotic gradient across the capillary wall[79, 80]. In these patients reduced UF was not associated with any change in AQP1 expression or density[78]. The pathological mechanisms of EPS are relatively poorly defined, only a very small proportion of all patients receiving PD go on to develop EPS. Therefore how findings in this group reflect patients that may never go on to develop EPS is unclear.

In conclusion, although the 3PM accurately describes solute and water movement in many circumstances it is frustrated by the lack of anatomical correlates. Newer models such as the pore-matrix model and the distributed model elaborate on the potential role for other anatomical features of the peritoneum in the peritoneal ‘barrier’ however neither has been shown to predict solute and water movement significantly better than the ‘simplistic’ 3PM. The

factors most influential in small solute transport in the healthy and ‘diseased’ peritoneum remain a subject of debate and research.

1.1.6 Clinical measurement of small solute transport

Small solute transport is measured clinically using the Peritoneal Equilibration Test (PET) as originally described by Twardowski[81]. The PET will be discussed in more detail in Chapter 2. The solute transport parameters most commonly derived from the PET are dialysate to plasma ratio of creatinine and the ratio of end to initial dialysate glucose, measured at the end of a 4 hour dwell using 2.27% glucose solution. Using these parameters patients are categorised as having high, high average, low average or low transport status. High transport represents rapid equilibration of small solutes across the peritoneal membrane. It is recommended that all PD patients have a PET within 6 weeks of commencing therapy and at least annually thereafter to monitor membrane function (Renal Association Clinical Practice Guideline – Peritoneal Dialysis – 2017). Currently there is no method for predicting peritoneal transport status prior to commencing PD.

Peritoneal transport characteristics help to guide decisions regarding length of dwells and modality of PD, changes in transport status over time give vital information about damage to the peritoneum caused by therapy and it’s complications. The importance of the peritoneal microvasculature in this process has been established however the exact nature of the barrier to transport faced by solutes is still debated.

1.2 Studying the systemic microcirculation in dialysis patients

1.2.1 Why study the microcirculation in dialysis patients

Patients with end stage renal disease (ESRD) who are on dialysis are at significant risk for systemic microvascular dysfunction. Uraemia is associated with endothelial cell activation[82], impaired endothelial repair[83], oxidative stress[84] and impaired nitric oxide bioavailability. Additionally, these patients exist in a state of chronic inflammation[85]. Levels of inflammatory mediators such as Interleukin-6 and tumour necrosis factor -α are strongly correlated with eGFR[86], both are associated with endothelial dysfunction[87, 88]. Other dialysis specific risk factors include repeated myocardial stunning and haemodynamic perturbation of vascular beds[89] during HD and exposure to non-physiological dialysis fluids in PD[89].

Multiple studies have demonstrated links between surrogate markers of endothelial dysfunction and CKD. These include; circulating endothelial surface layer components[90], markers of inflammation and amino acids released by the endothelial cells in response to damage [91, 92], peptides known to inhibit pro-atherogenic changes[93] and endothelial dysfunction as measured in larger vessels[94].

1.2.2 Techniques used to study microcirculatory dysfunction in dialysis patients

Techniques that directly and non-invasively study *in vivo* alterations in microvascular structure [94, 95] and function [90, 96-98] are increasingly being used to expand our knowledge of the relationship between CKD and microcirculatory dysfunction. Perturbation of microvascular function in patients

with CKD has been reproducibly demonstrated in different vascular beds, including skin[90, 98, 99], sublingual[89, 94, 100] and coronary[101]. The techniques for studying the cutaneous and sublingual circulations outlined here are discussed in detail in Chapter 2.

Cutaneous Microcirculation

The cutaneous microcirculation, the most easily accessible vascular bed, has been of interest in patients on dialysis since histological alterations were first demonstrated in these patients in the 1980s. Skin biopsies from haemodialysis patients without known macrovascular disease or diabetes demonstrated thickening of the basement membrane, endothelial activation and chronic inflammatory cell infiltrates in cutaneous capillaries [95]. The extent of these changes correlated with the haemodialysis vintage of the patients[96]. *In vivo* the nail-fold capillary bed is easily visualised microscopically. Morphological changes here have also been correlated with duration of dialysis [97]. Reduction in capillary numbers is important as it reduces the surface area available for exchange, jeopardising tissue health. Capillary rarefaction has been demonstrated in the nail-fold capillaries of paediatric HD patients [98] compared with healthy, 'height-age' matched controls. The paediatric population is interesting to study with regards the microcirculation as unlike their adult counterparts, they often have a single renal limited pathology. This helps to differentiate microcirculatory pathology attributable to uraemia and it's treatments from that attributable to other systemic pathologies for example diabetes. This finding of reduced nail-fold capillary density has been replicated in adult HD cohorts[99, 100] well matched for age, blood pressure and BMI with healthy controls.

Due to its role in temperature regulation, human skin has a high vasodilatory reserve and can change its flow more than a hundred-fold in response to metabolic, thermal and pharmacologic stimuli[101]. Relative changes in skin blood flow can be easily and non-invasively measured using laser Doppler based techniques[102] Figure 2.2. Even in the resting state oscillations in microvascular flow are modulated by multiple physiological factors. Spectral analysis can be used to sub-divide laser Doppler acquired recordings according to their frequency into those representing; endothelial activity, sympathetic activity, vascular myogenic activity, respiratory activity and heart activity [103]. Reports of baseline skin blood flow in dialysis patients did not initially seem to be significantly different to healthy controls[91, 104-107]. However, when examined in more detail subtle differences were apparent. Although the averaged flux was not different, 'hot spots' or distinct spots of high perfusion were reduced and significant impairments were noted in the frequency domains corresponding to endothelial, sympathetic and cardiac activity in dialysis patients compared with controls[108].

Maximal vasodilation of skin blood vessels can be achieved by localised heating to between 42-44°C [109]. A reactive hyperaemia can also be provoked by a brief period of arterial occlusion[110] Figure 2.5. Impairments in the maximal vasodilatory response to heating[104, 108] and maximal post-occlusive flow[91, 108] have been reported in HD patients compared with healthy controls. In their study of 63 HD patients and 33 healthy controls, Stewart and colleagues[108] reported a delay in the maximal vasodilatory response to heating in the dialysis patients compared with controls. However, they were only able to demonstrate a significant reduction in the size of the maximal post-occlusive flow compared with controls in those HD patients with known diabetes

and cardiovascular disease, not the cohort as a whole. A smaller study (16 HD patients versus 16 controls)[91], wherein all participants were 'free of concomitant diseases causing alterations in endothelium-dependant vasomotion', did report a reduction in maximal post-occlusive flow in the dialysis cohort compared with controls. As would be expected, their dialysis group were significantly more hypertensive than their healthy controls.

More direct interrogation of this apparent reduction in discreet aspects of microvascular function can be achieved by combining laser Doppler measurements with iontophoretic application of vasoactive substances [111] Figure 2.4. Impairments of both endothelial-dependant and -independent responses have been demonstrated in HD patients compared with age, sex and BMI matched healthy controls[91, 107] and pre-dialysis CKD patients with comparable cardiovascular burden[112].

Sublingual Microcirculation

The more recent introduction of sidestream dark field (SDF) imaging[98] allows for direct visualisation of flow in other vascular beds with a mucosal covering, Figure 2.6. The most commonly studied is the sublingual bed [113, 114]. To date the only published study using SDF to examine sublingual vessel density and flow in dialysis patients[115], reported a reduction in total and perfused vessel density plus increased vessel flow heterogeneity compared with controls. This was particularly pronounced in the very small vessels (diameter less than 20µm)[115].

The luminal surface of endothelial cells is coated with the glycocalyx, which acts as an interface between the blood and the vascular wall. As described above, it's delicate nature means that study of the glycocalyx is challenging. Historical approaches have included measurement of total volume using tracers, and measuring shed glycocalyx components in plasma. SDF-acquired images can now be combined with Glycocheck© software to analyse spatial and temporal variations in erythrocyte column width within the microvasculature[116], Figure 2.8. When the cell-impermeable glycocalyx is damaged, circulating red cells can travel closer to the endothelium. Using this approach, loss of glycocalyx has been demonstrated in a mixed cohort of HD and PD patients [90], and has been found to associate with diminished eGFR and with increased circulating levels of shed endothelial surface layer components syndecan-1 and thrombomodulin [117].

Coronary Microcirculation

The ability of the coronary microcirculation to adapt to changing demands is vital. Coronary flow reserve (CFR) is the maximum flow resulting from stress vasodilatation (with adenosine) of coronary arteries and the coronary microcirculation, measured using positron emission tomography or magnetic resonance imaging. In this context, 90% of myocardial blood flow takes place through vessels with diameter less than 150μm, which penetrate the walls of the myocardium [118]. CFR is therefore a test of both endothelial dysfunction and coronary microvascular reserve. It is expressed as the ratio of hyperaemic to basal diastolic peak velocities, with a value above two considered normal. Low CFR indicates a reduced ability to appropriately increase flow in response to increased oxygen demand. CFR was found to be significantly lower in dialysis patients compared with healthy controls who were well matched for age, sex,

BMI and blood pressure[119, 120]. In these patients with angiographically normal coronary arteries, 50% of the dialysis cohort were found to have CFR less than 2 compared with only 5% in the control group of non-dialysis patients with hypertension[119].

Caution must be exercised in attributing all the alterations observed in the above studies to renal failure and its treatments. Many patients with end stage kidney disease have co-morbid illnesses which may also affect the systemic microcirculation, most notably hypertension and diabetes mellitus. Several of the studies above exclude from their control group of 'healthy volunteers' those with these conditions but they are present in a large proportion of the dialysis group[108, 115]. In these studies, measured differences between groups are likely to represent the combined effects of chronic uraemia, dialysis and other co-morbidities.

Even in otherwise well matched cohorts, dialysis patients frequently have increased systolic blood pressure compared with their control counterparts[98, 115]. Therefore, in addition to their dialysis patients and healthy controls, Farkas and colleagues studied a third group of age-matched patients with essential hypertension[91]. They were able to demonstrate a significant reduction in both endothelium-dependant and independent vasodilatation in their dialysis patients compared with healthy controls and their "control" group of those with hypertension. Are microcirculatory changes associated with clinical outcomes?

Is there a link between microcirculatory changes and clinical outcomes?

A link between microvascular dysfunction and adverse CV outcomes has been demonstrated in other populations [121-124]. Vascular dysfunction in the skin has been demonstrated to correlate with coronary disease[125] and be an independent marker for CVD in patients with Type 2 diabetes[126]. As these techniques become better understood and increasingly used in renal cohorts, interest has turned to how they may be used as biomarkers to identify high risk patients and facilitate intervention at an earlier stage.

Coronary microvascular rarefaction has been postulated as a contributory factor to sudden cardiac death in the dialysis population[127]. In a cohort study of nearly 4000 individuals encompassing the whole spectrum of CKD, CFR was shown to be strongly associated with CV death independent of CKD stage[128]. Adjusting for CFR in CKD 4,5 and dialysis-dependant groups attenuated their risk of CV death by 10%, supporting the concept that coronary microvascular dysfunction may underlie some of the increased mortality associated with CKD.

In separate multi-variate regression analyses, endothelial impairment as measured by forearm post-ischaemic vasodilatation [129] and CFR[127] were found to be independently associated with all-cause mortality in haemodialysis patients.

Microvascular dysfunction of the coronary and peripheral circulations have also been correlated with outcome measures known to have negative prognostic implications such as hypoalbuminaemia[130, 131], right ventricular dysfunction[132] and microalbuminuria[133].

CKD mineral bone disease can cause large vessel calcification, a strong predictor of cardiovascular death in HD patients[134]. There is some evidence for an association between large vessel calcification and microvascular dysfunction in HD patients, those with femoral artery calcification exhibited lower maximal vasodilatory responses to ACh and SNP than both controls and HD patients without large vessel calcification [107]. There is also increasing evidence of a relationship between markers of worsening CKD mineral bone disease and microvascular abnormalities in the absence of large vessel calcification. Dermal capillary rarefaction and impaired CFR have been associated with increasing levels of both iPTH[98] and phosphorous [99, 128] in CKD cohorts. Even in cohorts with normal renal function serum phosphate concentrations have been negatively correlated with postocclusive capillary recruitment[135] and endothelial dysfunction in larger vessels[136].

Patients at risk of other non-CVD outcomes which significantly impact on morbidity and quality of life, such as wound healing have also been identified using these techniques. Those patients with lower skin blood flow both before and during HD, as measured by laser Doppler, have been shown to be at greater risk of developing wounds and skin defects[130]. All patients in this study who later went on to develop a skin defect had evidence of intradialytic 'critical perfusion' at the microvascular level in at least one measured area, although none exhibited intra-dialytic hypotension.

1.2.3 The effects of acute and chronic dialysis

Cardiovascular risk increases as patients progress through the stages of CKD and with time on dialysis[137]. Is microvascular impairment similarly related to stage of CKD and time on dialysis?

It has been found that even the creation of an arterio-venous fistula(AVF) in preparation for haemodialysis may have systemic microvascular effects. In pre-dialysis patients, successful formation of an AVF led to a reduction in endothelial dependant vasodilation in the fistula arm. Following fistula creation, these patients also exhibited a reduction in non-endothelium dependant vasodilation in the contralateral arm, indicating that localised changes to the structure of the macrocirculation can lead to widespread changes in the microcirculation. This was in contrast to those patients who had primary AVF failure, who exhibited no recordable local or systemic changes[138].

Cross-sectional studies also provide evidence for a relationship between stage of kidney disease and microcirculatory dysfunction. Plasma levels of shed glycocalyx components such as syndecan-1 and markers of endothelial activation such as angiopoietin-2 correlate inversely with eGFR [117]. Retinal microvessels also narrow progressively with each stage of CKD[139]. Additionally, there is histopathological evidence of endothelial activation and infiltration by inflammatory cells in dermal capillaries [95, 96] and circulating levels of adhesion molecules such as sVCAM-1 correlate with duration of dialysis [140]. The potential effects of renal replacement therapy itself on the microcirculation remain less well defined. Using SDF technology[117], Dane and his colleagues were able to demonstrate impaired glycocalyx integrity associated with worsening eGFR. However, in their ESRD group (n=23) no statistically significant difference was seen between the dialysis patients (n=9) and patients with ESRD who were not on dialysis(n=14)[117]. Common to many of the studies presented here small sample size in a heterogeneous cohort may have contributed to the lack of statistically significant findings.

A large cross-sectional study found that although coronary microvascular function assessed by CFR was 23% lower in dialysis patients compared with controls with preserved kidney function, this reduction occurred early in CKD, with a nadir being reached in CKD 4[128]. The authors found no additional reductions in stage 5 or 5D. However, it is important to note that the CKD 4 patients were on average 10 years older than the dialysis group and had a higher incidence of known ischaemic heart disease and oral nitrate use. It is possible in light of this that survivor bias has limited the apparent extent of microvascular dysfunction detected in the patients with CKD5 in this retrospective study. Some of these issues could be addressed by longitudinal studies directly investigating microvascular function in dialysis cohorts. INTHEMO is an ongoing two-year study primarily designed to assess the effects of haemodialysis intensity on micro and macrovascular parameters[141]. In a preliminary report, the investigators found no statistically significant change in glycocalyx parameters, measured using SDF images, at 6 months follow-up compared with baseline. They did however note significant heterogeneity in the degree and direction of change of calculated glycocalyx properties at 6 months, and data at study completion is awaited. One important limitation of historical studies may be the effect of the HD procedure itself. The microcirculation is inherently dynamic, and as described below, timing of measurements with regards HD therapy itself may have significant impact on results. Standardisation of timing of measurements with respect to HD therapy is an important consideration for future studies.

HD has been shown to cause varying degrees of macrohaemodynamic instability in patients often because of ultrafiltration (UF) of fluid, observed clinically as intradialytic hypotension. Recurrent intradialytic hypotension is

considered to have negative prognostic implications[142]. Studies of the sublingual microcirculation using SDF during a single HD session have demonstrated a reduction in microvascular flow and decrease in proportion of the microcirculation that is perfused through the course of the treatment[143, 144]. This reduced flow in all microvessels has been attributed to a reduction in circulating volume secondary to UF. This assertion is supported by data from studies showing reduced microvascular perfusion in patients undergoing isolated UF but not in those undergoing HD with linear UF [145] and partial correction of this reduced flow by a manoeuvre designed to increase central venous filling[143]. These microcirculatory changes were independent of macrohaemodynamic changes, for example blood pressure, implying an element of compensation by the microcirculation. Decreased intradialytic perfusion has also been demonstrated in the cutaneous circulation [130, 146]. However, in this context, hypervolaemic patients who were ultrafiltrated to normovolaemia had improved skin perfusion[147]. As this increased cutaneous perfusion was accompanied by a decrease in arterial and venous pressure the authors proposed a decreased myogenic response as a local auto regulatory effect. Another potential mechanism could be interstitial fluid removal with reduced external compression of vessels. How relatively acute changes in intravascular volume associated with HD change capillary flow remains unclear. This relationship is complicated by the myriad of vasoactive stimuli resulting from the HD process.

The endothelial glycocalyx plays an important role in mechanosensing and mechanotransduction[148]. It senses changes in shear stress and mediates biomolecular responses including flow-induced NO release[149]. The creation of vascular access required for haemodialysis (arteriovenous fistulas, grafts and

central venous catheters) as well as the process of haemodialysis itself result in disturbed fluid shear stress[150, 151]. Laminar shear stress is obligatory for the normal functioning of the glycocalyx. It promotes increased synthesis of hyaluronic acid and heparin sulphate[152, 153] and their incorporation into the glycocalyx layer[154], influencing the stability of the glycocalyx and its ability to sense shear stress. Prolonged laminar shear stress ultimately results in a quiescent endothelial phenotype[155], increased nitric oxide production and the down regulation of pro-proliferative and pro-inflammatory genes[156]. The absence of laminar shear stress and or disturbed shear stress results in decreased heparin sulphate expression[152] and decreased glycocalyx thickness and coverage[157]. A damaged glycocalyx results in disturbed mechanosignaling and reduced NO production in response to shear stress[158].

An increase in plasma shed glycocalyx constituents has been demonstrated over the course of a 4 hour dialysis session. However, this was not accompanied by a deterioration in sublingual glycocalyx parameters[159]. Importantly, the reliability of plasma shed endothelial components as a marker of endothelial damage in patients with significant renal impairment has been challenged, due to decreased renal excretion and unknown dialysis clearance[160].

It has been suggested that HD may not be entirely detrimental to the microcirculation. The process of HD results in the removal of circulating inhibitors of endothelial function such as; asymmetrical dimethylarginine, an inhibitor of endothelial NO production[161, 162]. Improvements in retinal microvascular function during single HD sessions have been demonstrated in several studies [163, 164]. However, these potentially beneficial effects appear

to be transient, returning to baseline within hours [161, 165]. This may however help to explain some of the heterogeneity in the literature. When studying patients on an intermittent therapy such as HD the timing of investigations with regards dialysis therapy is crucial. Timing varies greatly both between and within the studies outlined above.

The two main forms of dialysis offered to patients, HD and PD, are intrinsically different and likely to affect the systemic microcirculation in distinct ways. As a result of its acute haemodynamic effects and by virtue of the fact that they account for the large majority of the dialysis population, most microvascular work in dialysis patients has, to date, focused on HD. Studies investigating microcirculatory properties in PD patients lag behind their HD contemporaries. When PD patients are included in cohorts they are often analysed with the HD patients under the umbrella of 'dialysis requiring'. Attempts to analyse them as a sub-group are undermined by small numbers[90].

It is clear that PD patients have multiple risk factors for microcirculatory dysfunction including the cardiovascular effects of PD[166] that are both qualitatively and quantitatively different from those of HD. Studies examining the degree and nature of systemic microvascular dysfunction in PD patients compared with both healthy controls and patients with similar co-morbid profiles are lacking.

Peritoneal small solute transport, governed primarily by the peritoneal microcirculation as outlined above and measured by the PET, varies hugely between patients at initiation of PD[167]. In large epidemiological studies the only systemic factors convincingly correlating with transport status are age and

male sex[168]. There are a number of intra-peritoneal factors linked with transport, most importantly inflammation[70], but these cannot be assessed prior to commencement of therapy. Systemic microvascular dysfunction in dialysis populations has been demonstrated in multiple vascular beds. There is also evidence that it correlates with clinically relevant outcomes.

Only one previous study has attempted to investigate whether high peritoneal small solute transport reflects systemic endothelial dysfunction. They reported no correlation between transport of small solutes across the peritoneum and sublingual glycocalyx properties measured using SDF technology. However the conclusions of this study were hampered by several factors. Statistical power was limited by small numbers (n = 15) and this was a post hoc analysis of a sub group of patients recruited for a separate study. Perhaps most importantly the patients in this study had a median duration of PD treatment of 24 months[169]. As discussed above prolonged exposure to dialysate leads to local histological changes in the peritoneum that are not reflected in the systemic circulation. Clearly any relationship that may exist between the peritoneal and systemic microcirculation would be degraded over time and this question can therefore only be addressed in patients new to therapy.

In conclusion PD patients, like other patients with ESRD, are at significant risk of microvascular dysfunction. Little evidence exists as to the degree of systemic microcirculatory dysfunction in PD patients. This issue will be addressed in Chapter 3, a comparison of microcirculatory function in the skin of PD patients with both healthy controls and controls matched for significant vascular risk factors. Chapter 4 explores whether systemic microvascular function reflects small solute transport in incident PD patients.

Endothelial dysfunction may represent a significant non-traditional risk factor for cardiovascular morbidity and mortality in peritoneal dialysis, in combination with other factors it may explain the disproportionate cardiovascular risk experienced by these patients. It has been suggested that one of these additional risks may be metabolic related side effects of PD treatment. Specifically the systemic effects of absorbed glucose.

1.3 Metabolic consequences of peritoneal dialysis

1.3.1 Diabetes and the metabolic syndrome in dialysis populations

CV morbidity and mortality in dialysis cohorts is well in excess of the general population [170]. The majority of this excess risk is attributable to high prevalence of traditional CV risk factors and the consequences of chronic uraemia. However higher incidence of adverse CV outcomes has also in part been attributed to side effects of dialysis treatment. In the case of HD, repeated cardiac stunning and episodes of haemodynamic instability cause left ventricular dysfunction and are associated with sudden cardiac death[171]. Despite PD's less obvious effects on the circulation, adjusted mortality rates are broadly similar between HD and PD. This may be partially attributable to repeated exposed to supraphysiological levels of glucose in their dialysate.

Diabetes mellitus (DM) is the single most common cause of renal failure in Western populations [172]. Even in those patients without a diagnosis of DM there is a higher incidence of insulin resistance and hyperinsulinaemia than a population with preserved renal function[173], mainly as a consequence of uraemia, inflammation, metabolic acidosis and Vitamin D deficiency, all known risk factors for insulin resistance[174] . This forms part of a wider metabolic syndrome observed in dialysis patients including; obesity, hyperlipidaemia and

hypertension, which is known to predict cardiovascular mortality. Both HD and PD reduce uraemia and improve metabolic acidosis and therefore it has been suggested that insulin sensitivity in dialysis patients is improved compared with CKD5 [175]. However prevalence of the metabolic syndrome remains high in these patients. In the non-diabetic PD population prevalence of metabolic syndrome is estimated at between 39-51% [176, 177].

1.3.2 Evidence of new onset diabetes mellitus in PD

The prevalence of diabetes in dialysis cohorts is estimated at around 40%, multiple studies have reported high levels of undiagnosed diabetes and impaired glucose tolerance in dialysis and pre-dialysis cohorts [178, 179]. As outlined above dialysis patients have multiple risk factors for developing new onset diabetes, the incidence in dialysis cohorts is much higher than in the general population. Outcomes for dialysis patients with diabetes remain worse than those without[180].

New onset hyperglycaemia (fasting plasma glucose (FPG) >7mmol/L) has been reported in PD patients as soon as 4 weeks after commencing treatment[178]. Those particularly at risk have impaired fasting glucose prior to PD commencement[178, 181]. Others have reported new onset hyperglycaemia and metabolic syndrome but after longer exposure to PD (median onset of 11.4 months and 11.8 months respectively)[182]. The prevalence of metabolic syndrome in this cohort increased from 22.1% pre-PD to 69.2% ($p<0.01$) during this period.

However, large epidemiological studies comparing the risk of new onset diabetes in PD patients with their HD counterparts have produced conflicting

results. Over 6,000 PD patients were retrospectively identified from a National Health Insurance program in Taiwan between 2000 and 2010. They reported a higher incidence of new onset diabetes in PD patients compared with a propensity score matched cohort of HD patients, hazard ratio 1.61 (95% CI 1.32-1.97) [183]. This was maintained even after adjusting for the competing risk of death. Of note 44.6% of the patients included in this study had ever used 4.24% hypertonic glucose dialysate. This study was conducted between 2000 and 2010, Icodextrin was introduced in Taiwan in 2003 and there were strict rules about its prescription during this time. Since the widespread introduction of Icodextrin as a glucose sparing dialysate it is now very unusual to use such concentrated dialysate, therefore caution must be exercised in interpreting this data in the context of modern cohorts.

Using earlier data (1999-2005) from the same National Health Insurance Database the risk of new onset diabetes was found to be significantly associated with sex, age and baseline comorbidities. However they found no relationship between dialysis modality and new onset diabetes[180].

During a similar time period (1997-2005) data was collected from the Taiwan Renal Registry [184]. In this cohort of 2548 PD and over 10,000 propensity score matched HD patients they found that compared with PD patients, HD patients had a 41% increased risk of developing diabetes in the first 6 months of treatment and 2-fold increased risk thereafter. Neither of these last two studies comment on the composition of dialysate used by patients in this study however they do comment that as with the above study use of Icodextrin was minimal (<1%).

Using data from the United States Renal Data System over a 2 year period, Woodward also reported an increased rate of new onset diabetes in HD patients compared with PD (12.7% and 10.7% respectively)[185].

These retrospective, observational studies suffer with similar limitations, the nuances of which may explain differing findings in cohorts that often have a significant degree of overlap. Diabetes was diagnosed by the treating physicians using HbA1c or fasting plasma glucose. As discussed below the validity of these markers in dialysis populations is questionable. Additionally comparing outcomes between HD and PD patients is problematic as treatment modality is not randomly allocated. PD patients tend to be younger, more active and less co-morbid. In Woodward's study[185] the PD and HD groups were not well matched in terms of known risk factors for diabetes, the HD group contained significantly more men and more black people. Two of the studies used comparison propensity score matched HD groups to attempt to circumvent this issue. Propensity scoring aims to replicate randomisation but has several limitations[186]. Both studies use pre-existing data sets, so are limited by the data that has been collected. It is therefore possible that there are unmeasured confounders that will affect their analysis. Additionally different factors appear to have been used to generate the propensity score in these studies. This is also a population at high risk of events that may preclude the occurrence of the primary endpoint. For example the Wang paper[183] that reported higher risk in PD patients accounted for the competing risk of death in their analysis whilst Chou and colleagues[184] who reported higher risk in HD patients do not mention competing risks in their analysis.

The applicability of these findings to UK PD cohorts is also not certain. You will note that the majority of these studies have been conducted in Asian countries. In addition to the differing dialysis regimes used it may be that the higher propensity for DM in these populations makes their results difficult to apply to Western populations. Incidence in this cohort was 12% whilst most studies report a new onset diabetes incidence rate of between 4-6% per year in PD [176, 181, 185]. In Woodward et al's [185]study patients were taken from a list of those wait-listed for renal transplantation, this will result in a significantly skewed cohort including only those deemed fit enough for transplantation and, as this was conducted in the US those with sufficient health cover for such an operation.

1.3.3 The role of glucose-based dialysate

Commercially available dialysis solutions contain one of 3 osmotic agents; glucose, icodextrin or amino acids. Icodextrin and amino acid based solutions are licensed to be used for only one of the dwells in a 24 hour period and therefore glucose is still the most widely used osmotic agent. In general terms, glucose provides adequate ultrafiltration and is safe and cheap. The effects of conventional glucose based dialysate on the structure and function of the peritoneal membrane itself have been well documented[187]. However as a result of it's small molecular size glucose is freely absorbed from the peritoneal cavity, this results in loss of the osmotic gradient but also net absorption of glucose estimated at 100-300g per 24 hours[188]. The systemic consequences of this glucose absorption, especially in non-diabetic cohorts, is not well understood.

The amount of glucose absorbed per day from dialysate was first studied in the 1980s. At this point it was viewed as a potentially positive side effect of PD in 'undernournished' dialysis patients[188]. In the intervening years data on the high prevalence of hyperglycaemia, insulin resistance and diabetes in PD cohorts has changed our collective perspective on the contribution of the absorbed glucose in contemporary cohorts.

In observational studies dialysate glucose exposure is calculated as the total grams of unhydrated glucose within the 24 hour dialysate regime (e.g. 2 litres of 1.36% glucose based dialysate = 2 x 13.6gs = 27.2g)[179]. It is estimated that 60-80% of the daily dialysate glucose load is absorbed[189], the actual amount of glucose absorbed will vary between patients dependant on the transport characteristics of their membrane and length of dwell.

Using data from the multi-ethnic GLOBAL fluid cohort, higher random plasma glucose correlated positively with dialysate glucose exposure in non-diabetic patients with a median duration of PD of 1 year[179]. There was no such relationship in their incident patient group. Others have reported an association between dialysate glucose exposure and new onset metabolic syndrome in non-diabetic cohorts, this occurred at a median time of 11.8 months after starting PD[182]. This may therefore represent a time-point when a change in insulin sensitivity/glucose homeostasis is occurring.

Of interest the GLOBAL investigators found no relationship between random plasma glucose and either transport status (measured as D/P_{Cr} at 4 hours) or PD modality (CAPD v APD). This is in keeping with others who have

demonstrated no correlation between fasting plasma glucose or insulin resistance and transport status [178, 181, 182].

In contrast to the above, other studies found no correlation between dialysate glucose absorption and development of new onset diabetes[181] or insulin resistance[190]. The study by Dong and colleagues was a particularly rigorously conducted prospective study[181]. The diagnosis of diabetes was made in patients who had been orally and peritoneally fasted and they measured actual glucose absorption by subtracting glucose in the drained dialysate from that in the instilled. They found that the factors most predictive of new onset diabetes were age, BMI and CRP. Systemic inflammation has also been shown to be an independent predictor of metabolic syndrome development [182] and is likely to be an important covariate to consider in the interaction between dialysate glucose exposure and glucose homeostasis.

The use of random and fasting plasma glucose have limitations in this context as discussed in detail below, this is especially true in cohorts with mixed PD modalities. Due to the differing daily patterns of glucose exposure, the degree of hyperglycaemia in the overall population could be overestimated in studies comprised predominantly of CAPD patients, measuring random plasma glucose or cohorts comprised predominantly of APD patients, measuring fasting plasma glucose. In the GLOBAL cohort APD use was 6.5% in incident patients, it's use had increased in the prevalent patients as would be expected but only to 15.8%. Use of APD varies widely between countries, it is used extensively in developed countries and minimally in developing countries. UK APD usage in prevalent patients is estimated at ~40%[191]. Therefore findings in the GLOBAL cohort may not fully reflect patterns in UK patients.

All the above studies are observational and therefore their results require adjustment for confounding factors. The prescribed dialysate glucose load may be a proxy for a wider systemic issue such as membrane failure and fluid overload. Objective assessment of fluid status is difficult to document and therefore difficult to adjust for in large observational cohort studies.

1.3.4 Glucose-sparing regimes

Evidence from observational studies of a link between the glucose in dialysis treatment and glucose metrics is unclear. To overcome the biases inherent in observational studies interventional studies are required. Direct comparison with HD in the form of an RCT would be extremely challenging. However a number of studies have examined the impact on metabolic parameters of reducing dialysate glucose exposure using glucose sparing regimes.

The combined results of two large, multi-national, interventional studies (IMPENDIA and EDEN) in diabetic PD patients demonstrated the potential systemic benefits of reduced dialysate glucose exposure[192]. During a 6 month study period participants were randomised to treatment with either a glucose sparing regime (using icodextrin and amino-acid based dialysate for two of the daily exchanges) or standard all-glucose based dialysate. In an intention to treat analysis, HbA1c fell in the intervention group but remained unchanged in the control group (0.5% difference between groups, 95% CI 0.1% to 0.8% p=0.006). The separation between the two groups was observed as early as 3 months and persisted to the 6 month study end point. This corresponded with a reduction in VLDL cholesterol and serum triglycerides in the intervention group. However important to note was the increased incidence of treatment-related adverse events in the intervention group especially relating

to complications of volume overload. Subsequently the IMPENDIA[193] investigators reported that this finding could be replicated using fructosamine corrected for albumin (glycated serum protein), as a marker of glycaemic control not affected by red blood cell life span (which cause problems in the interpretation of HbA1c in this group) .

Glucose sparing regimes have also shown improvement in metabolic parameters in non-diabetic PD patients[194]. This multi-centre randomised trial of APD patients, assigned the intervention group to 2L of icodextrin for the long dwell versus 2L of 2.5% glucose dialysate in the control group. At 3 months follow-up patients in the intervention group had lower levels of insulin resistance (measured as HOMA index) compared with controls (1.49 95% CI 1.23-1.74 versus 1.89 95% CI 1.62-2.17 p=0.036). This was reflected in reduced insulin levels but of interest there was no significant difference in fasting plasma glucose or HbA1c between the groups.

1.3.5 Glucose metrics in dialysis populations

Evidence from observational studies examining the relationship between dialysate glucose and metabolic parameters is somewhat contradictory and seemingly at odds with evidence from interventional studies. A proportion of this can be explained by the need to adjust for multiple contributory factors in the observational studies. Another important consideration is the glucometabolic endpoints used and their interpretation in an ESRD population.

The American Diabetes Association requires one of the following criteria for a diagnosis of diabetes[195];

fasting plasma glucose >7mmol/l (fasting is defined as no calorific intake for at least 8 hours) OR

2 hour plasma glucose >11.1mmol/l, during a standard 75g oral glucose tolerance test OR

HbA1C >48mmol/mol OR

in the presence of classic symptoms of hyperglycaemia, random plasma glucose >11.1 mmol/l

UK diagnostic criteria mirror these with the caveat that in the absence of symptoms diabetes should not be diagnosed on a single laboratory sample but repeated on a separate day.

One should note that these definitions have changed over time especially when analysing historical cohorts. Additionally a number of difficulties exist in using these standard definitions in a renal cohort and more specifically in a PD cohort.

HbA1c

Glycated haemoglobin (HbA1c) is a measure of glycaemic control over the previous 3 month period, the average life span of an erythrocyte. The issues with the use of HbA1c are common to the whole ESRD population. Although HbA1c has been shown to correlate well with risk of developing complications of diabetes such as retinopathy and neuropathy, no patients with significant renal

disease were included in these studies [196]. The majority of dialysis patients are anaemic with reduced erythrocyte lifespan meaning less time exposed to ambient glucose and thus less glycation. Consequently in these patients HbA1c levels are lower for any given range of plasma glucose levels[197]. Duong found in a cohort of diabetic PD patients that HbA1c >8% (64mmol/mol) was associated with higher all cause mortality, however this association disappeared in patients with Hb <11g/dl[198] . Standard treatments for anaemia such as recombinant human erythropoietin and intravenous iron have all been shown to result in a falsely low HbA1c[199, 200]. KDIGO currently recommend monitoring HbA1C in diabetic patients with renal disease. They acknowledge it's limitations and the lack of high level evidence supporting it's use, however they comment that no alternative biomarker of chronic glycaemia has yet been identified with better evidence with regards to long term outcomes (KDIGO clinical practice guidelines on diabetes management in chronic kidney disease 2019).

Random plasma glucose, fasting plasma glucose and the Oral Glucose Tolerance Test

The draw-backs of the various options for measuring plasma glucose are more particular to treatment with PD. The main issue here is with the definition of fasting. PD is a continuous therapy, ensuring peritoneal as well as oral fasting would require interruption of the usual dialysis regime. Duong was able to ensure peritoneal and oral fasting of their participants[181] although this is not always practical and is impossible in retrospective cohorts. The effect of continuous intraperitoneal glucose on fasting plasma glucose and the results of an oral glucose tolerance test (OGTT) is not well studied. In fact the effects may vary dependant on several factors including degree of peritoneal glucose

exposure and transport status. The results of these investigations are much more difficult to interpret in this context than in standard patients who have been fully fasted for the requisite 8 hours.

Random plasma glucose is potentially even more problematic as even though PD is a continuous therapy glucose exposure (peritoneal and systemic) is not equivalent throughout the 24 hour period. Glucose absorption is greatest during the initial portion of a dwell when the concentration gradient is highest and different dialysate compositions (glucose and non-glucose containing) are used at different time points during the day. Patients on APD and CAPD are systematically exposed to glucose containing fluids during different time portions of the day.

Poor glycaemic control as measured using the above metrics has been associated with poor outcomes in non-renal cohorts[196]. This relationship has been more difficult to define in renal populations, partially as a result of the limitations listed above.

Fasting plasma glucose measured 4 weeks after commencing PD was reported to significantly correlate with risk of all cause mortality[178]. In multi-variate analysis every 0.6 mmol/L increase in fasting plasma glucose conferred 1.6% excess hazard of all-cause mortality. The Global Fluid study investigators also reported higher mortality associated with higher random plasma glucose but this association disappeared in incident patients after adjustment for confounders[179]. After adjustment in their prevalent group there was a trend towards increased mortality but this did not reach statistical significance.

Duong also reported a positive association between HbA1c and serum glucose and all cause mortality[198]. However this relationship was only present in the

case of significant hyperglycaemia, HbA1c >8% (63.9mmol/mol) or random plasma glucose > 300mg/dl (16.7mmol/l) and as mentioned above disappeared in anaemic patients.

Several retrospective, observational studies have reported positive correlations between dialysate glucose exposure and all-cause mortality[201, 202]. Although they adjusted for a number of covariates known to be important, the demographic differences between their high and low glucose exposure groups were significant and therefore the potential for residual confounding is high.

1.3.6 Evidence from physiological studies

As we have seen there is no strong evidence from large observational studies of a link between dialysate glucose and development of new metabolic derangement. However this conflicts with evidence from interventional studies of glucose sparing regimes showing improvements in glucose homeostasis.

Smaller physiological studies are able to look in more detail at individual dwells. As might be expected only modest increases in plasma glucose have been demonstrated using 1.36% (weakest strength) glucose dialysate[203, 204]. However, in a cross-over study of 8, non-diabetic patients, plasma glucose levels and consequently plasma insulin levels were demonstrated to rise significantly from baseline during a 2 hour dwell with 3.86% glucose dialysate[204] (Figure 1.7). This was true for conventional dialysate and biocompatible dialysate (pH neutral bicarbonate/lactate-buffered, with low glucose degradation products). In 3 of the 8 patients plasma glucose levels exceeded 11mmol/l during the 3.86% dwell. Interestingly this hyperglycaemia

and hyperinsulinaemia was accompanied by an acute elevation of blood pressure that was not seen during the 1.36% dwell. In this study peritoneal transport characteristics did not appear to affect the magnitude or rate of change in plasma glucose. However with only 8 study patients their ability to detect such a correlation will be limited.

In a larger study of 34 patients they were able to demonstrate a correlation between transport status and serum glucose levels at 15, 30 and 60 minutes into a 4 hour dwell with 4.25% glucose solution ($r=0.535$ $p=0.001$; $r=0.529$ $p=0.001$; $r=0.406$ $p=0.008$ respectively)[205].

In a mixed cohort of diabetic and non-diabetic patients significant increases in plasma glucose, compared with baseline were demonstrated in response to the more commonly used 2.5% glucose dialysate[206]. In this study the pattern of response to the same dialysate glucose load was noted to be different in the diabetic and non-diabetic groups. Blood glucose and insulin responses were delayed in the diabetic patients compared with the non-diabetics.

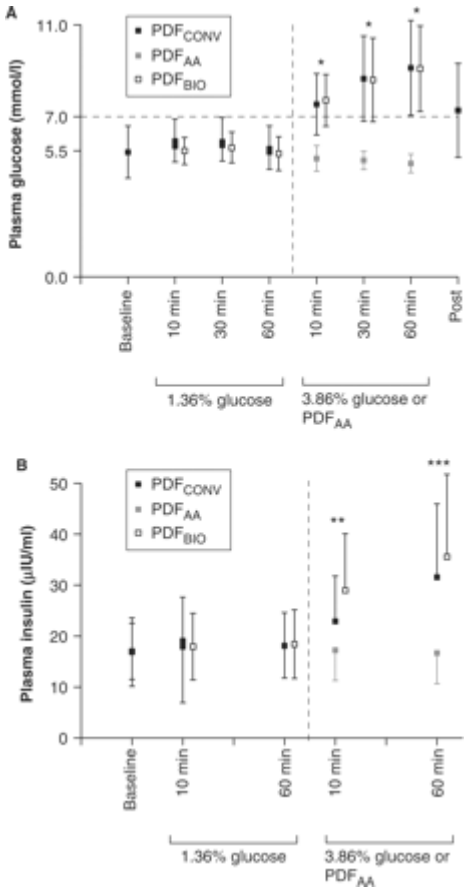

Figure 1.7 Plasma glucose (A) and insulin levels (B) during dwells with 1.36% and 3.86% glucose reproduced with permission from Selby et al 2007 *NDT* 22:3 870-879

Plasma and insulin levels rose significantly during 3.86% dwells compared with baseline and dwells with amino acid based dialysate (PDF_{AA}). PDF_{CONV} = conventional dialysate, PDF_{BIO} = biocompatible dialysate. Data are mean ±SD. *p<0.001 vs baseline, **p<0.05 vs PDF_{AA}, ***p<0.05 vs baseline, p<0.001 PDF_{CONV} vs PDF_{AA} and p<0.01 PDF_{BIO} vs PDF_{AA}.

As seen in the larger cohorts all these studies reported a high prevalence of insulin resistance in the PD patients without a diagnosis of diabetes.

There remains some difficulty in comparing these studies as none were peritoneally fasted and all used different composition dialysates the night before the test, potentially affecting both the fasting blood results and the response to the ‘tested’ solutions.

1.3.7 The role of continuous glucose monitoring and glycaemic variability

Evidence from single dwell studies has been extended using continuous glucose monitors. These studies benefit from the ability to analyse the effect of clinically used regimes (as opposed to the hypertonic solutions used in some of the above studies) and the interaction with real-life factors such as oral energy intake and exercise.

First introduced in diabetes care at the turn of the millennium, continuous glucose monitoring systems (CGMs) have proven to be useful in a number of research settings[207]. CGMs measure glucose levels in interstitial fluid via a subcutaneous sensor, with a measurement interval between 5 and 20 minutes. In the context of PD they allow evaluation of short-term, peri-exchange glycaemic changes. Results can be analysed in the context of the 24 hour dialysis regime over-coming the issues with random and fasting plasma glucose outlined above. Measurements allow calculation of mean glucose values but also oscillations in blood glucose over the 24hour period - glycaemic variability (GV).

There are numerous measures of GV outlined in more detail in Chapter 2. As would be anticipated there is a high degree of correlation between the different measures. The gold standard measurement is standard deviation of glucose (SDG) as nearly every other measure is strongly correlated with this [208]. Although there is strong laboratory evidence of increased GV causing harm[209] there remains much debate within the diabetic community as to it's role as a predictor of diabetic complications[210, 211]. However, given the variability in prescribed regimes and lack of true fasting status, GV may be more instructive in the PD population than average glucose.

Early CGM work in diabetic PD patients demonstrated several important points; blood glucose levels were well above the recommended levels for large portions of the day and finger-prick glucose testing as routinely carried out by diabetic patients missed many hours of very high readings[212].

The exact relationship between dialysate glucose and plasma glucose is again debatable. In a cross-over study of 8 diabetic patients Marshall et al[213] found that a glucose sparing regime was associated with reduced mean glucose and reduced glycaemic variability compared with traditional glucose containing regimes. In contrast Mori[214] found no correlation between 24hour peritoneal glucose absorption and standard deviation of glucose but a strong correlation between standard deviation of glucose and small solute peritoneal transport ($r=0.71$ $p=0.031$). Skubala[215] also reported a relationship between transport status and mean glucose levels, however only patients with high and high-average transport were represented in this cohort and the unusually high incidence of peritonitis during the study period (14/30 patients) makes their data almost uninterpretable.

Multiple studies reported no correlation between HbA1c and mean CGM glucose levels [214, 216, 217].

In the majority of the large studies above patients undergoing APD and CAPD have been analysed together despite the very different pattern of their glucose exposure. Okada[218] demonstrated that this different exposure results in differing patterns of plasma glucose which would not be appreciated by time averaged measures such as HbA1c. They reported a significantly lower standard deviation of glucose and difference in the maximum and minimum blood glucose over 72 hours in APD patients compared with CAPD despite their comparable HbA1cs.

These studies have significant limitations on the conclusions they can draw. Small patient numbers limited their statistical power although some have adopted a cross-over design to improve this. Other important factors especially in small cohorts is heterogeneity of population, for example of the 8 patients in Marshalls study [213] 5 had Type 2 diabetes and the other three had Type 1 diabetes, Skubala[215] included diabetes and non-diabetics in their cohort. With regards people with diabetes there will be significant variation in the medications prescribed, all studies contained patients on a mixture of oral hypoglycaemic agents and insulin. Additionally, a number of the studies appear to have been conducted retrospectively using data from patients who had worn CGMs, therefore their results may be biased towards those with poor control. There is also limited baseline metabolic profiling of the patients which may limit their applicability to wider populations.

Despite their significant limitations, small physiological studies can play an important role in our greater understanding of this interaction. Data from these studies can be used in larger studies to inform protocol decisions for example; the most appropriate outcome measure, when samples should be taken in relation to dialysis treatment, representative daily patterns within different subgroups and the degree of improvement that might be expected in any interventional trials. However currently we are lacking robustly designed studies using CGMs in diabetic PD patients and to my knowledge no studies have addressed this issue in a purely non-diabetic cohort.

Compared with the general population, PD patients are at significantly increased risk of metabolic syndrome and diabetes mellitus, both of which

predispose to cardiovascular morbidity and mortality. The role of glucose, absorbed from peritoneal dialysate, in this pathology is unclear. Results from large observational studies are contradictory and differ from findings in small interventional and physiological studies. This may be partially attributable to difficulties in choosing and interpreting outcome measures. In Chapter 5 CGM technology is used address the issue of how glucose absorbed from dialysate affects average glucose levels, glucose oscillations and 24 hour glucose profiles in PD patients compared with a pre-dialysis cohort.

1.4 **Aims and hypotheses**

The overall aim of this thesis is to address the role of some of the putative non-traditional risk factors in excessive levels of cardiovascular morbidity and mortality in peritoneal dialysis patients. Additionally, how systemic changes such as microvascular dysfunction can influence dialysis therapy and how dialysis therapy, specifically high dialysate glucose concentrations, may provoke systemic changes.

To explore this I will test the following hypotheses;

- Patients on peritoneal dialysis will exhibit a greater degree of systemic microvascular dysfunction than healthy controls and controls with similar cardiovascular and metabolic profiles
- Incident peritoneal dialysis patients with a high rate of small solute transport will exhibit a greater degree of systemic microcirculatory dysfunction than those with slower rates of small solute transport
- Stable, non-diabetic patients on peritoneal dialysis will exhibit a greater degree of glycaemic variability compared with non-dialysis controls

Chapter 2 Detailed methods

To aid the flow of the results chapters this chapter contains detailed methods of the techniques used in all of the studies contained in this thesis. These techniques will be given context within individual chapters. Unless otherwise stated all procedures were performed by myself.

2.1 The skin as a model of the microcirculation

A number of the techniques used in the studies outlined in Chapters 3 and 4 examine reactivity of the skin microcirculation and therefore I will briefly outline the role of the human cutaneous circulation as a model of generalised microvascular function. The structure of the skin vasculature is unique and highly specialised (Figure 2.1). Arterioles and venules are connected through two horizontal plexuses. The upper horizontal plexus is in the papillary dermis within 1mm of the skins surface, it is from this that the capillary loops arise. The arterioles that control blood flow to the capillary loops are typically 10-100μm in diameter, highly innervated and comprised of an inner lining of endothelial cells encircled by a dual layer of vascular smooth muscle cells[219]. In contrast, the capillary loops have a much smaller diameter of 8-10μm, are not innervated and do not contain a smooth muscle layer. The lower plexus is at the dermal-hypodermal interface. These vessels are typically of greater diameter with 4 or 5 layers of vascular smooth muscle[220]. This anatomical arrangement differs subtly between skin regions. In glabrous (non-hairy) skin arteriovenous anastomoses bypass resistance vessels thereby directly connecting arterioles and venules, these lie deeper in the dermis than capillary loops and therefore are less efficient for thermoregulation.

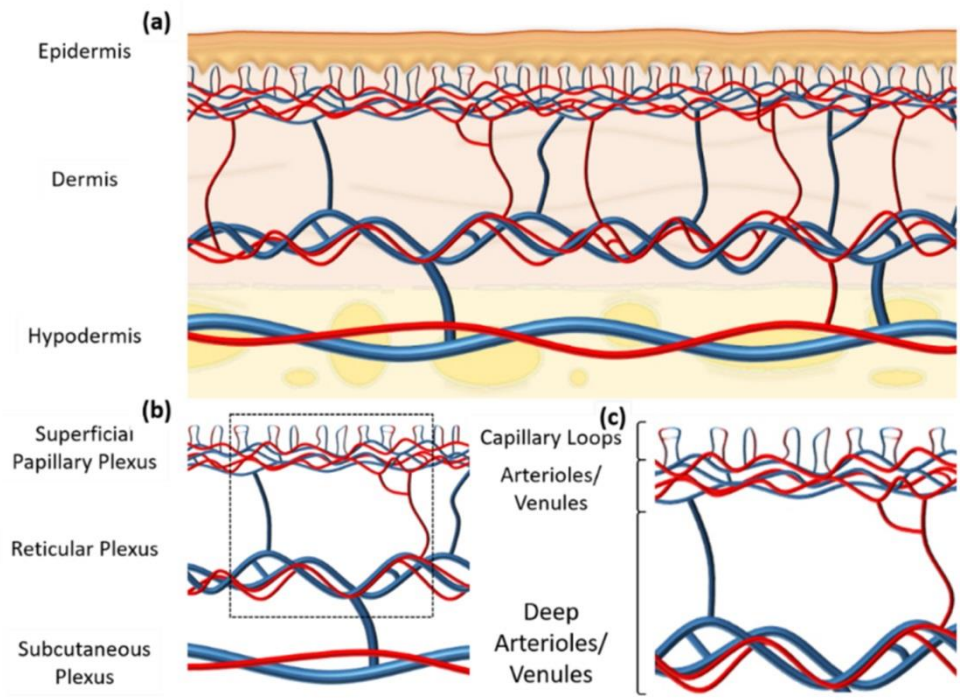

Figure 2.1 The cutaneous microcirculation (reproduced from Deegan and Wang 2019 with permission[221]). a) Cartoon depicting the 3 primary layers of the skin: the epidermis, dermis and hypodermis b) cartoon highlighting the vasculature of the skin in 3 layers; superficial papillary plexus, reticular plexus and subcutaneous plexus. The perforated black square highlights the vessels predominantly, but not exclusively, the focus of cutaneous imaging modalities c) closer look at the papillary and reticular plexuses – capillary loops and smaller arterioles and venules of the papillary plexus and the deeper, larger arterioles and venules of the reticular plexus.

The cutaneous microcirculation is unique in its role as the main effector organ of thermoregulation. It also has to perform the duties expected of microvasculature elsewhere namely; delivery of oxygen and nutrients to tissues and subsequent removal of waste products, fluid homeostasis and facilitation of the inflammatory response. Skin blood flow can vary hugely, from 250ml/min at rest [222] up to 8L/min in conditions of severe heat stress[223]. Variations in skin blood flow are precisely controlled and result from complex interactions between; arteriolar myogenic responses, endothelium dependant flow-induced vasodilation, metabolic and neurovascular interactions[102]. The inter-play of the mediators in these pathways are understood to varying degrees and depend

on the provoking stimuli and the position of any given vessel in the vascular tree, as discussed below.

There are a number of factors that make the cutaneous circulation an attractive model for the study of systemic vascular function in humans. Perhaps of greatest importance is that the superficial capillary loops are located within a millimetre of the skins surface making it the most easily accessible vascular bed in humans. In addition, as a result of its role in thermoregulation it has significant potential for vasodilatation in response to numerous stimuli Consequently a number of minimally-invasive techniques have been developed to study it's function[110], several of which will be discussed in detail later in this chapter. The forearm or lower leg are the sites most commonly studied however other sites can be accessed depending on the specific research question[224]. In essence these techniques utilise metabolic, thermal and pharmacological stimuli to manipulate putative signalling pathways associated with vascular pathology and measure the vasodilatory response using laser Doppler technology (discussed below). Using these techniques, pathology induced microvascular dysfunction of the skin has been demonstrated in patients with a wide variety of conditions including; hypertension[225], Type 2 diabetes[133], coronary artery disease[121] systemic sclerosis[226] and CKD [108]. The skin has also been used as a predictor of global vascular health as changes in cutaneous microvascular structure and function have been shown to correlate with increased risk of cardiovascular events[125, 227].

There are however several challenges associated with using the skin as a translational model to investigate vascular dysfunction in humans. Many of the techniques used to stimulate vasodilatation do so via multiple mechanisms

which are not specific to vascular signalling pathways. This is a common problem with the majority of *in vivo* work rather than being exclusive to the skin vasculature and necessitates the complimentary use of *in vivo* and *ex vivo* work to refine our knowledge of specific physiological pathways. Due to the superficial nature of the capillary bed it is easily influenced by additional stimuli, most notably in this case, ambient room temperature. Due to it's role in thermoregulation the skin is acutely sensitive to changes in ambient temperature, especially in the extremities. This can result in significant changes in cutaneous vascular flow[228]. Consequently it is vital that temperature in the study area is maintained (usually at 21-24°C) and changes in participants' skin temperature are noted and considered when evaluating the results.

2.2 **Laser Doppler**

Changes in blood flow within the skin's microcirculation can be measured using laser Doppler flowmetry. The Doppler principle, named after mathematician and physicist Christian Johann Doppler, describes a shift in the observed frequencies of waves derived from moving objects compared with static objects. This principle applies to both sound and light waves. When the coherent monochromatic light emitted by the laser Doppler transducer encounters moving red blood cells (RBCs) in the microcirculation it undergoes a change in wavelength. Light hitting static objects, such as constituents of the skin, remains unchanged. The combined non-shifted and Doppler shifted beams are reflected back and measured by a photodiode, Figure 2.2. The change in magnitude and frequency distribution is related to the number and velocity of the RBCs[102]. This is most often recorded as red blood cell flux and expressed in perfusion units or arbitrary units. Although this is not a direct measure of blood flow, a linear relationship between flux and flow has been demonstrated[229]. It can

also be considered an indirect measure of vasodilatation, which results in increased flow or flux in the studied area.

Most laser Dopplers have a penetration depth of 1-1.5mm, therefore the flux signal will reflect activity in arterioles, capillaries, post-capillary venules and venules[230]. Laser Doppler can be used to measure relative changes in red blood cell flux over time at a single point or over a larger area. Laser Doppler flowmeters (LDF) traditionally have one transmitting and one receiving optical fibre. Newer probes have a single laser Doppler fibre surrounded by several receiving fibres. They have a high sampling frequency of around 32Hz but sample over a small volume of around $1mm^3$. This level of penetration usually results in flux recordings from the sub-epidermal papillary loops and the arterioles located in the superficial and median derma[110]. Due to the high sampling frequency LDF has excellent temporal resolution, making it ideal for monitoring reactive flow, however the small area of measurement results in poor spatial resolution and contributes to problems with reproducibility.

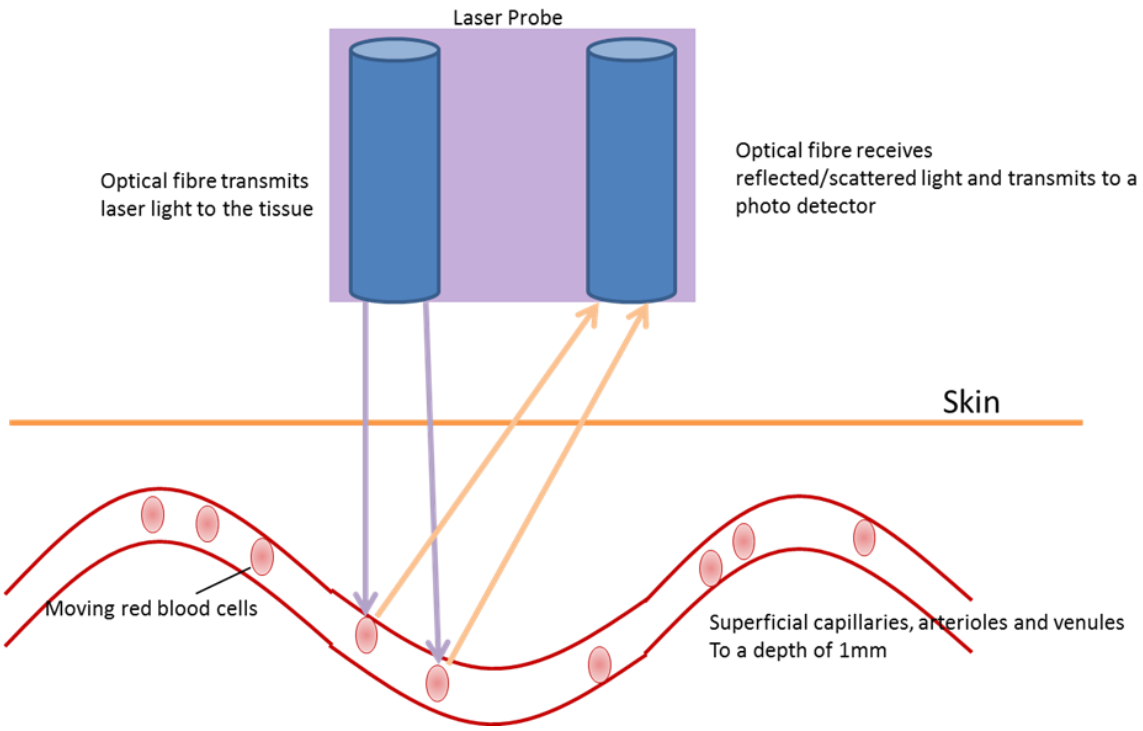

Figure 2.2 Schematic representation of the principles of laser Doppler measured flux.

Measurements over a greater area are conducted using Laser Doppler Perfusion Imaging (LDPI). The laser beam is emitted above the skin and reflected by a computer driven mirror. It progressively scans the area and produces a 2D perfusion map for that area Figure 2.3. This enables mapping of a large number of adjacent sites on the skin. In addition to absolute flux values this larger scan area also gives an indication of vascular flow heterogeneity. The spatial resolution is superior and site-to-site variability is improved compared with LDF however as each scan takes up to 10 seconds the temporal resolution suffers.

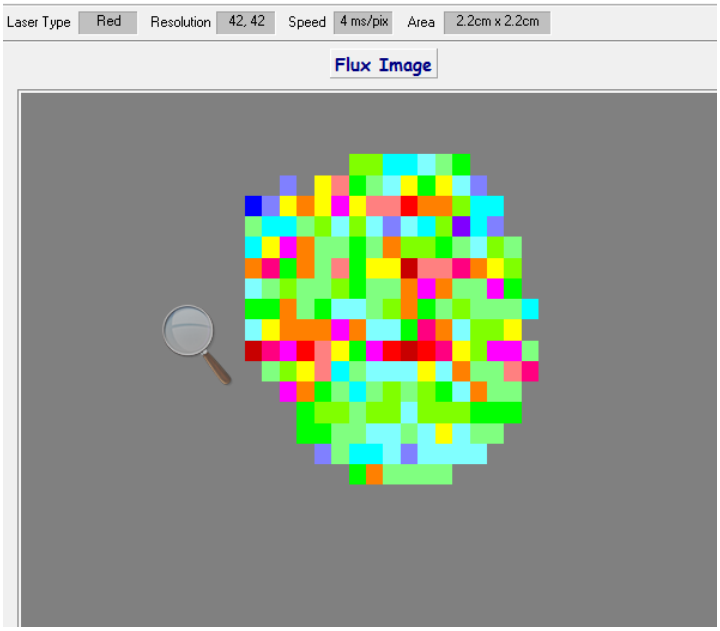

Figure 2.3 2D perfusion image generated using laser Doppler perfusion imaging. Brighter colours indicate greater flux.

There are several important potential limitations to consider when conducting and interpreting studies using laser Doppler technology. Perfusion units are arbitrary and not standardised units. Consequently a disadvantage of laser Doppler based studies is that it is difficult to compare absolute flux values between studies using different probes or brands of device. Outcomes are often reported as a percentage of change from the biological zero (during arterial occlusion), resting baseline or maximal vasodilatation, usually induced by local heating. Care must be taken in presenting data only as a percentage change or percentage of maximum as this may conceal differences in absolute skin blood flow between different groups[222].

Even when perfusion is absent flux does not reach a zero value. This is a consequence of Brownian motion of macromolecules in the interstitial space.

This absolute value is usually small but best practice dictates that the flux at biological zero be subtracted from the flux values.

The emitted light can be scattered by any moving object and therefore the flux signal from red bloods cells is easily disrupted by other artefactual signals such as patient or probe movement, both of which can cause flux signals larger than the blood flux[231]. Strict protocols must be adhered to in order to limit potential movement artefact and any movement noted must be recorded contemporaneously to avoid misinterpretation of the data at a later stage.

Another limitation of Doppler based techniques is they provide no morphological information for example on capillary density. Capillaroscopy is often performed as a complementary technique for a fuller assessment of the microcirculation.

Laser-based techniques can be used to assess skin microcirculation either purely observationally (baseline flow) or by examining the response to a physical or pharmacological challenge. The area measured by the Doppler probe is usually small and there is inherent heterogeneity in microcirculatory structure and flow. Therefore site-to-site and day-to-day comparisons of flux can be highly variable[232]. Reproducibility of reactivity tests is better than baseline cutaneous flow[233]. The major source of day-to-day variability is the site of the measurement, when the site is standardised, and raw data is normalised against biological zero or maximal vasodilatation, the reproducibility of reactive tests such as post-occlusive reactive hyperaemia and iontophoresis of acetylcholine are improved and compare favourably with other vascular techniques such as flow mediated dilatation of the brachial artery (the standard test of endothelial function in larger vessels)[234].

In summary the reactivity of the skin microcirculation in combination with laser Doppler imaging has been widely used as a model for investigating vascular dysfunction in humans. It's major advantages include; non-invasive real-time assessment of flow, however it has some important limitations that need to be taken into consideration when designing an appropriate protocol to answer specific research questions. I have utilised two techniques combining laser Doppler with stimulatory tests of cutaneous microcirculation in my PhD work and they are outlined below.

2.3 Iontophoretic application of vasodilators

Background

Iontophoresis

Iontophoresis allows minimally invasive trans-dermal delivery of charged molecules to a localised area of cutaneous microcirculation. Iontophoresis is based on the principle that a charged molecule in solution will migrate across the skin under the influence of a low-intensity electric current[111] Figure 2.4. In research settings it is used primarily for the delivery of vasoactive substances to the cutaneous microcirculation. Consequent vasodilatation is measured using Laser Doppler Perfusion Imaging.

The quantity of drug delivered depends on a number of factors; the magnitude and duration of the current, the concentration and pH of the solution used and the barrier properties of the skin.

The application of anodal and cathodal charges alone, without chemical vasodilators, has been demonstrated to result in non-specific vasodilatation.

This current mediated vasodilatation is especially pronounced following a cathodal current[235]. Although the exact underlying mechanism is unclear, it is speculated that this is the result of activation of local sensory nerves via an axon reflex, as application of topical anaesthesia prior to iontophoresis inhibits non-specific vasodilatation[235]. The iontophoretic charge can be delivered either in the form of a continuous current or multiple current pulses separated by current-free intervals. The amplitude of the current-induced vasodilatation depends on the delivered electrical charge and the delivery pattern[236]. Recommendations are to use a low intensity current and limit current and charge density as far as possible[237]. It has been suggested that the contribution of current induced vasodilation to the final value may be accounted for by subtracting the response to the vehicle substance measured at a control site from the response to the active substance[235]. However when the vasoactive substance is not present in the vehicle solvent the ionic composition of the solution is changed, this will potentially affect the size of the non-specific vasodilatation exhibited[238]. For this reason coupled with the spatial and temporal heterogeneity of skin perfusion, indiscriminate use of this approach may produce erroneous results[234].

The use of non-polar solvents such as deionized water enhance iontophoretic delivery of the active substance by limiting the number of competing ions however it induces more current-induced vasodilatation. Conversely saline solutions will reduce the degree of iontophoretic transport, sodium and chloride having greater electrical mobility than larger molecules and will therefore account for a larger fraction of the current, but also the current-induced vasodilatation [219]. Iontophoretic protocols are designed to balance avoidance

of non-specific vasodilatation with the need to reach a plateau in the drug-response curve.

The barrier properties of the skin vary between individuals but also between different skin areas depending on the density of epidermal appendages such as sweat glands and hair follicles. The pathway taken by a given molecule will depend on the properties of that particular molecule. For charged drugs, such as are used commonly in microvascular studies, the predominant route appears to be via hair follicles and sweat ducts as opposed to through intercellular spaces in the stratum corneum[239]. Inherent barrier properties of the skin favour the delivery of small cationic drugs as the skin carries a net negative charge at physiological pH. Measurement of skin resistance is possible by connecting a voltmeter in parallel and correcting for skin resistance in outcome reporting improves reproducibility [240] however in practice this is rarely done. Although there is little that can be done to standardise anatomy, standard good practice would dictate mild epidermal stripping with adhesive tape and an alcohol swab to reduce resistance from build-up of elements on the skins surface and to minimise the iontophoresis of those elements [237].

The above factors in combination mean that, unlike direct injection, the exact quantity of drug delivered is unknown and will vary between individuals, even when using identical protocols. Microinjection of these vasodilators delivers a more consistent dose. It also avoids the confounding effect of the current, however non-specific vasodilation is provoked by needle insertion trauma and this technique is much more invasive and therefore less acceptable to participants.

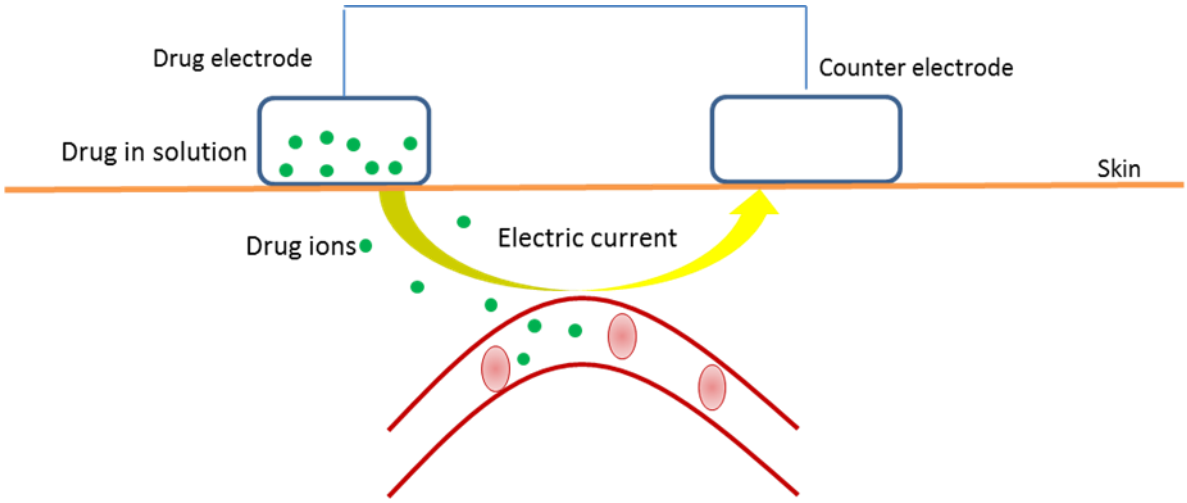

Figure 2.4 Schematic representation of iontophoretic delivery of vasoactive substances

Pharmacological vasodilatation

The most commonly used vasoactive agents are acetylcholine (ACh) and sodium nitroprusside (SNP), which are used to induce endothelium-dependant and non-endothelium dependent vasodilatation respectively. Positively charged ACh is driven by an anodal current and negatively charged SNP is driven by a cathodal current.

ACh is a muscarinic receptor agonist. Cholinergic vasodilatation is dependant on a functioning vascular endothelium and may induce vasodilatation via multiple pathways including prostanoids[235], nitric oxide (NO)[241] and endothelium derived hyperpolarising factor dependant pathways[242]. The relative contributions of these mediators varies between tissues and levels of the vascular tree. Approximately 50% of total forearm vasodilatation in response to an infusion of ACh is thought to be mediated by NO derived from the endothelium. However at the level of the cutaneous microcirculation it's role has

been debated. Several studies have proposed a minimal role for NO[243, 244]. In a study of ACh application using intradermal microdialysis, thus avoiding any current induced vasodilatation, inhibition of nitric oxide synthase did not significantly attenuate ACh mediated dilatation of human cutaneous vessels [242]. However, also using intradermal microdialysis, Boutsiouki and colleagues[245] reported a 30% decrease in induced blood flux after NOS blockade. In healthy male volunteers, inhibiting prostanoid production with Aspirin was shown to result in a 53% reduction in the response to iontophoretically applied ACh[243]. However this has also been disputed [235]. It has been suggested that the NO and prostanoid pathways are capable of enzymatic cross-talk such that inhibition of one leads to upregulation of the other[246]. Combined inhibition of both pathways results in an inhibitory effect greater than the sum of inhibiting them individually[247]. It seems likely that a complex interaction between these pathways is responsible for the observed vasodilatory response to ACh.

SNP is essentially exogenous delivery of NO to the vascular smooth muscle cells. SNP reacts with tissue sulfhydryl groups to produce NO, which acts directly on the smooth muscle, circumventing the endothelium and producing non-endothelium dependant vasodilatation[238].

Iontophoresis derived outcomes

The major reported outcome measures for iontophoresis of ACh and SNP are peak response and total response (area under the curve for the total monitoring time). As discussed above these are measured by laser Doppler and recorded in arbitrary or perfusion units. A common reporting method is to describe the peak response as a percentage change in perfusion from baseline or

percentage of maximum perfusion. However total reliance on baseline measures risks obscuring differences in absolute skin blood flow between study groups. Incorporation of the maximum perfusion response, usually measured as a response to localised heating, assumes that the experimental maximum is a true representation of physiological maximum[238].

Iontophoresis coupled with laser Doppler perfusion imaging allows the local administration of very small amounts of active substances to the cutaneous microcirculation without affecting the systemic circulation. The complexity of the underlying mechanism and other limitations as outlined above must be acknowledged. However, when assessed over a relatively large area using LDPI the reproducibility of ACh and SNP iontophoresis is good[240] and it is a useful tool for interrogating the cutaneous microcirculation in multiple pathological conditions.

A reduced response in the skin microvasculature to iontophoretic application of ACh has been demonstrated in patients with coronary artery disease[125], type 2 diabetes and obstructive sleep apnoea[248] as well as patients on haemodialysis treatment[91]. Impairments in the response to ACh have been shown to reflect impaired endothelial function in other vascular beds including the coronary microcirculation[249].

Measurement protocol

All participants were asked to attend for a morning study visit having fasted for at least 8 hours overnight and without any traditional glucose based dialysate in situ. All participants were given a standardised breakfast of 2 pieces of brown bread toast with butter and a glass of water.

All studies were performed at a neutral room temperature of 23±1°C. All subjects were allowed to acclimatize in this temperature for 30 minutes prior to investigations beginning. Patients were studied in a semi-recumbent, supine position. The flexor aspect of the right arm was used unless the participant had an arterio-venous fistula on that arm, in which case the flexor aspect of the left arm was used. Presence of a fistula in either arm was recorded, as microvascular changes have been demonstrated both in the fistula arm and contralateral arm[138]. The forearm was gently wiped with alcohol and then sterile water. For each test substance separate Perspex direct electrode chambers (30mm total diameter x 3mm height, inner chamber 10mm diameter) were attached to the flexor surface using an adhesive ring. Care was taken to avoid cross-contamination between these chambers which were cleaned thoroughly with alcohol and water prior to each use. Care was taken to avoid visible large vessels or skin anomalies.

Endothelium-dependant vasodilatation

A direct electrode chamber was filled with the ACh vehicle substance (3% w/v mannitol in water, prepared by Royal Devon and Exeter Hospital Pharmacy). A glass cover slip was placed over the chamber. An indifferent electrode was attached to the flexor aspect of the wrist. An anodal current of 0.1mA was applied for 5 20 second bursts with 60 seconds between currents. In a different direct electrode, this process was then repeated at a separate site using ACh (1% w/v Miochol-E, Novartis, Camberly, UK).

Endothelium-independent vasodilatation

A direct electrode chamber was filled with SNP vehicle substance (0.45% w/v saline, Baxter UK). A glass cover slip was placed over the chamber. An

indifferent electrode was attached to the flexor aspect of the wrist. A single cathodal current of 0.2mA was applied for 60seconds. Flux at this site was recorded immediately prior to the current application and at 60 second intervals after for 5 minutes. In a different direct electrode chamber, this process was then repeated at a separate site using SNP 0.25% w/v (25mg/ml Hospira Pty Ltd, Melbourne) dissolved in 0.45% saline.

Measurements of forearm skin temperature were documented throughout the study.

Measurements of flux were taken using a solid-state laser Doppler perfusion imager (LDPI), Moor LDI (Moor Instruments, Axminster, Devon). The head of the LDPI was positioned 50cm above the Perspex chamber. The area scanned was 2.2cm x 2.2cm. The current was delivered by a battery-powered iontophoresis controller (Moor iontophoresis controller, MIC2, Moor Instruments, Axminster, Devon). The LDPI was interfaced with a computer equipped with MoorLDI laser Doppler imager software Version 5.3.

Prior to every study visit the laser Doppler was calibrated using the manufacturers sealed calibration boxes. The flux value of the sealed boxes did not change significantly over the study period.

Scans were analysed using the same MoorLDI software. The region of interest was defined manually by selecting the area that represented the inner chamber on the scanned images. Data was recorded as peak response during the monitoring period in arbitrary units (AU) and total perfusion of the area of interest (area under the curve), adjusted for baseline.

Reproducibility

4 healthy non-smoking volunteers participated in my reproducibility study: 3 female and 1 male. They each attended for 3 separate study visits under standardised conditions as outlined above (Table 2.1 - Table 2.4). The overall inter-day coefficients of variation (CV) were; ACh peak response 9.7%, ACh AUC 12.2%, SNP peak response 16.3% and SNP AUC 22.5%.

Table 2.1 Peak response to ACh iontophoresis (arbitrary units) measured on 3 occasions in 4 subjects

Subject	Visit 1	Visit 2	Visit 3	Mean	CV (%)
1	406	474	473	451	8.64
2	705	622	563	630	11.32
3	547	584	497	542.7	8.05
4	383	454	470	435.7	10.63

Table 2.2 Area under the curve of ACh iontophoresis (arbitrary units) measured on 3 occasions in 4 subjects

Subject	Visit 1	Visit 2	Visit 3	Mean	CV (%)
1	88050	117480	115960	107163.3	15.5
2	192010	153770	136860	160880	17.6
3	123340	125110	117780	122076.7	3.1
4	82410	106370	98490	95756.67	12.8

Table 2.3 Peak response to SNP iontophoresis (arbitrary units) measured on 3 occasions in 4 subjects

Subject	Visit 1	Visit 2	Visit 3	Mean	CV (%)
1	356	421	432	403	10.2
2	342	544	359	415	27
3	348	392	338	359.3	8
4	356	368	249	324.3	20.2

Table 2.4 Area under the curve of SNP iontophoresis (arbitrary units) measured on 3 occasions in 4 subjects

Subject	Visit 1	Visit 2	Visit 3	Mean	CV (%)
1	56520	72800	75780	68366.7	15.2
2	65080	114000	61840	80306.7	36.4
3	59360	71960	54840	62053.3	14.3
4	52890	57950	35500	48780	24.1

This is in line with reproducibility data obtained by other researchers in the department, (average CV of 20% across all parameters) and also with published figures from other groups[250].

2.4 Post occlusive reactive hyperaemia (PORH)

Background

PORH is a method of measuring the microvascular response to a brief period of arterial occlusion which results in a transient increase in cutaneous flow that gradually degrades back to baseline[110]. Figure 2.5

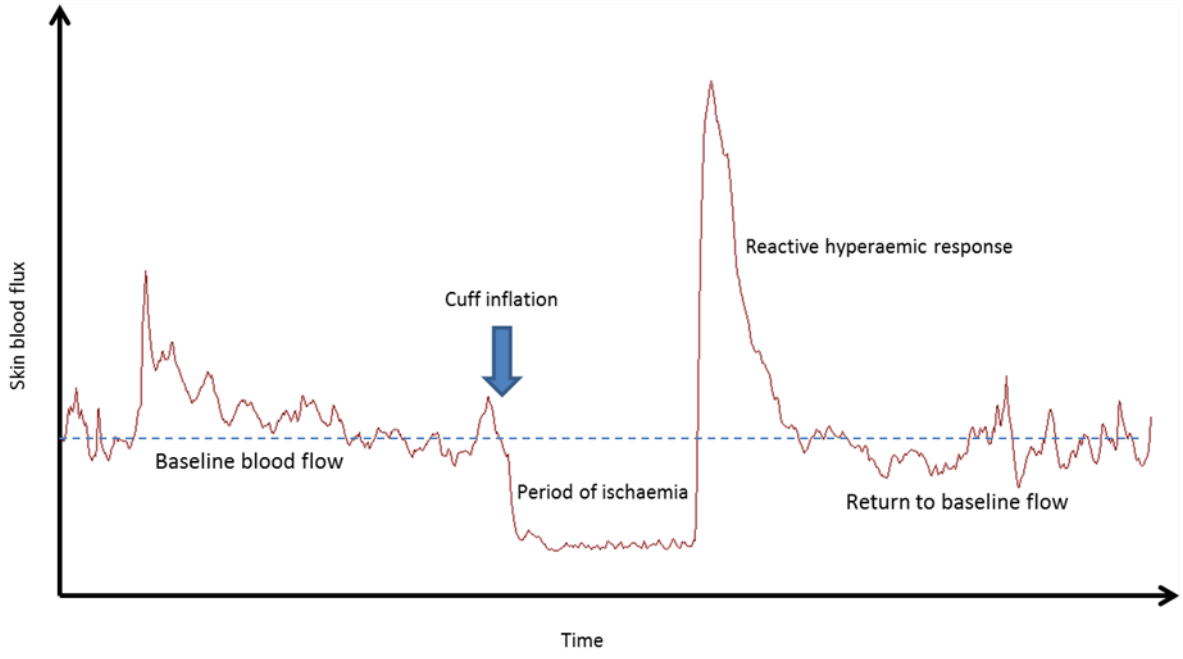

Figure 2.5 Representative laser Doppler trace obtained before and after a brief period of arterial occlusion

A reactive hyperaemia can be induced by inflating a cuff placed around the upper arm or leg and then releasing the pressure in the cuff. Upon release of the cuff the response takes the form of a post-ischaemic flow initially many times faster than baseline followed by exponential decay to baseline.

PORH is said to provide a global assessment of cutaneous microvascular function, however it is a complex response, the result of endothelium dependant and independent mechanisms. Sensory nerves are thought to play a significant

role in the reactive vasodilatation via local axon reflexes, contributing to both the height of the peak and the time course of the reaction[251, 252]. The return of flow into a previously occluded vessel lined with healthy endothelium causes release of multiple vasodilating substances from the endothelium. In contrast to flow mediated dilatation in larger vessels such as the brachial artery[253] it has been suggested that NO plays a limited role in the post occlusive hyperaemic response in cutaneous microcirculation. Blocking NO synthesis by inhibiting NO synthase does not significantly alter PORH in the skin [254, 255]. In support of this NO levels measured in the forearm did not rise significantly in response to increased skin blood flow elicited by PORH[256].

There is conflicting evidence surrounding the role of prostanoids[257-259]. A meta-analysis conducted in 2015[257] of studies investigating the effects of COX inhibition on skin peak PORH reported significant heterogeneity between studies but concluded overall a neutral effect of COX inhibition. They commented that differences in drug administration, oral versus microdialytic, may account for discrepancies in the literature.

In combination with the local axon reflex, endothelium derived hyperpolarising factors (EDHF) produced through the cytochrome epoxygenase pathway are thought to be the most significant mediators of the hyperaemic response[260, 261]. The role of EDHFs is postulated to be more important in the time course of the reaction rather the peak[261]. Blockade of sensory nerves and EDHF mediated vasodilatation individually was shown to result in a significant reduction in the peak response and AUC curve responses[260]. In half these subjects, blockade of either pathway reduced peak response by 70-95% and in

some subjects combined blockade of both pathways completely abolished the hyperaemic response.

In contrast to iontophoresis, the requirement for high temporal resolution in recording the post occlusive response which lasts only a few seconds, means measurements are recorded using single-point LDF. This compromises on spatial resolution and impacts the reproducibility of the above outcome measures. Most studies are conducted using the volar aspect of the forearm and reproducibility varies mainly depending on how stringently the location of the laser probe is recreated. When the site is marked, ensuring exactly the same site is measured day-to-day CVs of between 6% and 22% are recorded[262].

PORH derived outcomes

The most commonly reported outcome measure is peak hyperaemic flux. As discussed above this can be expressed as a raw value or more commonly as a function of baseline e.g. percentage change from baseline. However it has been reported that absolute values have a lower inter and intra-day variability than data expressed as a percentage of baseline[263]. The most reproducible measure appears to be peak flux expressed as a percentage of the maximum vasodilation, achieved usually by local skin heating[228]. Another commonly reported outcome is extent of the hyperaemic response, area under the curve, either for the whole reactive phase or the first minute after cuff deflation. Time to peak perfusion is frequently quoted however it's physiological significance remains to be established[237].There is no consensus in the literature on parameter selection.

As with iontophoresis a major issue when attempting to directly compare outcomes across studies is heterogeneity of protocols. There is significant variability in the duration of arterial occlusion, ranging from 1 to 15 minutes. This is of importance as there is a positive correlation between the size of the hyperaemic response and the duration of arterial occlusion[254, 263]. The most commonly used occlusion time is 5 minutes, probably as this is the standardised time used in flow mediated dilatation of the brachial artery, although no such standardisation method exists for the evaluation of microvascular function[237].

PORH has been used extensively to investigate generalised cutaneous microvascular function. An attenuated hyperaemic response has been demonstrated in patients with hypertension[264], peripheral arterial disease[265], coronary artery disease[121] and Type 2 diabetes[126]. The average peak flux post occlusion has been shown to be reduced in patients on HD [91, 108]. Impairment in this cutaneous hyperaemic response have been shown to correlate with changes in other vascular beds including increased renal resistive index[266] and increasing albuminuria[267].

Measurement protocol

All subjects were asked to attend for a morning study visit having fasted for at least 8 hours overnight and without any traditional glucose based dialysate *in situ*. All participants were given a standardised breakfast of 2 pieces of brown bread toast with butter and a glass of water. All studies took place in a temperature controlled room (23±1^{o}C). All participants were acclimated to this temperature for 30minutes prior to investigations commencing. Participants were studied in a semi-recumbent, supine position. Participants' right arm was

used for the study unless there was a fistula present in which case the left arm was used.

Flux was captured using a Moor instruments DRT4 laser Doppler perfusion and temperature monitor (Moor Instruments, Axminster Devon). This was calibrated prior to every study visit using a calibration box containing motility standard solution. The flux values obtained from this box were compared to the laboratory standard to ensure they were within the acceptable range. The single point laser probe was attached to the flexor aspect of the forearm using an adhesive disk. Care was taken to avoid visible large vessels and skin anomalies.

A multi-outlet, blood pressure cuff attached to a pressure reservoir, was placed around the upper arm. Baseline flux was recorded for 10 minutes. The pressure reservoir was primed to 200mmHg. After the 10 minute baseline recording the cuff was rapidly inflated to 200mmHg. This pressure was maintained for exactly 4 minutes. It is crucial that all occlusions were of the same duration as there is a linear correlation between amplitude of response curve and length of occlusion [237]. Complete arterial occlusion was ensured by monitoring the flux trace. After 4 minutes the cuff was rapidly deflated. Flux was continually monitored until it returned to baseline or for a further 10 minutes. Participants were asked to remain as still as possible throughout the study to limit movement artefact. Particular attention was paid to movement of the study limb at the point of cuff deflation as this could introduce significant artefact which would affect the analysis process. Any movement at this time point was recorded.

Skin temperature was measured and recorded throughout the study. Offline analysis was performed using Moorsoft DRT4 Version 2.0. With the time constant set to 3s the following parameters were calculated; baseline flux, biological zero (during cuff inflation), height of peak flux, time to peak flux, time to return to baseline flux (Figure 2.5). MoorVMS-PC Version 3.1 software was used to calculate the area under the reactivity curve, this was done for the whole reactive phase i.e. till return to baseline and also the first minute after cuff release.

Reproducibility

4 healthy, non-smoking volunteers participated in my reproducibility study: 3 female and 1 male (Table 2.5 and Table 2.6). They were studied under standardised conditions as above. The studies were carried out following the above protocol however for the reproducibility data the leg was used instead of the arm. The probe was adhered to the dorsum of the foot and the cuff was placed around the calf. For the research study the limb was changed as it was discovered that many of the potential participants suffered with lower limb oedema and it was therefore felt to be unacceptable from a patient perspective to cuff the leg. Additionally variable degrees of limb oedema would likely compromise the results obtained as the penetrative depth of the laser would vary.

Table 2.5 Time to peak post occlusive hyperaemic response (seconds) measured on 3 occasions in 4 subjects

Subject	Visit 1	Visit 2	Visit 3	Mean	CV (%)
1	29.3	18.9	12.6	20.3	41.6
2	69.2	134.2	89.9	97.8	34
3	39.4	17.1	42.8	33.1	42.2
4	34.7	36	39.9	36.9	7.3

Table 2.6 Height of peak post occlusive hyperaemic response (arbitrary units) measured on 3 occasions in 4 subjects

Subject	Visit 1	Visit 2	Visit 3	Mean	CV (%)
1	40.3	65.7	55.1	53.7	23.8
2	33.8	33.3	89.1	52.1	61.6
3	49.7	46.5	45	47.1	5.1
4	55	37.2	28	40.1	34.3

Overall coefficients of variation were; baseline flux 24.2%, biological zero 18.8%, time to peak flux 31.3%, height of peak flux 31.2% and time to return to baseline 13.7%. These are in keeping with standards within our laboratory and with published values for this technique [268, 269].

2.5 **Sidestream darkfield imaging**

Background

The use of capillaroscopy to examine superficial vessels of the microcirculation had been largely confined to the nail-fold capillaries until the introduction of

handheld vital microscopes. Modern hand-held devices use sidestream dark-field (SDF) imaging to capture high contrast images of the microcirculation in real time.

Green light-emitting diodes are placed concentrically at the tip of the probe, with a sensor at the centre, this arrangement minimises surface reflections which may interfere with the image. The principle of SDF and its precursor orthogonal polarization spectroscopy is that green light illuminates down into a tissue bed, the scattered light is within the wavelength of the haemoglobin absorption spectrum (530nm) and therefore is absorbed by the haemoglobin contained in red blood cells (RBCs)[270]. Reflected light is captured on a charge-coupled camera. RBCs are visualised as black or grey bodies on a white background. Penetration of the light is up to 3mm, superficial capillaries and venules that contain RBCs are visualised (Figure 2.6). The vessel walls themselves are not directly visualised, their imaging depends on the presence of RBCs within them. White blood cells are also visible as refringent bodies

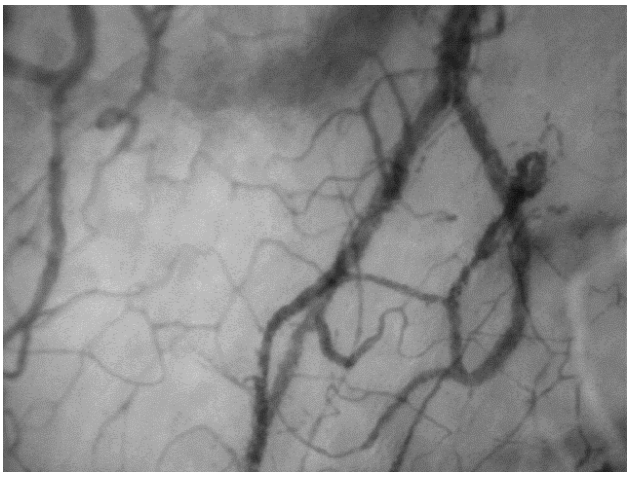

Figure 2.6 An SDF image of the sublingual microcirculation

SDF imaging can be used in any tissue with a mucosal covering. The sublingual mucosa represents a highly vascularised area, supplied by sublingual arteries, branches of the external carotid artery. Due to its ease of access, thin covering of epithelium and embryological and metabolic similarities to splanchnic mucosa, it is the circulation that has been most widely studied using SDF [113, 271]. Guidelines from the original round table conference of 2007[114] and the updated second consensus of 2018[272] on evaluation of the microcirculation using SDF, exemplify the sublingual bed.

Several studies have demonstrated relationships between SDF measured changes in the sublingual circulation and changes in other vascular beds. In animal models of significant haemodynamic compromise, sepsis and cardiac arrest, significant correlation between SDF measured microcirculatory changes in the sublingual bed and perfusion indices in the gut[273, 274] and kidneys[275] have been reported. In a human study of patients following major trauma there was a significant negative correlation between SDF measured microvascular perfusion indices and serum lactate levels, a marker of global hypoperfusion (PVD and lactate $r=-0.416$ $p<0.01$)[276]. Systemic sclerosis is characterised by widespread microvascular changes in the cutaneous circulation, in stable patients with systemic sclerosis there is reported to be a good correlation between nailfold capillary density assessed using capillaroscopy and sublingual capillary density assessed using SDF[277].

SDF has been most widely utilised as a research tool in the intensive care setting. Marked alterations in the microcirculation of septic patients including; reduced vascular density, reduced capillary perfusion and increased heterogeneity of perfusion of the smallest vessels (<20μm), was first

demonstrated by De Backer in 2002[278]. The observed dysfunction also seemed to have prognostic value in severely septic patients admitted to an ICU, dysfunction was greater in non-survivors than survivors[278, 279]. Sublingual microcirculatory changes have also been demonstrated in a number of chronic disease states compared with healthy controls including; coronary artery disease[280], malaria[281], cerebrovascular disease[282] and pre-eclampsia[283]. SDF has predominantly been used in dialysis settings to demonstrate impairments in microvascular perfusion associated with intra-dialytic haemodynamic instability [143-145]. However reductions in sublingual microcirculatory density and impairments of microcirculatory perfusion have been demonstrated in dialysis patients compared with controls[115].

Measurement protocol

All participants were asked to attend fasted overnight for at least 8 hours and without any traditional glucose based dialysate *in situ*. All participants were given a standardised breakfast of 2 pieces of brown bread toast and butter with a glass of water. All studies were conducted in a temperature regulated room at 23^{o}C ±1. Participants were left to acclimate in this room for 30 minutes prior to investigations. All participants were examined in the supine position (Figure 2.7).

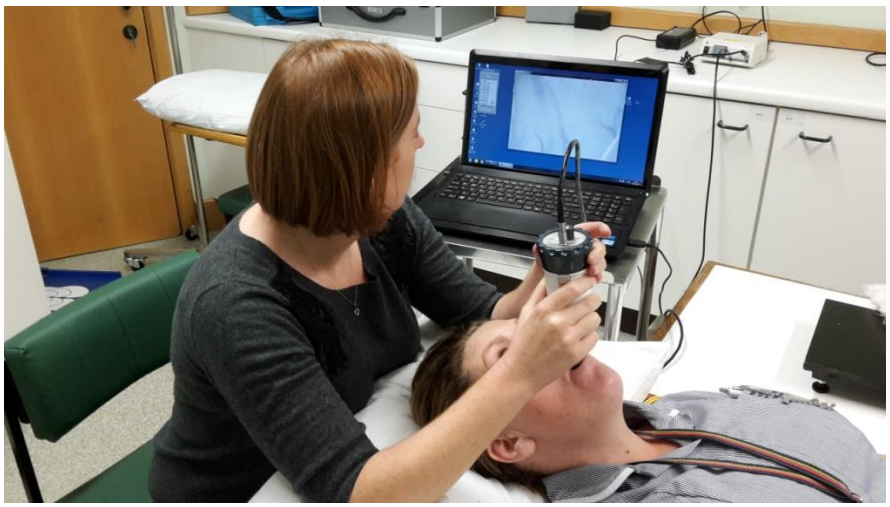

Figure 2.7 Acquisition of SDF images

The instrument was set-up according to manufacturer's instructions. Images were acquired using a hand-held device and SDF imaging (Microscan, Microvision Medical, Amsterdam, Netherlands). This instrument has a 5x objective lens and a numerical aperture of 0.16. A single use plastic cap was applied to the instrument prior to use. The operator placed the Microscan sublingually on the floor of the mouth avoiding the frenulum. The probe was held as perpendicular as possible. Care was taken to avoid compression artefact by real time inspection of the recordings for unidirectional flow[284]. Areas where the capillary network formed loops were avoided as this has been shown to represent lingual rather than sublingual vasculature [113]. The operator was able to alter illumination levels and focus during the examination in order to achieve the optimal images. Good image capture technique is essential for subsequent analysis; RBCs should be seen clearly within the capillary, the image should be evenly illuminated, well focused and free of pressure artefact. Areas containing bubbles were also avoided as they occlude the optical path and cause light scattering outside the region of interest.

Video sequences of 20 seconds duration were recorded using AVA 3.1 software (Academic medical centre, University of Amsterdam, Netherlands). These sequences contained a mixture of capillaries and venules and if possible arterioles. A captured image sequence that is suitable for later analysis should span sufficient duration such that if stability or pressure artefacts are present this could be adequately determined[285]. A sufficient number of recordings were captured such that the operator was confident of having 3 recordings that could be reliably analysed off-line at a later time, as per the European Society of Intensive Care Medicine recommendations[272].

Method of analysis

The video sequences captured as above were later analysed off line. Analysis was performed using a semi-automated vascular analysis (AVA 3.1) software (Academic medical centre, university of Amsterdam, Netherlands). Recordings were reviewed sequentially until 3 video sequences sufficient for analysis were identified. Some movement is inevitable due to the large magnification, therefore recordings were processed using the AVA image stabilisation feature. This reduces inter-image displacement caused by small movements of the camera during image acquisition. Although this stabilisation is necessary for ease of analysis care must be taken as peripheral parts of the image not seen on successive images when movement occurs are lost during the stabilisation process. Consequently the final image is smaller than the original and the software does not readily supply data on percentage reduction from original. The final outcome variables as described below are calculated per mm length of examined vessel and therefore the effect of this reduction in image size on the quality of data is minimised. The first roundtable consensus guideline recommends a minimum of 10 seconds of continuous and steady capture[114].

Software is available for automated identification and categorisation of vessels, however we in keeping with other groups use the approach of drawing vessels by hand and scoring by eye[285], the result of an earlier departmental analysis concluding that automated software analysis is not yet sufficiently reliable. All vessels in the sequence were identified manually and segmented from the background tissue by drawing the vessel centreline and lumen boundary using the AVA software. Vessels were then allocated to one of three groups; small (<20µm), medium (20-50µm) or large (50-100µm). These were denoted on the screen by different line width and colour.

The flow in each vessel was then semi-quantified by the person analysing the record as; continuous, sluggish (flow for 50-100% of the recording), intermittent (flow for <50% of the recording) or no flow. The AVA software uses these data to calculate a number of different indices as below. Full examination is usually only made of small and medium vessels as analyses of the larger vessels are of limited interest and there are very few in the image. These large vessels are however useful as a control measure to ensure excessive pressure is not being applied to the area during the recording stage.

SDF derived outcomes

The first round table consensus of examination of the microcirculation, mandates that scoring of the microcirculation should include; an index of vascular density, assessment of capillary perfusion and a heterogeneity index[114].

Vessel density is assessed by two methods;

Total vessel density (TVD) (mm/mm^2) – total length of all vessels divided by the total area studied. This represents capillary distance or diffusive capacity.

De Backer density or count density (1/mm) – 3 equidistant horizontal and vertical lines are drawn over the image. The total microvessel count is derived by the number of times these lines were crossed by small, medium or large vessels. Count density is reported as number of vessels crossing/total line length.

Three indices of perfusion are calculated;

Perfused vessel density (PVD) (mm/mm^2) – total length of perfused vessels divided by the total surface area. Perfused vessels are defined as those with continuous or sluggish flow. This is a measure of both diffusive capacity and red blood cell velocity or convective capacity.

Proportion of perfused vessels (PPV) (%) – total number of perfused vessels/total number of vessels X 100. This has a good degree of reproducibility but it is the index most sensitive to the change in image size during optical magnification outlined above.

Microvascular Flow Index (MFI) - microvascular flow is semi-quantitatively measured. The image is divided into 4 quadrants. Flow in each of these quadrants is characterised according to the predominant type of flow (as defined above) and allocated a number; continuous (3), sluggish (2), intermittent (1) or no flow(0). The values of the quadrants are averaged to

produce the MFI. This has also been demonstrated to be a very reproducible measure. The main disadvantage of this score is it does not give information about functional capillary density. As this is an ordinal score care must be taken in the interpretation of changes in the score following therapeutic interventions as a change from 0 to 1 may not have the same implications as a change from 2 to 3. Latterly an additional category of hyperdynamic flow has been added. However, it is recommended that this only be included in pathophysiological states where hyperdynamic flow is anticipated[272]. All participants in this study were clinically stable and therefore not anticipated to have hyperdynamic flow.

Most often these scores are interpreted collectively to give a comprehensive measure of the perfusion characteristics of the microcirculation in the studied vascular bed at that time point. The smallest vessels (<20μm) are considered to be the most important physiologically, for oxygen and nutrient exchange, and therefore analysis of microvascular flow using SDF typically focuses on these vessels.

As with other techniques evaluating the microcirculation, spatial and temporal heterogeneity of vessel perfusion and anatomical variations contribute to variability in day-to-day measurements. However this can be attenuated by recording sequences from at least 3 adjacent areas and taking the mean to be representative of that individuals microvasculature[113]. Using this technique reproducibility in the smallest vessels (<20μm) compares favourably with other techniques (total vessel density coefficient of variation 13.5% and proportion of perfused vessels coefficient of variation 12.9%)[113].

Reproducibility and quality control

To assess competency in data analysis of recorded images, I followed the department's standard procedure which is to analyse 3 previously recorded video sequences. My values for these videos were within the data range previously recorded by other members of the group and were therefore deemed to be acceptable. It has previously been demonstrated in our lab that inter-observer variability is less than 13% for parameters pertaining to the smallest vessels (<20µm)[113].

My reproducibility data were collected from 4 healthy volunteers (2 male and 2 female). Each visit was conducted as per the protocol above. Three 20 second recordings taken on each of 3 separate visits were analysed.

Table 2.7 Total vessel density of the smallest vessels (mm/mm^2) measured on 3 occasions in 4 subjects

Subject	Visit 1	Visit 2	Visit 3	Mean	CV (%)
1	18.78	19.42	16.79	18.33	7.48
2	19.08	18.39	17.84	18.44	3.37
3	14.94	19.91	14.85	16.57	17.48
4	20.29	18.55	20.17	19.67	4.94

Coefficients of variation for the means of all the calculated parameters in the smallest vessels were less than 10% (TVD 8.3%, PVD 9.67%, PPV 0.18%). This was comparable to previously published results in healthy volunteers[113].

2.6 **Glycocheck©**

Background

As discussed in Chapter 1, examination of the endothelial glycocalyx *in vivo* remains challenging. The structure of the glycocalyx is too fragile to survive standard microscopic processing and can only be imaged by electron microscopy. Previously, studies of the glycocalyx had been limited to measurements using radiolabelled markers and shed glycocalyx components in serum. Recently developed technology allows non-invasive, indirect examination of glycocalyx properties *in vivo*. This technology utilises SDF imaging combined with Glycocheck© software. As it utilises SDF imaging, this technique can be used in any vascular bed with a thin enough mucosal covering. For the reasons already stated the most commonly studied area is sublingual.

As described above haemoglobin in RBCs absorbs green light and therefore SDF images portray RBCs as black/grey objects on a white background. The glycocalyx does not absorb the scattered light in this way and therefore cannot be directly visualised by SDF. It is also not possible to visualise the endothelial cells themselves with SDF imaging. The parameters produced by the Glycocheck© software are based on the principle that an intact endothelial glycocalyx has a cell permeable layer and a cell-impermeable layer. Consequently the outer edges of the microvascular lumen (from the surface of the endothelial cell to the most luminal aspect of the proposed cell-impermeable layer) are relatively devoid of RBCs. If the endothelial surface layer is damaged then RBCs are able to gain greater access to this usually cell-impermeable region. Consequently RBCs are able to deviate further from their central region of flow towards the endothelial cell surface. This degree of deviation is

calculated by the Glycocheck as the perfused boundary region (PBR) and used to make inferences about glycocalyx properties, outlined in detail below.

Although Glycocheck© produces calculated and not directly measured glycocalyx parameters there is strong evidence of their association with other measures of glycocalyx integrity. In mouse experiments[117] degradation of the glycocalyx with hyaluronidase resulted in reduction of the distance between endothelial cells and the red blood cell column width (presumed to represent the glycocalyx) as measured using Glycocheck. Administration of an oral glycocalyx precursor (sulodexide) to patients with Type 2 diabetes over 2 months led to an improvement in Glycocheck parameters coincident with a reduction in systemic vascular permeability[286]. Glycocheck parameters strongly correlated with systemic glycocalyx volume measured using glycocalyx permeable tracers (dextran-40) in patients with diabetes ($r=0.73$, $p=0.01$)[287] and with sublingual microvascular perfusion in patients undergoing cardiac surgery ($r = -0.65$ $p = 0.002$)[288].

Using Glyocheck©, impairment of the glycocalyx has been demonstrated in multiple disease states including; sepsis[289], coronary artery disease[290], stroke[291] and diabetes[287]. In renal cohorts Glycocheck has been used to demonstrate loss of glycocalyx barrier properties in both haemodialysis and peritoneal dialysis patients compared with healthy controls[90, 117].

Measurement protocol

All participants were asked to attend fasted overnight for at least 8 hours and were given a standardised breakfast of 2 pieces of brown bread toast and butter

with a glass of water. They were also asked to attend without any glucose based dialysis fluid *in situ*.

All studies were conducted in a temperature regulated room at 23^{o}C ±1, participants were left to acclimate in this room for 30 minutes prior to investigations. All participants were examined in the supine position. A single use cap was applied to the instrument prior to use.

The instrument and software were set-up according to manufacturer's instructions. Images were acquired using a hand-held SDF video microscope (KK technology, Honiton, Devon UK). This was connected to a laptop computer running Glycocheck© Glycocalyx Measurement Software (Glycocheck BV, Maastricht, The Netherlands).

The operator placed the microscope sublingually on the floor of the mouth avoiding the frenulum. Pressure on the tissue was avoided to ensure normal flow. The operator optimised the image to allow the software to take valid readings; minimising motion artefact, maintaining adequate light intensity and adjusting focus. Once these criteria were satisfied the software automatically collected sufficient information to calculate the parameters described below. Air bubbles interfere with image collection and analysis, therefore if they were encountered the camera was repositioned.

Per measurement approximately 10 movies of 40 consecutive frames were recorded.

Method of Analysis

Analysis was done in a fully automated fashion within 5 minutes of collecting the images, by the Glycocheck software and involves 4 steps (Figure 2.8). The first frame was used to automatically identify all available microvessels with a diameter between 5 and 25µm. Every 10µm along the length of the detected vessels marker lines were placed perpendicular to the direction of the vessel (Figure 2.8B). Each marker line represents the start of a single vessel segment for which 21 parallel intensity profiles were obtained i.e. every 0.5µm. Each intensity profile was tested for the presence of red blood cells and signal quality. Quality checks were performed throughout the analysis process. The software only selected vascular segments of which at least 11 of the 21 line markers were positive for the presence of RBCs i.e >50% perfusion. This is intended to minimise the influence of haematocrit on the outcome variable[117]. For each intensity profile the red blood cell column width (RBCW) was determined. This was done for all 40 frames, generating 840 measurements per vessel segment, which were then used to generate a cumulative distribution curve to determine median RBCW (Figure 2.8E). The cumulative distribution curve was also used to establish the outer edge of the RBC perfused lumen (Dperf) by linear extrapolation between the 25^{th} and 75^{th} percentiles (Figure 2.8F). The major outcome variable for Glycocheck was the perfused boundary region (PBR). The PBR reflects how far red blood cells can deviate from their median column width and penetrate the usually cell-impermeable area (Figure 2.8D). Larger PBRs represent greater lateral movement of RBCs and imply greater penetration of RBCs into the glycocalyx. The PBR for vessels between 5-25µm, was then calculated as Dperf – median RBCW/2 (as the PBR is present on both sides of

the RBC column). The calculated PBRs were averaged to provide a single PBR for each participant.

Reproducibility

Reproducibility data was collected on 4 healthy individuals (2 male and 2 female)**.** They each attended on 3 separate occasions. The protocol was followed as above. The average day to day coefficients of variation across the 4 individuals for PBR of vessels 5-25μm was 10.84%. This is in keeping with others in our group and published figures.

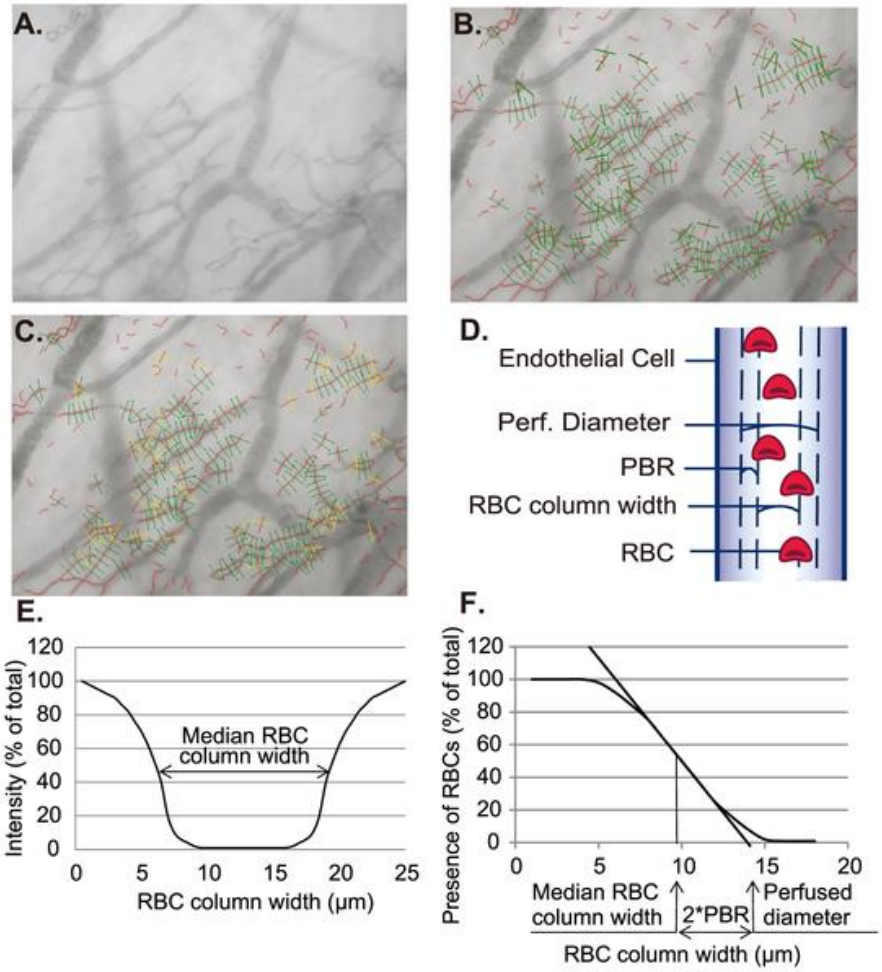

Figure 2.8 Glycocheck algorithm

A) Images captured using sidestream darkfield imaging. B) The software automatically places vascular segment markers (green) every 10µm along the vessels. C) The software selects vascular segments with sufficient quality for analysis, invalid segments are marked (yellow). During the 40 frames the percentage of time in which a particular vascular segment has RBCs present is used to calculate the RBC filling percentage. D) Cartoon depicting the concept of glycocalyx permeability by lateral movement of RBCs, dotted lines represent the edges of the cell impermeable and cell permeable areas E) For each vascular segment the intensity profile is calculated to derive median RBC column width. F) The distribution of RBC column widths is used to calculate the perfused diameter, median RBC column width and ultimately the perfused boundary region (PBR). Reproduced with permission Lee et al 2014 [292].

2.7 **Peritoneal equilibration test**

Background

There is considerable inter-patient variability in the transport of small solutes across the peritoneal membrane. Data on rate of small solute transport is used by clinicians to aid prescription of dialysis. Intra-patient changes over time provide vital information on functionality of the membrane and also potential for long term complications such as encapsulating peritoneal sclerosis. A number of tests of peritoneal function have been developed however the most commonly performed Peritoneal Equilibration Test (PET) is that described by Dr Zbylut Twardowski in 1987[81].

The peritoneal transport rate of small solutes is assessed by measuring the rate at which they equilibrate between the blood and dialysate. The concentration of small solutes in the blood and dialysate are measured at defined times during a standardised 4 hour dialysis exchange to calculate the dialysate to plasma ratio (D/P ratio). This method can be used to measure the transport rate of any solute that passes from the capillary into dialysate. There is significantly less variability in the transport rate of small solutes such as urea and creatinine compared with larger solutes, probably as a consequence of the increased role of convective transport in the movement of larger molecules[293]. There is a strong correlation between the rate of transport of most small solutes. For example, for creatinine and sodium transport the correlation coefficient is r = 0.956 [81]. Consequently, conventionally only urea and creatinine are measured/reported.

These ratios are used to classify the patient into 1 of 4 clinical categories (Figure 2.9). This standardised equilibration curve is still based on the one

created by Twardowski[81] using his data obtained conducting 103 PETs in 86 patients. The line dividing high-average from low-average represents the mean equilibration ratio for his population. The cut-offs between high-average and high and low-average and low are set one standard deviation up or down from the mean. The outer-edges represent the maximum and minimum values recorded in that population.

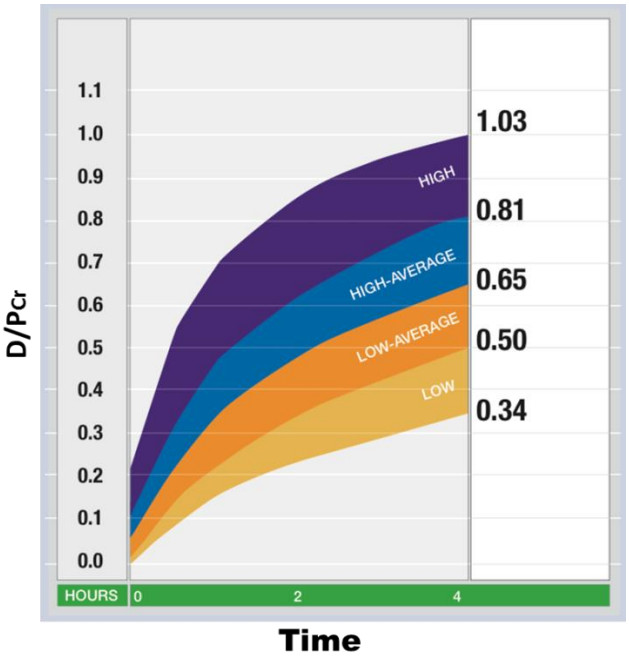

Figure 2.9 Standardised Equilibration curve of Dialysate to Plasma Ratio of Creatinine (D/P_{Cr}) measured during a 4 hour PET. Patients are assigned to 1 of 4 categories according to their D/P_{Cr} at 4 hours; high transport = greater than 0.8, high-average = 0.65-0.8, low-average = 0.5-0.64, low = less than 0.5[81]

When conducted under standardised conditions (as outlined by Twardowski, and in detail below) and when the patient is free of complications e.g. peritonitis, the short term reproducibility (within 3 months) of the 4 hour D/P ratio for small solutes is excellent ($r = 0.94$, $p = <0.001$)[294]. Additionally the PET is relatively simple to perform and therefore useful in clinical practice.

The PET can be used to calculate how quickly glucose is absorbed from the dialysate and consequently how quickly the osmotic gradient is lost. This is done by calculating the ratio of glucose in the dialysate at a given time (t) compared with time zero (G_t/G_0) (Figure 2.10).

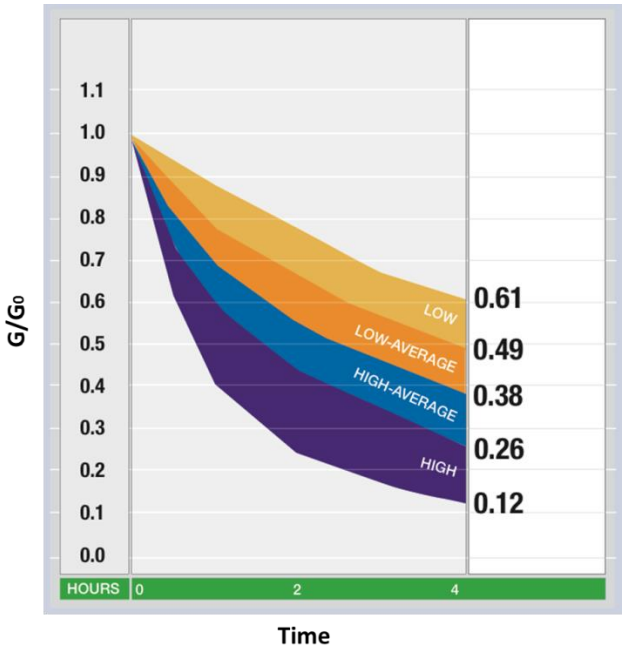

Figure 2.10 Standardised Equilibration curve of ratio of absorbed glucose (G/G_0) measured during a 4 hour PET. Patients can also be assigned a transport status based on speed of glucose absorption, the categories correspond to those of D/P_{Cr}

The PET can also be used to detect ultrafiltration failure and measure free water transport although these will not be discussed in detail here.

The equilibration curves described by Twardowski[81] and still used clinically were based on an unselected cohort of patients from a single centre. Therefore it seems unlikely that this will represent a 'normal range' in wider populations. Little data is given on the demographics of this original cohort; age range was 19-84years and it included 18 patients with diabetic nephropathy. Attempts to reproduce Twardowski's equilibration curves in other populations have yielded

remarkably similar results. In a cohort of 100 PD patients[294] Davies and colleagues reported a mean D/PCr of 0.65 with standard deviation of 0.16 compared with Twardowski's 0.67±0.14, leading them to conclude that the PET was a robust technique for measuring small solute transport.

Although transport categories are widely reported their relevance as comparators between populations and studies is debatable[293]. With regards their use in the study presented in Chapter 4, $D/P_{Cr}4H$ and G_4/G_0 ratios will be presented as continuous variables in a correlation analysis to define the strength of any relationship between solute transport and systemic microvascular variables within our study population. Reference will be made to transport category as defined by the clinically used normogram only as a means of adding clinical context.

Another consideration when interpreting the results of the PET is that assays used to measure creatinine in standard laboratories often experience interference at high glucose levels (>50mmol/L)[295]. This is not usually an issue with blood samples as patients rarely exhibit blood sugar levels as high as this. However, given the high concentrations of glucose in dialysate, Twardowski demonstrated that when using a picric acid based assay this introduced significant artefact into the creatinine measurements, especially in those samples taken at T_0. He therefore applied a correction factor in his creatinine levels. Variation in biochemical analysis especially with regards correction for glucose is also an issue encountered by contemporary studies comparing the results of PETs performed at different sites [70]. In the Global fluid study[70] they also employed a mathematical correction for these 'centre effects'.

The Twardowski PET is still used widely in clinical practice. National and international guidelines recommend testing the peritoneal membrane using the PET within 6 months of commencing PD treatment and then annually thereafter to monitor the functional status of the membrane and assess required dialysis dose [293]. It is also the most commonly used test of small solute transport in clinical research settings[70, 296, 297].

Measurement Protocol

All PETs were conducted as part of patients' routine clinical care and were therefore carried out by members of the clinical team who were trained according to local protocols. Patients with diabetes were asked to ensure that their capillary blood glucose was less than 13mmol/L on the morning of the test. Standard PETs were conducted as per Twardowski's original description, summarised in Figure 2.11. Participants were asked to perform an overnight exchange of no less than 6 hours prior to attending, using 2L of 2.27% dialysate. It has been shown that the PET curve becomes substantially steeper if preceded by a dwell with icodextrin or a dry dwell[298]. Participants attended with this fluid *in situ.* With the participant in the upright position the overnight exchange was drained in a time not exceeding 25 minutes. The volume drained and length of dwell were recorded. A 10ml sample of the drained fluid was obtained.

2L of warmed dialysate (2.27%) was then infused over 10 minutes. This is the concentration of dialysate used by Twardowski and now enshrined in the PET methodology, however subsequent studies have shown no significant effect on 4 hour D/P_{Cr} or categorisation into transport group when higher or lower osmolality solutions were used[299, 300]. When all the fluid had been infused this was noted as time zero. At time zero and at 120 minutes, 200ml of dialysate

was drained into an empty dialysate bag, the sample was thoroughly mixed by inverting the bag. All except 10mls was then reinfused. The 10ml sample was taken from the medication port using an aseptic technique. Between each sample the set was capped off and the participant was free to ambulate.
A blood sample was also obtained at 120minutes.
At the end of the dwell (240 minutes) the dialysate was drained, the bag was agitated and a 10ml sample was obtained from the drained fluid. The total drained volume was recorded.

Blood and dialysate samples were analysed contemporaneously at the Royal Devon and Exeter biochemistry laboratory for; creatinine, urea and glucose. Additional dialysate and plasma samples were stored at -80^{o}C for later analysis. For creatinine measurements the assay used was Creatinine Jaffe Gen.2 by Cobas. Product literature states that glucose levels <120mmol/L do not interfere with the results using this assay. Although some of the T_0 dialysate samples had glucose concentrations in excess of this, the outcome measure for this study was dialysis to plasma ratio of creatinine at 4 hours. All 4 hour dialysate samples had glucose concentrations well below this threshold and therefore no significant artefact would be anticipated.

At the Cardiff site patients underwent a ‘fast PET’ whereby dialysate samples were only taken at the end of the 4 hour dwell. Excellent agreement between the results of the D/P_{Cr} at 4 hours measured using the fast PET and standard PET has been demonstrated ($r = 0.77$ $p = 0.0001$)[301].

As a different creatinine assay was used at the Cardiff laboratory, samples taken at the Cardiff site were frozen and re-processed in the Exeter lab using the above assay to avoid the centre effect described elsewhere[70].

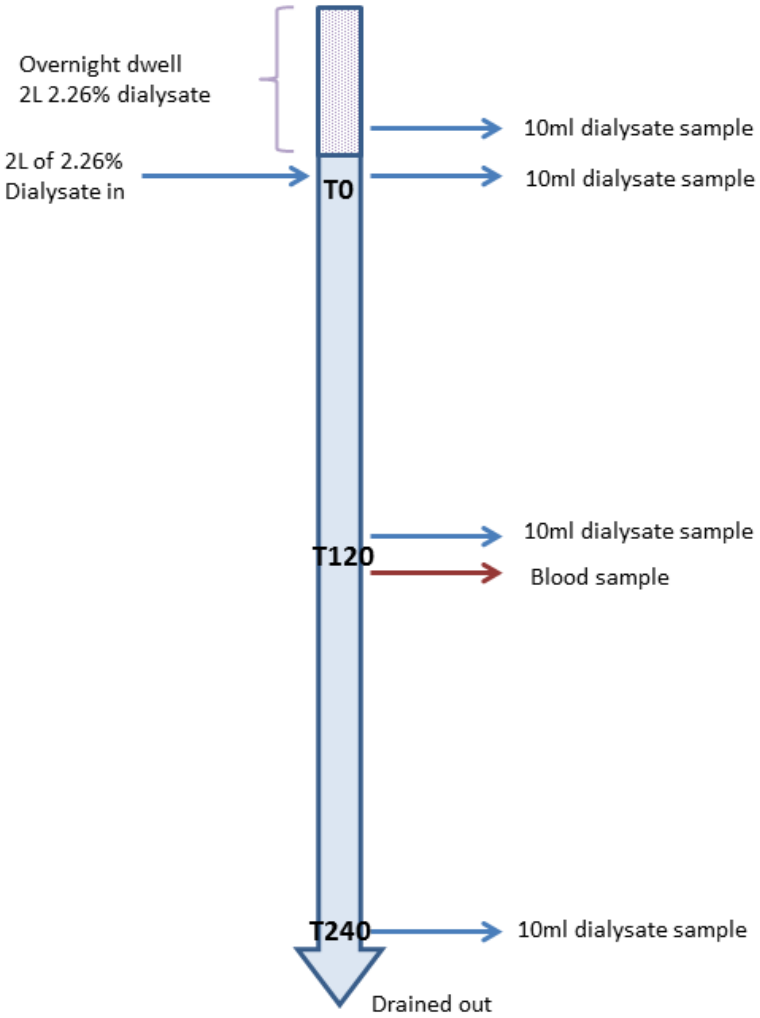

Figure 2.11 Flow diagram summarising the timing of blood and dialysate samples during a standard 4 hour PET (T0 to T240 minutes).

2.8 Continuous glucose monitoring

Background

For patients with diabetes mellitus and their health care professionals, the ability to measure glucose levels on a daily basis is crucial to guide therapy. Daily venous samples are impractical and therefore traditionally patients recorded capillary glucose levels at home using a point of care glucose meter. Glucose is measured in capillary blood usually from a finger-prick. There are obvious limitations to the utility of this data obtained from intermittent readings. Most importantly patients choose when to test glucose levels and therefore the readings recorded are likely to be biased with a high risk of missing periods with significantly high or low glucose levels.

Continuous glucose monitoring (CGM) became commercially available in 2000. Traditional CGMs employ a sensor with an enzyme (glucose oxidase) tipped electrode which is placed subcutaneously in the interstitial fluid of the abdominal wall. The oxidation of glucose by this enzyme generates an electrical current in the electrode which is transmitted from the sensor to a monitor. Measurements of interstitial glucose are automatically taken at regular intervals ranging from 5-20minutes depending on the device used. These devices are available for personal use, providing real-time glucose information to the patient or professional use, in which the patient is blinded to the readings, most often used in clinical research.

Accuracy and utility of CGMs

CGMs are measuring glucose in a different compartment (the interstitium) to capillary blood glucose monitors. Interstitial glucose is related to blood glucose via a diffusion process resulting in a co-dependency which allows blood glucose

to be deduced from interstitial glucose[302]. Traditional CGMs therefore require 8 hourly calibrations against capillary glucose measurements. There is a physiological lag, the time taken for interstitial glucose to equilibrate with blood glucose. This lag is most marked during times of rapid change in blood glucose. Consequently the relationship between interstitial glucose and blood glucose is least well defined during periods of rapid flux[303]. Accuracy and precision of CGMs have improved significantly since they were first introduced. Mean absolute relative difference values of glucose compared with the Yellow Springs Instrument (the gold-standard bed side analyser) are now <10%[304, 305].

There is also a technological lag, the time taken for the sensor information to be processed. In early sensors this lag was considerable (up to 15 minutes) however improvements in algorithms have reduced this to only a few minutes[207]. Physiological and technological lags influence the action that can/should be taken based on a single reading but have a less marked impact on the utility of sensors to analyse glucose trends.

CGMs allow capture of continuous streams of data, however they require regular calibration against capillary glucose measurements. For data sets to be considered valid calibrations have to occur at least 8 hourly. Subsequent data cleaning entails confirming the calibration frequency, identifying errors with paired sensor values and reviewing missing data points[306].

CGM derived outcomes variables

The continuous data obtained using CGMs allows calculation of mean 24 hour glucose levels and glycaemic variability. In addition it allows interpretation of individual glucose readings in the context of direction of change and

appreciation of the amplitude of oscillations in relation to food, medication and exercise.

Dozens of metrics have been proposed for glycaemic variability, the most commonly used are defined in Table 2.8.

Table 2.8 Glycaemic variability metrics

Modified from Bergenstal, *Diabetes Care* 2015[210]

GV metric	Definition	Clinical implications
Standard deviation (SD)	Amount of variation/dispersion of a data set. SD = √variance	Measure most familiar to clinicians Easy to calculate Most accurate if values are normally distributed around the mean
Coefficient of variation (%CV)	The extent of variability in relation to the population mean CV = 100 x SD/mean	Less influenced when comparing data sets with widely different mean values
Interquartile range (IQR)	The spread of values 25% above and 25% below the median (the middle fifty)	Plotting the IQR on a modal day profile makes it easy to spot what time of day has the most GV and needs attention
Mean amplitude of glycaemic excursions (MAGE)	Average of all blood glucose excursions greater than 1SD of all measures for that profile	Used for many years. Can be applied to self monitored glucose levels and CGMs
Continuous overall net glycaemic action (CONGA) 1-24hr	Within day glycaemic variation. SD of the differences of glucose readings for a defined period of hours	No clear benefit compared with the above 4 more commonly used measures

There is no consistency in the literature regarding the metrics that should be reported[307], however nearly every measure is highly correlated with overall standard deviation (SD) of glucose[208]. As there are so many indices of glycaemic variability, examining each of them for possible relationships with any

given factor risks generating out comes with statistical significance as a result of multiple comparisons. Therefore it is recommended that primary outcomes are specified *a priori* [308] and chosen based on the research question being asked.

The reproducibility of daily average measures and glycaemic variability calculated using CGM data compares favourably to more traditional indices. When conducted correctly, fasting plasma glucose has a CV of between 5-7%[309, 310] and the 75g oral glucose tolerance test 12.7 - 14.4%[309, 311, 312]. Coefficients of variation of average daily glucose, SD of glucose and $CONGA_{1hr}$ have been reported as 3.9%, 16.3% and 7% respectively[312].

CGMs in renal populations

An accurate reflection of glycaemic control in patients with CKD is difficult to achieve for the reasons outlined in Chapter 1. The European Renal Best Practice Guideline Development Group on Diabetes in Advanced CKD reviewed all current methods of measuring glycaemic control in dialysis patients and concluded that although future studies are needed to assess whether their use can result in better control and lower mortality, CGMS are probably the only method to correctly evaluate glycaemic control[313].

CGMs have been successfully used in diabetic haemodialysis patients to demonstrate differing glycaemic control on dialysis versus non-dialysis days[314] and previously underappreciated levels of hypoglycaemia in the hours following a dialysis session[315]. A number of studies have used CGMs to describe blood glucose patterns in diabetic PD patients[212, 214, 216, 218, 316, 317]. In this patient group they reported excellent agreement between

interstitial glucose measured by the CGM and venous glucose measurements (r^2=0.82 p = <0.0001)[317].

Flash glucose monitors

Recent years have seen the introduction of flash glucose monitors (FGM). FGMs, such as Freestyle Libre (FSL) (Abbott Diabetes Care, Witney, Oxon, UK), provide the user with a real-time glucose recording whenever the sensor worn by the patient is brought into close proximity with a hand-held reader, in addition to automatically capturing interstitial glucose levels at 15 minute intervals.

Like traditional CGMs, the FreeStyle Libre single-use sensor uses wired enzyme technology (osmium mediator and glucose oxidase enzyme) to monitor interstitial glucose levels. Data is transferred by radio frequency identification from the sensor to the reader (Figure 2.12), which is able to store historical data for up to 90 days [318]. The sensor is able to store a maximum of 8 hours of data at any given time and therefore the reader and sensor must interact in this way at least every 8 hours otherwise data is lost. Unlike CGMs the Freestyle Libre is worn on the back of the upper arm and can remain in situ for up to 14 days. However, the major difference between the FSL and traditional CGMs is the need for external calibration has been removed, as the FSL is factory calibrated.

Figure 2.12 FreeStyle Libre sensor and reader (Freestylelibre.co.uk)

The mean absolute relative difference (MARD) of glucose between the FSL and capillary blood glucose meters has been reported at 10% and between FSL and Yellow Springs Instrument at 10.7%[319]. This is comparable to traditional CGMs[207]. As with the CGMs, FGMs are least accurate during times of rapid rate of change of glucose and at extremes of measurement[320]. The mean lag time between sensor reading and YSI is 4.5 minutes [321]. A number of multi-centre studies have concluded that in a clinical setting it is a safe and effective replacement for intermittent capillary glucose monitoring[318, 322].

Icodextrin, a commonly used glucose sparing dialysate is metabolised to maltose and absorbed from the peritoneal cavity. Maltose has been shown to interfere with some capillary blood glucose monitoring systems specifically those that use test strips containing glucose dehydrogenase-pyrroloquinolinequinone or glucose dye oxidoreductase, resulting in erroneously elevated glucose levels[323]. Maltose does not interfere with the glucose oxidase enzymatic method used in CGMs and FSL[324].

The main advantage to using the FSL in my non-diabetic PD study cohort is the lack of capillary glucose calibration. The 8 hourly finger-prick calibrations required with traditional CGMs may have rendered them unacceptable to this group unused to self-measurement.

The main criticisms of both CGMs and FGMs have been that readings become less reliable during times of rapid change[325] and at extremes of measurement[326], calculation of glycaemic variables can be limited by erratic patterns. Studies in other non-diabetic cohorts with normal renal function have not observed rapid changes in glucose levels [327, 328]. In my cohort of non-diabetic patients, who do not use glucose-lowering therapies rapid changes in glucose levels or readings at the extremes of measurements are unlikely. Documenting the influence of dialysate on these metrics is the purpose of this study and therefore this factor cannot be fully accounted for.

There are no published studies using FSL in dialysis cohorts. However, as outlined above CGMs have been validated in PD cohorts, the FSL system uses identical enzymatic assays to traditional CGMs and shows good agreement with CGMs in other cohorts[329].

Measurement Protocol

Continuous glucose monitoring was conducted using the FreeStyle Libre flash glucose monitoring system (Abbott Diabetes Care, Witney, Oxon, UK). Application of the FreeStyle Libre was conducted in accordance with the guidelines produced by the manufacturer.

Application of the sensor was carried out by myself. An area on the back of the upper arm was chosen for sensor application. The area was chosen as one that

generally stays flat during normal daily activities and areas with scars, moles, stretch marks or lumps were avoided. The code on the sensor was checked against the code on the applicator. The chosen area was cleaned with the 2 alcohol wipes provided. Once the alcohol had dried the sensor was applied using the pre-loaded applicator. After checking that the sensor was securely applied it was activated with a reader. The LOT number of the sensor and the reader used were recorded. Participants were fully briefed on the use and care of the sensor emphasising that ‘scanning’ of the reader needed to take place at least 8 hourly to avoid missing data. They were also issued with a user information booklet provided by the manufacturer. The FSL output was not ‘masked’ from participants, when the reader is ‘scanned’ against the sensor a real time reading is displayed on the screen. The participants were also able to retrospectively access the readings from the monitoring period. Participants wore the sensor for 72hours after which it was removed and data from the reader was uploaded using FreeStyle Libre Glucose Data Management Software Version 1.0. Analysis of the FSL derived data is discussed in detail in Chapter 5.

Chapter 3 Cutaneous microcirculatory dysfunction in peritoneal dialysis patients

3.1 Introduction

Patients on peritoneal dialysis have multiple risk factors for microcirculatory dysfunction. These relate to their uraemia and renal replacement therapy but also the constellation of co-morbidities often present in this group including hypertension and diabetes. Although multiple studies have assessed the microcirculation *in vivo* in HD patients none have looked directly at systemic microcirculatory function in PD patients. A limitation of previous studies comparing dialysis patients with controls is a lack of adjustment for co-morbidities and demographic factors known to negatively impact the microcirculation.

3.2 Aims and objectives

I aimed to test the hypothesis that patients on PD would exhibit a greater degree of systemic microvascular dysfunction compared with both healthy controls and controls with similar cardiovascular and metabolic profiles.

Objectives

To study multiple aspects of cutaneous microvascular function in stable PD patients.

To generate 2 control groups from a cohort of patients previously studied in our laboratory using identical techniques; one healthy control group and one control group matched for factors known to influence microvascular function.

To compare cutaneous microvascular function in PD patients with the control groups

3.3 **Methods**

This analysis was performed in a cohort of 28 PD patients recruited for a study of the relationship between systemic microcirculatory function and peritoneal transport (Chapter 4). The study was approved by East Midlands – Leicester Central Research Ethics Committee (REC ref 16/EM/0395) and conducted according to the declaration of Helsinki. Participants were recruited from patients attending Royal Devon and Exeter Hospital and University Hospital Wales for their dialysis care. Potential participants were identified by the clinical care teams, offered a participant information sheet and with their agreement they were referred to the research team.

Inclusion criteria:

Aged 18 years or older

Less than 6 months of PD therapy

Exclusion criteria:

Unable to give informed consent

Use of calcineurin inhibitor

History of Raynauds disease

Major vascular event within preceding 3 months

Participants were not studied within 4 weeks of any inflammatory illness. Participants were studied at the Diabetes and Vascular Research Centre, Exeter or at the Clinical Research Facility, University Hospital of Wales.

Participants attended for their study visit in the morning, orally fasted for at least 8 hours overnight. Traditional peritoneal dialysate contains high levels of glucose which is absorbed into the systemic circulation at a variable rate. Participants were therefore asked to attend ‘dry’ i.e. without any dialysate in situ. Their overnight dialysis regime the night before was conducted as normal, details of the normal dialysis regime were documented, these are documented in the demographics table in Chapter 4. Fasting bloods were collected. A brief clinical examination was performed including; blood pressure and a subjective assessment of fluid status (weight and clinical assessment of fluid status). Information was recorded on; demographic details, past medical history, current medications and dialysis regime.

To reduce the influence of food intake on microvascular parameters, participants were given a standardised meal of two pieces of brown toast with butter and a glass of water. Due to the homeostatic role of skin microcirculation, all investigations were performed in a temperature controlled room in which participants had acclimated for 30 minutes, as described in Chapter 2. All investigations were performed by myself.

Control participants were selected from a cohort previously recruited and studied in the Diabetes and Vascular Research Centre, Exeter. These participants were part of the SUMMIT study (SUrrogate markers for Micro and Macro-vascular hard endpoints for Innovative diabetes Tools, http://www.imi-summit.eu/). These participants were recruited between 2010 and 2013 and studied by trained members of the Diabetes and Vascular Research team using identical equipment and protocols to the PD patients. The ‘healthy control’ group were pair matched to PD patients for sex and age +/- 5 years, they had

no documented major co-morbidities, nor took any regular medications. The 'matched control' group were pair matched to the PD patients strictly for sex, diagnosis of diabetes and presence of 1 or more documented vascular events (myocardial infarction, unstable angina, coronary revascularisation procedure, stroke or transient ischaemic attack) and for age +/- 5 years. Both diabetes and coronary vascular disease have been associated with a reduction in dermal microvascular function[330, 331].

Data used in this analysis were collected and analysed using the following tests, described in detail in Chapter 2;

- The reactive hyperaemic response to a 4 minute period of arterial occlusion of the arm measured using laser Doppler flowmetry (PORH)
- The response to iontophoretic application of acetylcholine (ACh) 1% dissolved in mannitol and sodium nitroprusside 0.25% (SNP) dissolved in 0.45% saline, to measure endothelium dependant and endothelium-independent vasodilation respectively. The degree of response was measured using laser Doppler perfusion imaging.

Statistics

Statistical analysis was performed using StataSE-16 (Stata Corporation, Texas, USA). Graphical analysis was performed using GraphPad Prism 8 software. All variables were tested for normality by visual inspection of a histogram plot and by Shapiro-Wilks test. For normally distributed data the differences between

group means were analysed using a one-way ANOVA. Where significance was observed an unpaired t-test was used to explore differences between 2 means. For non-normally distributed data differences between group means were analysed using the Kruskal-Wallis test. Where significance was observed the Mann-Whitney rank sum test was used to explore differences between 2 medians. A result was considered to be statistically significant if $p \leq 0.05$.

In a study of endothelial function in larger vessels Van Guldener and colleagues reported a 5SD difference between their PD patients and controls[332]. Farkas and colleagues compared HD patients to controls using PORH and iontophoresis, and observed a 3-5SD difference between the groups[91].

28 completed participants in each group would allow detection of a 0.75SD difference between means with power of 80% and 2-sided $\alpha=0.05$.

3.4 **Results**

Data in this section are presented as mean ± standard deviation for normally distributed data and median (interquartile range) for non-normally distributed data.

The characteristics of patients, matched-controls and healthy controls are outlined in Table 3.1. Healthy controls had no medical history and did not use any medication. There were no significant differences in age, sex, blood pressure, BMI or smoking status between the 3 groups. In addition the matched-control group was well matched with the PD patients with regards diabetes and known cardiovascular disease, although there was significantly less use of inhibitors of the renin-angiotensin-aldosterone system (RAAS) in the

matched controls. Both the healthy controls and matched controls had significantly higher haemoglobin than the PD patients. Mean serum creatinine in the healthy controls was 83µmol/L and 81.5µmol/L in the matched-controls.

Table 3.1 Characteristics of study participants

Data are presented as mean ± SD *p<0.05 versus PD patients
CAPD = continuous ambulatory peritoneal dialysis, RAAS = renin angiotensin aldosterone system, EPO = erythropoetin

	Healthy Controls	**Matched Controls**	**PD patients**
Age (years)	65.4 ±11.7	66.2 ± 13.1	66.5 ± 15.9
Male (%)	19 (68)	19 (68)	19 (68)
Ethnicity Caucasian (%)	28 (100)	28 (100)	26 (93)
Systolic blood pressure (mmHg)	138 ± 14	142 ±13	140 ± 22
Diastolic blood pressure (mmHg)	78 ± 8	78 ± 6	82 ± 13
BMI	24.9 ± 4	26.8 ± 4.8	27.9 ± 5.7
Haemoglobin (g/L)	138.5 ± 11.7*	138.3 ± 11.7*	118.5 ± 11.7
Diabetes n(%)	0*	9 (32)	9 (32)
Smoking n(%)	1 current 10 previous	0 current 12 previous	1 current 9 previous
History of cardiovascular disease n(%)	0*	8 (28.6)	8 (28.6)
Inhibitors of RAAS n(%)	0*	3 (10.7)*	11 (39.3)
CAPD n(%)			13 (46.4)
Time on PD (weeks)			15.7

Previous peritonitis n(%)	2 (7)
EPO usage n(%)	17 (60.7)
Presence of arteriovenous fistula n(%)	2 (7)
Parathyroid hormone (pmol/L)	28.16

Comparison of post occlusive reactive hyperaemia between PD patients and controls

25 of the recruited PD patients had adequate data for PORH. Data obtained were non-normally distributed and therefore non-parametric tests were used to analyse the results, as outlined above.

The primary effect parameter for PORH was absolute peak flux measured in arbitrary units (AU). Peak hyperaemic flow was significantly lower in the PD group than in both the healthy controls and matched controls [median (interquartile range) 57.4AU (45.2-91.9), 90.45AU (60.9-135.6) and 74AU (58.8-136.9) respectively p= 0.03 healthy controls versus PD, p = 0.04 matched controls versus PD] Figure 3.1. Although the maximum hyperaemic flow in the matched controls was lower than the healthy controls this was not statistically significant.

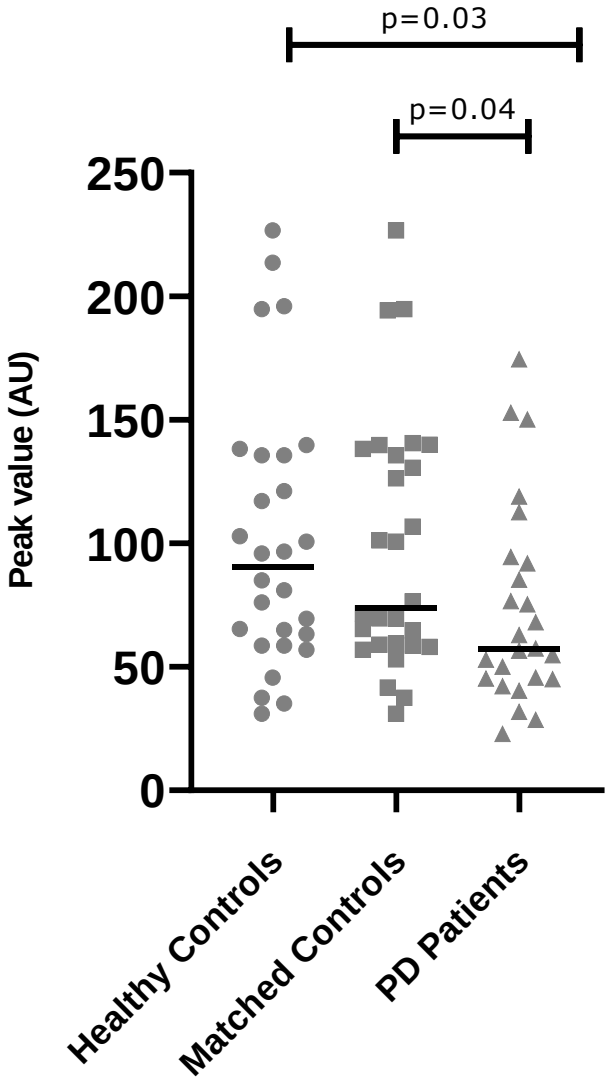

Figure 3.1 Peak post-occlusive hyperaemia in arbitrary units (AU) by group.
A line is drawn at the median value; healthy controls 90.45 (60.9-135.6), matched controls 74 (58.8-136.9) and PD patients 57.4 (45.2-91.9). Significant statistical differences between groups are shown.

The time taken to reach maximum post-occlusive hyperaemia was not significantly different between groups (healthy controls 17.75s (12.45-26.6), matched controls 13.6s (10.8-19.4) and PD patients 14.8s (11.2-16.8) p=0.12). However the time taken to return to baseline flow was significantly shorter in the PD patients, 130.6s (96.3-147.1) versus 173.15s (142.1-218.7) in healthy controls and 161.7s (126.7-212.6) in matched controls (p=0.0005 versus

healthy controls, p=0.0074 versus matched controls). Consequently the total hyperaemic response was reduced in the PD patients.

Comparison of endothelium and non-endothelium dependant vasodilatation between PD patients and controls

23 of the recruited PD patients had data deemed adequate for analysis. Data were normally distributed and therefore parametric tests were used.

Responses to administration of the vehicle substances (mannitol as the ACh vehicle and 0.45% saline as the SNP vehicle) were not different between groups.

The primary effect parameters for response to iontophoretic application of ACh and SNP were peak flux value measured in arbitrary units (AU) and area under the curve (AUC) subtracting the baseline for the total monitoring period. The peak response to ACh trended towards being greater in the healthy controls compared with the matched controls and the PD patients however this did not reach statistical significance (562 AU ± 185 in healthy controls, 486 AU ± 126 in matched controls and 473AU ± 179 in PD patients p = 0.16 ANOVA), Figure 3.2. The total perfusion response to ACh as determined by area under the curve also trended towards being higher in the healthy controls but was not statistically significant (116769AU ± 62181 healthy controls, 92843AU ± 43225 matched controls and 89175AU ± 55021 PD patients p = 0.245 ANOVA), Figure 3.3.

Both the peak response and total perfusion response (AUC) to SNP were significantly higher in the healthy controls than the PD patients (peak response

498 ±131 healthy controls versus 402 ± 144 in PD patients p = 0.02 and AUC 83043 ± 29648 healthy controls versus 58532 ± 32616 PD patients p = 0.01). There was no statistically significant difference between the matched controls and the PD patients in their response to ACh or SNP, Figure 3.2 and Figure 3.3.

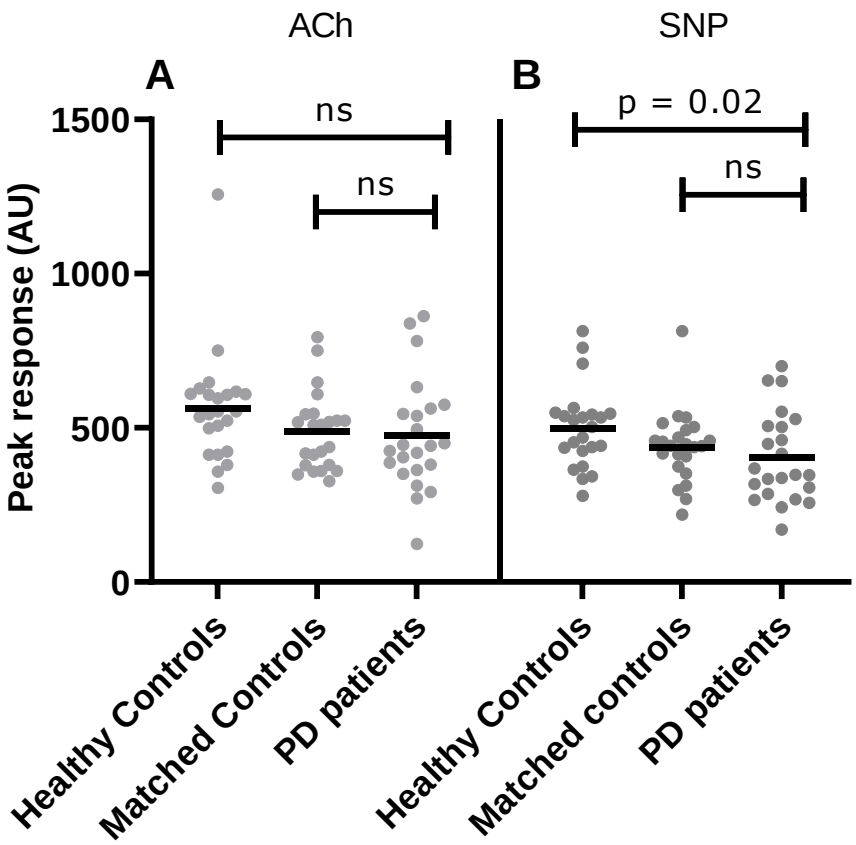

Figure 3.2 Peak response (in arbitrary units) following iontophoretic delivery of ACh (A) or SNP (B). Line drawn at the mean value. P value indicated where ≤0.05

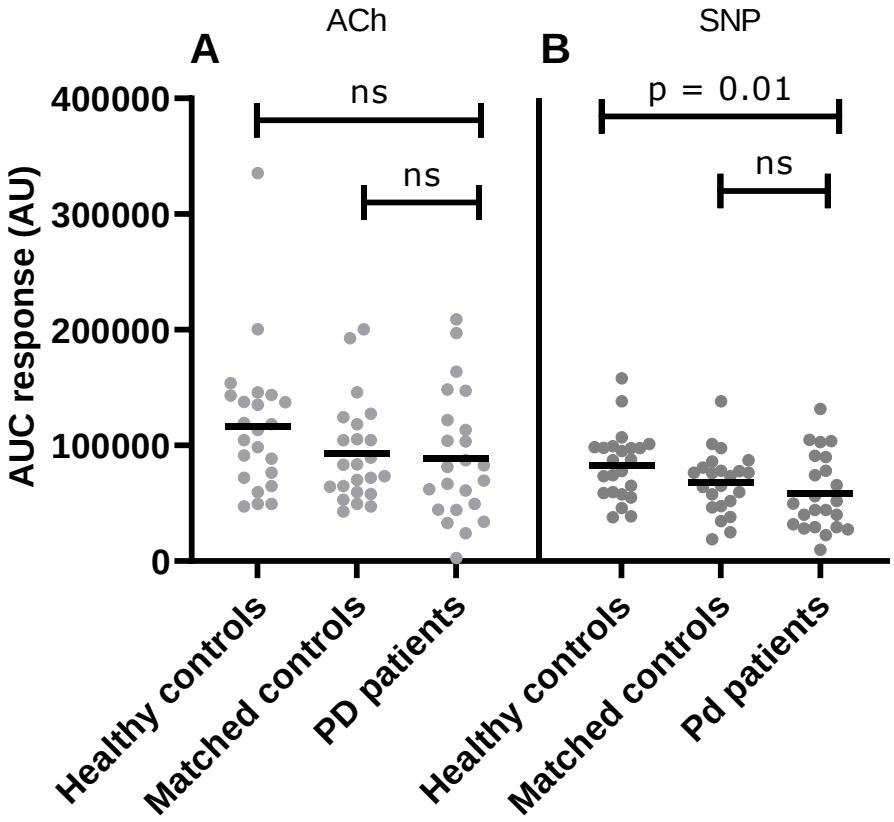

Figure 3.3 Total response (depicted as area under the curve) to iontophoretic application of ACh (A) or SNP (B). Line drawn at the mean value. P value indicated where ≤0.05

Comparison of responses in patients taking a renin angiotension aldosterone blocker versus those who were not

The number of patients taking renin angiotensin aldosterone system blockers was significantly higher in the PD group compared with the matched controls (Table 3.1). Visual inspection of the data did not reveal any obvious systematic influence of these medications on cutaneous microvascular reactivity in this cohort (Figure 3.4 and Figure 3.5).

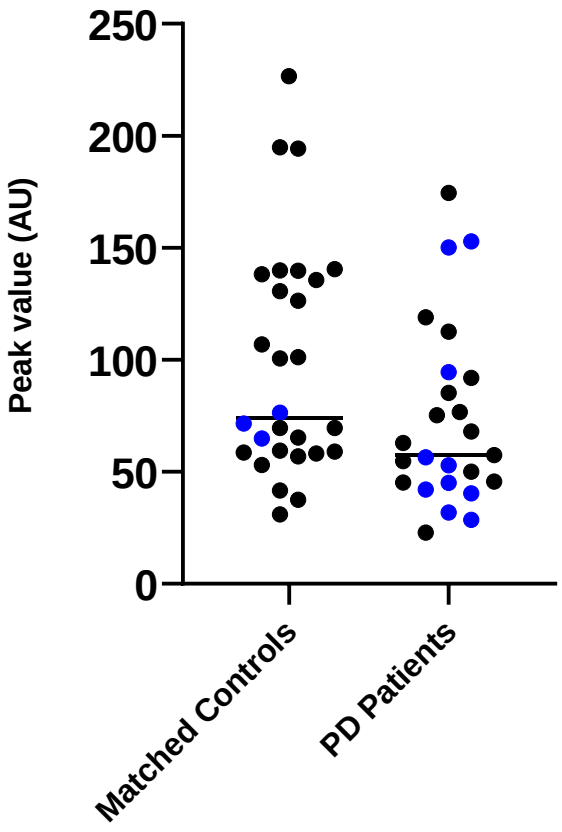

Figure 3.4 Peak post-occlusive hyperaemia in arbitrary units (AU) for matched controls and PD patients. Patients taking a RAAS blocker are highlighted in blue, no obvious systematic effect of RAAS blockers in our cohort.

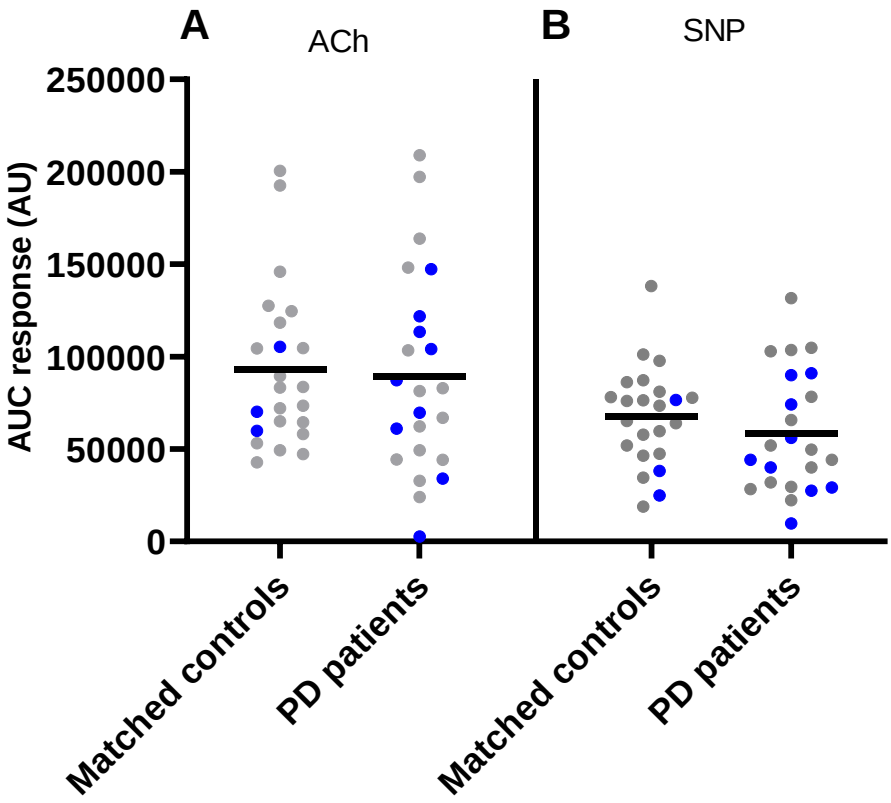

Figure 3.5 Total response (depicted as area under the curve) to iontophoretic application of ACh (A) or SNP (B). Patients taking a RAAS blocker highlighted in blue.

Table 3.2 Summary of cutaneous microvascular responses in PD patients compared with controls

	Healthy Controls	Matched Controls	PD patients
Post-occlusive reactive hyperaemia			
Peak response (AU)	90.45 (60.9-135.6)[a]	74 (58.8-136.9)[b]	57.4 (45.2-91.9)
ACh-mediated vasodilatation			
Peak response (AU)	562 ± 185	486 ± 126	473 ± 179
Total response (AUC)	116769 ± 62181	92843 ± 43225	89175 ± 55021
SNP-mediated vasodilatation			
Peak response (AU)	498 ± 131[a]	437 ± 117	402 ± 114
Total response (AUC)	83043 ± 29648[a]	67754 ± 26820	58532 ± 32616

[a]p=<0.05 healthy controls versus PD patients, [b]p=<0.05 matched controls versus PD patients

3.5 **Discussion**

The results of this study indicate impairment of cutaneous microvascular reactivity in patients with end stage renal disease on peritoneal dialysis compared with healthy controls and controls matched for significant microvascular co-morbidities, as summarised in Table 3.2.

Skin microvascular reactivity is regulated by mechanisms that include; arteriolar myogenic response, endothelium dependant flow-induced vasodilatation, metabolic and neurovascular interactions[102]. Patients with CKD have multiple risk factors for endothelial dysfunction including; disturbed bone mineral metabolism, inflammation, oxidative stress and accumulation of uraemic toxins. Microvascular dysfunction in renal patients is most often attributed to reduced nitric oxide (NO) bioavailability[333, 334]. This is a plausible hypothesis given that accumulated uraemic toxins such as asymmetric dimethylarginine (ADMA) reduce NO availability by competitively inhibiting endothelial NO synthase[335]. Oxidative stress and the accumulation of reactive oxygen species also result in the scavenging of NO[336]. A reduction in flow mediated dilatation (FMD) of the brachial artery (an NO dependant reaction) has been demonstrated in patients with ESRD[337] and patients on PD [94]. In larger vessels, such as the brachial artery, the chief stimulus to NO production is shear stress hence the NO dependency of FMD[253]. The role of NO further down the vascular tree is less well characterised, it has been suggested that the role of NO in the endothelium dependant control of vascular tone is inversely associated with the calibre of the arteries[338, 339]. In resistance arteries endothelium-dependant hyperpolarizing factor (EDHF) has been postulated as the predominant mediator of endothelium dependant dilatation[340]. The relative contributions of

NO and EDHF to endothelial dysfunction in CKD patients remains disputed. Ex-vivo studies in human uraemic resistance arteries have shown reduced EDHF and NO type responses[339, 341], of note these vessels are larger than those we anticipate to be measuring a response in (200μm in diameter compared with an approximately 80μm diameter).

My study demonstrated a significant attenuation of the post occlusive reactive hyperaemic response in PD patients compared with both healthy controls and those matched for covariates known to influence microvascular function. This reactive hyperaemia measured in the microcirculation in response to a brief period of arterial occlusion represents global microvascular function, the actions of endothelium dependant and endothelium independant pathways on the vascular smooth muscle. However at this level of the vascular tree it is not thought to be primarily an NO dependant reaction as inhibition of nitric oxide synthase does not seem to significantly attenuate the cutaneous hyperaemic response[254]. The more important determinants of the post occlusive hyperaemic response at a microvascular level in human studies, appear to be the release of endothelium-derived hyperpolarising factors [261] and the action of sensory nerves via local axon reflexes[251].

ADMA and other uraemic toxins such as advanced glycation end products and indoxyl sulphate have also been shown to impair endothelial function by non NO dependant mechanisms. These include inhibiting the regeneration of injured endothelium by reducing the differentiation, mobilization and function of endothelial progenitor cells[342], impairing cell-cell interactions[343] and endothelial activation[344]. Loss of EDHF-type vasodilatation of the carotid artery has been demonstrated in a rat model of ESRD (5/6

nephrectomised)[345] and in isolated human resistance arteries[340]. The relevance of these findings in larger vessels, to *in vivo* function in human microvessels is debatable. Additionally, rat models of CKD have only shown modest increases in ADMA compared with the very large increases reported in humans[346], casting doubt on their applicability to human CKD pathology. The *in vivo* role of EDHF in vasodilatation has only been studied in larger vessels in renal populations[347]. In contrast to the animal and ex vivo work Passauer and colleagues concluded that in haemodialysis patients, reduced ACh-induced vasodilatation of the forearm was primarily attributable to reduced stimulation of NO. As discussed above how this relates to vessels further down the vascular tree, such as were measured in my study, is unclear. My results suggest at the microvascular level, vasodilatory pathways independent of NO are also affected in PD patients, the exact nature of this impairment remains unclear.

The local application of vasodilatory substances by iontophoresis allows analysis of more discrete aspects of the vasodilatory response. Application of SNP is essentially the exogenous delivery of NO to smooth muscle cells and therefore represents a test of endothelium independent reactivity. In this study the smooth muscle response to SNP was significantly reduced in the PD patients compared with healthy controls. This reduced total dilative capacity to NO is likely to be either the result of smooth muscle cell dysfunction in the microvessel wall, reduced bioavailability of delivered NO or inability of the vessel wall to dilate due to other restrictive components. My findings of reduced response of the cutaneous microcirculation to application of SNP are in keeping with other *in vivo* studies in patients with CKD[348] and those on HD[91, 107]. In contrast, studies of *ex vivo* uraemic human resistance arteries[339] and *in vivo* forearm blood flow in patients with CKD[349], showed no significant

reduction in the response to SNP compared with controls. This further emphasises that vasodilatation is likely to be mediated by different pathways at different levels of the vascular tree.

Disordered homeostasis of calcium and phosphate in dialysis patients results in deposition of calcium in the medial layer of larger blood vessels, containing the vascular smooth muscle cells. Vascular smooth muscle cells cultured in a high phosphate medium exhibit phenotypic changes usually seen in mineralised tissues such as bone[350]. In larger vessels this results in vessel rigidity[351] and has been correlated with cardiovascular morbidity and mortality[134].Cutaneous microcirculatory dysfunction has been shown to correlate with this calcification of larger vessels[107]. Histological examination of cutaneous vessels in HD patients has demonstrated; basement membrane thickening and reduplication of the basal lamina in venules and arterioles[352]. Calciphylaxis is a rare but serious complication of renal replacement therapy, where-in calcium accumulates in the medial wall of small blood vessels and leads to significant morbidity and mortality. Histopathological studies of the cutaneous vessels of patients with calciphylaxis have demonstrated circumferential calcification of the small and medium sized vessels[353]. However skin biopsies from HD patients without a diagnosis of calciphylaxis haven’t demonstrated significant vascular wall calcifications[96]. The relationship between mineral bone disease and the microcirculation is more complex than simply vessel rigidity and I will discuss this in more detail in Chapter 4.

Rigidity of the medial layer and alterations to the responsiveness of smooth muscle cells to NO or reduced bioavailability may represent defects in an

endothelium independent vasodilatory mechanism and explain the reduced response to SNP application that I and others observed in the cutaneous microcirculation *in vivo*.

In contrast, iontophoretic application of ACh causes vasodilatation in an endothelium-dependant manner primarily via the actions of endothelium derived hyperpolarising factors[354] prostanoids[355] and nitric oxide[242]. Although in this study there was a trend towards a reduction in the ACh response in the PD patients compared with healthy controls, this did not reach statistical significance. *Ex-vivo* work has suggested that in human resistance arteries exposed to a uraemic milieu, vasodilatation mediated at kinin receptors is more impaired than muscarinic receptors[339]. We tested only one specific agonist, disease specific risk factors may differentially affect receptors and their regulatory pathways. In this study we did not demonstrate any significant difference in the vasodilatory response to ACh or SNP in PD patients compared with the control group matched for significant co-morbidities. The design of my study means that abnormalities of endothelial function less than 0.75 SD difference from the mean might still be present in PD patients.

There are no published studies examining cutaneous microvascular function exclusively in PD patients for direct comparison. Most previous work has focused on HD patients, where PD patients were included they were analysed as a single group with the HD patients[348]. Farkas and colleagues compared 16 HD patients with 16 healthy controls and 16 patients with essential hypertension[91], all 3 groups were well matched for age and sex. During the PORH test they also reported a reduction in peak post occlusive flow following a 3 minute occlusion in the HD and hypertensive groups compared with healthy

controls. However they found no statistically significant difference between the hypertensives and the dialysis patients. In keeping with my results in PD patients, they reported a statistically significant reduction in response to SNP in the dialysis group compared with both controls and hypertensives. Additionally they reported a significant reduction in the response to ACh in both HD and hypertensive groups compared with controls but also between the hypertensive and HD patients. The aim of Farkas' study was to analyse the effect of blood pressure on microvascular reactivity, consequently the mean blood pressures in their hypertensive group and dialysis group were significantly higher than the controls (systolic blood pressure controls 122mmHg, hypertensives 152mmHg and HD patients 152mmHg). The hypertensive group had significant hypertension having been recruited from a hospital clinic where they had been referred for uncontrolled hypertension, with a 13-14 year history of hypertension. Additionally their protocol called for withdrawal of all antihypertensive medications 72 hours prior to the study investigations, although the reason for this is unclear and 72 hours is unlikely to be a long enough wash-out period for many drugs. In contrast, the 3 groups in my study were well matched for systolic blood pressure and the average for all 3 groups was at least 10mmHg lower than the participants in Farkas' study. Previous studies in CKD have suggested that hypertension has a more deleterious effect on endothelial dependant vasodilatation than uraemia[356, 357]. Several studies in hypertensive patients with normal renal function, have shown improvements in PORH following blood pressure reduction [264, 358]. Therefore we may have seen a larger difference between groups in the endothelial dependant pathways if blood pressure was less well controlled in the dialysis group. However the aim of including a matched control group in

addition to a healthy control group was to assess the level of microvascular impairment attributable to uraemia and dialysis therapy independent of other co-morbidities.

Several other studies have demonstrated significant reductions in response to both ACh and SNP in HD patients compared with healthy controls[107] and patients with ESRD on conservative management [356]. Several factors may account for their significant findings with regard ACh. Dialysis patients in these studies had a dialysis vintage of at least 5 years in comparison to my PD patients (mean duration of treatment = 15 weeks) and they may therefore represent a cohort with increased exposure to vascular insults. In common with the Farkas study [91] participants in these studies were on average 10 years younger than in our study[107, 356]. It has been suggested that age is inversely associated with microvascular function in healthy subjects but that this relationship is lost in advanced CKD[348]. Consequently this may be reflected in proportionately better microvascular health in the younger control groups in these studies than reported here.

Another factor to consider in these studies of HD patients in comparison to my PD cohort is residual renal function. In Farkas' study [91] 6 out of the 16 study participants were anuric, the remainder were described as having 'very low residual renal function'. Residual renal function has been shown to be independently associated with endothelial dysfunction at least in the form of reduced FMD[359]. Residual renal function in my PD group varied but all patients in this study had a urine output of at least 500ml/day.

An acknowledged difficulty in the interpretation of in vivo microvascular studies is variation in protocol[110]. The protocols for iontophoresis and PORH in the above studies were all subtlety different from each other and from the one used here making direct comparisons between the above studies and mine even more challenging.

In this study I was able to demonstrate a significantly reduced response to PORH in the PD patients compared with both healthy controls and matched controls. I also demonstrated a significantly reduced response to SNP in the PD patients compared with healthy controls but compared with matched controls the reduction was not significant. No significant reduction in the ACh response was demonstrable compared with either healthy or matched controls. As we have seen above studies in other dialysis cohorts (mainly comprised of HD patients) have been able to demonstrate larger and more significant reductions compared with controls. A number of factors may contribute to this disparity. The dialysis population is significantly skewed in favour of HD and therefore recruitment of large numbers of PD patients is difficult. The 28 patients in each of my three groups would allow detection of a 0.75SD difference between groups. The difference in response between PD patients and healthy controls was 0.48SD and therefore this study was not adequately powered to detect this level of difference. Another contributory factor, especially in small groups is heterogeneity. To allow an adequate representation on our PD cohort I included in my study patients with diseases known to effect microcirculatory function e.g. diabetes mellitus. In Farkas' study[91] they were able to demonstrate a difference of 4SDs between their HD patients and controls and 3.7SDs between their HD patients and hypertensives. In addition to the factors outlined in the above paragraphs they excluded patients with diseases known to affect the

microcirculation, resulting in a more homogenous cohort and reduced variability in their outcome measurements.

Previous studies of cutaneous microvascular reactivity to ACh and SNP have struggled to show a difference between healthy controls and patients with CKD who are not on dialysis[356, 360]. One explanation for the relatively modest differences I observed in my PD cohort, who were relatively new to dialysis therapy, is that they sit on a continuum between patients with CKD and HD patients who are receiving a more haemodynamically stressful therapy. In Thang's study comparing dialysis patients with controls[348] they studied both PD and HD patients but analysed them as a single group commenting that the 'responses to ACh and SNP were similar' although no figures are given. Of note this study included 20 HD patients and 16 PD patients and therefore would not have been powered to detect a difference of less than 1SD between these patients. There are no other published studies examining the difference between HD and PD patients using these techniques.

The control group was well matched with the PD patients for age, sex, blood pressure, diabetes and CVD. However there were some important differences between the groups that may have influenced microvascular reactivity. Use of medications that inhibit the renin angiotensin aldosterone system (RAAS) was significantly higher in the PD patients compared with controls. It has been suggested that ACE inhibitors may have additional benefits for the microcirculation above and beyond their blood pressure lowering effects. In a study of hypertensive renal transplant recipients randomized to nifedipine or Lisinopril, the ACE inhibitor group had a significantly better post occlusive hyperaemic response after 21 months of treatment[269]. There was also a trend

towards increased response to iontophoresis of ACh in the Lisinopril group, but this did not reach statistical significance. However, a shorter study in hypertensive patients found no effect on endothelial function measured by iontophoresis of ACh or FMD after 12 weeks of Ramipril versus doxazosin[361]. The exact effect of RAAS inhibition on the reactivity of the cutaneous microcirculation is unclear. Although there was significantly more RAAS blockade use in my PD group the distribution of results don't seem to indicate that it systematically skewed the PORH results or reaction to vasoactive drugs (Figure 3.4 and Figure 3.5). If RAAS inhibition does improved cutaneous microcirculatory function then the true difference in peak post occlusive hyperaemic response may be larger than reported here and it may explain the lack of significant differences between the Ach response in the PD group and the matched controls.

The other major difference between the groups was haemoglobin levels. As would be expected the PD group were significantly more anaemic than the other two groups. The impact of this on the measurements taken by laser Doppler is unclear. Even at normal haemoglobin levels, red blood cell filling in the microcirculation is highly variable[292] and how this is reflected in perfusion will to some degree be dictated by adaptations at the microvascular level to chronic anaemia[362]. To my knowledge no studies have looked directly at the impact of anaemia on laser Doppler measurements. However as demonstrated in previous studies[356] although haemoglobin and haematocrit were significantly lower in the PD patients than controls no significant difference in baseline flow as measured by LDF was observed (mean ± SD 23±16.5 in healthy controls, 20.7±13.4 in matched controls and 22.4±15.2 in PD patients p=0.569).

The main causes of anaemia in CKD are iron deficiency and deficient erythropoietin production in renal tubular cells. Red blood cells modulate microvascular tone by releasing NO which stimulates vasodilatation[363], anaemia will result in reduced NO bioavailability. Recombinant human erythropoietin (EPO) is a common treatment for renal anaemia. Clinically EPO has been shown to increase systemic vascular resistance[364], however in cultured human endothelial cells[365] and rodent models of CKD[366, 367] it was shown to increase NO availability via it's actions on eNOS thereby potentially improving microvascular function. 60% of our PD patients were receiving EPO treatment, there were no significant differences in reactivity between those taking EPO and those not. Anaemia and it's treatment have a complex interaction with microvascular reactivity and may have introduced further variability into the data.

This study is the first to attempt to evaluate cutaneous microvascular reactivity *in vivo* in a cohort of exclusively PD treated patients compared with healthy controls and appropriately matched controls. The strengths of the study are; 3 well characterised groups, well matched for factors known to affect microvascular health. All the microvascular tests were conducted in a single department and therefore rigorously standardised. With regards the PD patients standardisation of the oral and peritoneal intake at the time of the investigations adds to the rigor of the protocol. The study has a number of limitations; this is a secondary analysis of PD patients who were recruited for a different study, consequently mean duration on PD is short. It is a cross-sectional study and not longitudinal. Control participants were selected from a cohort recruited and analysed at an earlier date, however the single centre nature of this study and continuity of protocol and equipment and rigorous training of operators will help

to limit variability. Whether the impairments demonstrated are the result of chronic uraemia, PD treatment or a combination of both cannot be elucidated from this study as there was no control group with advanced renal disease. More information on any potential impact of PD therapy on the microcirculation could be gained from a longitudinal study of patients as they progress through their PD career. However experience from conducting the present studies has shown that in a single centre it is difficult to recruit and retain sufficient numbers of PD patients for meaningful statistical numbers.

This is the first study to examine cutaneous microvascular reactivity in PD patients. I have shown a reduction in multiple aspects of cutaneous microvascular reactivity in patients with end stage renal disease on peritoneal dialysis compared with healthy controls and patients with similar metabolic and cardiovascular risk profiles. This impairment appears to be a result of deficiencies in multiple vasodilatory pathways not only NO dependant pathways and attributable to chronic uraemia or an aspect of PD therapy and not to co-morbidities.

Chapter 4 The relationship between systemic microcirculatory function and small solute transport in incident peritoneal dialysis patients

4.1 Introduction

Although the role of some of the anatomical components of the peritoneum in the peritoneal transport barrier are still the subject of debate, the peritoneal microvascular endothelium is generally acknowledged to be the greatest determinant of the rate of small solute transport in PD. As demonstrated in Chapter 3 patients on PD have impaired systemic microcirculatory function compared with healthy controls. The relationship between the systemic and peritoneal microcirculations is likely to be complex, wherein both are influenced by demographic and biochemical factors including age, sex, co-morbidity and inflammation. Progression of CKD and it's sequelae e.g. mineral bone disorders also impact microvascular health. The relationship between systemic and peritoneal microvascular structure and function on commencement of PD therapy remains relatively unstudied. One previous study attempted to compare *in vivo* markers of systemic microvascular function with rate of small solute transport[169]. However, their conclusions with regards any relationship between the peritoneal microcirculation and the systemic microcirculation were limited by the fact that their PD cohort had a mean duration of dialysis of 24 months.

4.2 Aims and objectives

Primary Aim

To test the hypothesis that incident patients with a high rate of small solute transport would exhibit a greater degree of systemic microcirculatory dysfunction than those with slower rates of small solute transport.

Secondary Aims

To examine whether peritoneal transport of larger molecules is associated with systemic microvascular structure or function

To examine whether intraperitoneal or systemic inflammation is associated with rate of small solute transport in this cohort

To examine whether severity of mineral bone disease is associated with impairments in systemic microvascular structure or function.

Primary Objectives

To recruit a cohort of patients who were new to PD (on therapy less than 6 months).

To study multiple aspects of systemic microvascular structure and function in recruited patients.

To measure small solute transport rate in recruited patients, using the peritoneal equilibration test.

To compare small solute transport rate with systemic microvascular structure and function in incident PD patients.

Secondary Objectives

To measure serum and intraperitoneal albumin and calculate peritoneal clearance of these.

To compare transport of albumin with systemic microvascular structure and function.

To measure systemic and intraperitoneal inflammation.

To compare small solute transport rate with systemic and intraperitoneal inflammation.

To measure serum parathyroid hormone (PTH), phosphate and calcium as markers of CKD mineral bone disease.

To compare serum markers of mineral bone disease with systemic microvascular structure and function.

4.3 **Methods**

This was a cross-sectional, observational study of incident PD patients. I aimed to recruit 46 participants across two research sites. The study was approved by East Midlands – Leicester Central Research Ethics Committee (REC ref 16/EM/0395) and conducted according to the declaration of Helsinki. The aims, objectives and conduct of the study were reviewed by the local lay steering committee (Peninsula Research Bank steering committee). All patient facing material was reviewed by the general steering committee and additionally by patients with an interest in renal research, feedback was incorporated into the final drafts of these documents.

Participants were recruited from patients attending Royal Devon and Exeter Hospital and University Hospital Wales for their dialysis care. Potential participants were identified by the clinical care teams, offered a participant information sheet and with their agreement they were referred to the research team.

Inclusion criteria:

Aged 18 years or older

Less than 6 months of PD therapy

Exclusion criteria:

Unable to give informed consent

Use of calcineurin inhibitor (CNI)

History of Raynauds disease

Major vascular event within preceding 3 months

All participants gave written informed consent.

Peritoneal Permeability

All participants underwent a test of peritoneal permeability. These formed part of the participants' routine clinical care and were therefore conducted by appropriately trained members of the clinical team, according to local protocols. Participants recruited from the Royal Devon and Exeter Hospital underwent a standard Peritoneal Equilibration Test (PET) as described in detail in Chapter 2. Participants recruited at the Cardiff site underwent a 'fast PET' according to their local practice, whereby dialysate samples are only taken from the overnight dwell and at the end of the 4 hour dwell. All other aspects of the PET including the preceding night's dwell were as for the standard PET and excellent agreement between D/P_{Cr} at 4 hours for standard PET and fast PET has been shown ($r = 0.77$ $p = 0.0001$)[301].

As described in Chapter 2 differences in laboratory creatinine assays can influence results and therefore all samples collected during the PET at the Cardiff site were re-analysed in the Exeter laboratory, to allow comparison.

Systemic Microvascular Tests

Participants were not studied within 4 weeks of any inflammatory illness. Participants underwent the microvascular tests at the Diabetes and Vascular Research Centre, Exeter or at the Clinical Research Facility, University Hospital of Wales. Participants attended for their study visit in the morning, orally fasted for at least 8 hours overnight. Traditional peritoneal dialysate contains high levels of glucose which is absorbed into the systemic circulation at a variable rate. Participants were therefore asked to attend 'dry' i.e. without any dialysate in situ. Their overnight dialysis regime the night before was conducted as normal. Fasting bloods were collected. A brief clinical examination was performed including; blood pressure and a subjective assessment of fluid status (weight and clinical assessment of fluid status). Information was recorded on; demographic details, past medical history, current medications and dialysis regime.

To reduce the influence of food intake on microvascular parameters, participants were given a standardised meal of two pieces of brown toast with butter and a glass of water. Due to the homeostatic role of skin microcirculation, all investigations were performed in a temperature controlled room in which participants had acclimated for 30 minutes, as described in Chapter 2. All microvascular investigations were performed by myself.
Participants underwent 4 tests of microvascular structure and function, described in detail in Chapter 2;

- The reactive hyperaemic response to a 4 minute period of arterial occlusion of the arm, measured using laser Doppler flowmetry (PORH)

- The response to iontophoretic application of acetylcholine (ACh) 1% dissolved in mannitol and sodium nitroprusside 0.25% (SNP) dissolved in 0.45% saline, to measure endothelium dependant and endothelium-independent vasodilation respectively. The degree of response was measured using laser Doppler perfusion imaging.
- Measures of sublingual microvascular density, perfusion and flow characteristics, captured using sidestream darkfield imaging (SDF)
- Measurement of glycocalyx barrier properties in the sublingual circulation captured using SDF imaging and analysed with Glycocheck© software

Analysis of blood, dialysate and urine samples

Blood and dialysate samples collected at the time of the PET and during the microvascular visit were frozen at -80^{o}C for later analysis.

Residual creatinine clearance was calculated from a 24 hour urine collection. Creatinine measurements in blood and dialysate were conducted in the biochemical laboratory at the Royal Devon and Exeter Hospital using the Creatinine Jaffé Gen.2 by Cobas. The manufacturers state that glucose levels <120mmol/L do not interfere with this assay.

IL-6 was measured in blood and dialysate (samples were taken from the overnight dwell the night before the PET) at the immunoassay biomarker core laboratory, University of Dundee. Samples were measured in duplicate using IL-6 Meso Scale Discovery V-PLEX assay, the lower limit of detection for this assay is 0.06pg/ml.

Albumin was measured in the 4 hour dialysate sample at the biochemistry laboratory at the Royal Devon and Exeter Hospital using Roche immunotrubidimetric microalbumin assay and expressed as mg/L. Peritoneal albumin clearance was calculated using the formula

$$\text{Peritoneal alb Cl} = D_{alb} / P_{alb}$$

Where D_{alb} = dialysate albumin content, P_{alb} = plasma albumin concentration, and expressed as ml per 4 hour dwell.

Statistics

Statistical analysis was performed using StataSE-16 (Stata Corporation, Texas, USA). Graphical analysis was performed using GraphPad Prism 8 software. All variables were tested for normality by visual inspection of a histogram plot and by Shapiro-Wilks test. For normally distributed data correlations between variables were assessed using Pearsons correlation coefficient. For non-normally distributed data correlations between variables were assessed using Spearman's rank correlation coefficient. A result was considered statistically significant if $p \leq 0.05$.

46 completed participants would have allowed detection of a moderate association between peritoneal small solute transport and systemic microvascular factors, correlation coefficient of 0.4 with power of 80% and 2-sided $\alpha = 0.05$.

4.4 **Results**

Recruitment targets were based on an average of 40 new starters on PD per annum at each of the recruitment sites, a recruitment period of 2 years and an assumption that 50% of new starters would agree to participate. It was also assumed that recruitment would be higher at the Exeter site as a consequence of my regular presence and input. Participants at the Cardiff site were recruited and consented by the clinical team during clinic visits, data is not available on the numbers approached versus those who consented.

Recruitment for the study is summarised in Figure 4.1. At the Exeter site there were 63 new starters during the recruitment period. 10 were deemed not suitable for PET testing, this was either a consequence of frailty or medical issues which resulted in them losing their PD catheter prior to their first PET (e.g. peritonitis). Of those persons who declined to take part the most common reasons given were high burden of existing hospital visits and travelling time/distance. A proportion of potential participants who were approached agreed in principle but intervening illness prevented them from attending for the consent/study visit.

In total 37 participants were consented across the two sites. 8 participants withdrew or were withdrawn from the study prior to the microvascular investigations. Reasons for withdrawal were; 2 withdrew for social reasons, 2 were withdrawn for intervening acute medical issues, 2 were withdrawn as they were unable to attend for their visits within 6 months of starting PD, 1 changed to HD and 1 was withdrawn as they were subsequently found to be on a CNI (exclusion criteria). The challenges, general and specific to this study,

associated with recruitment and retention in this population are discussed in more detail in Chapter 6.

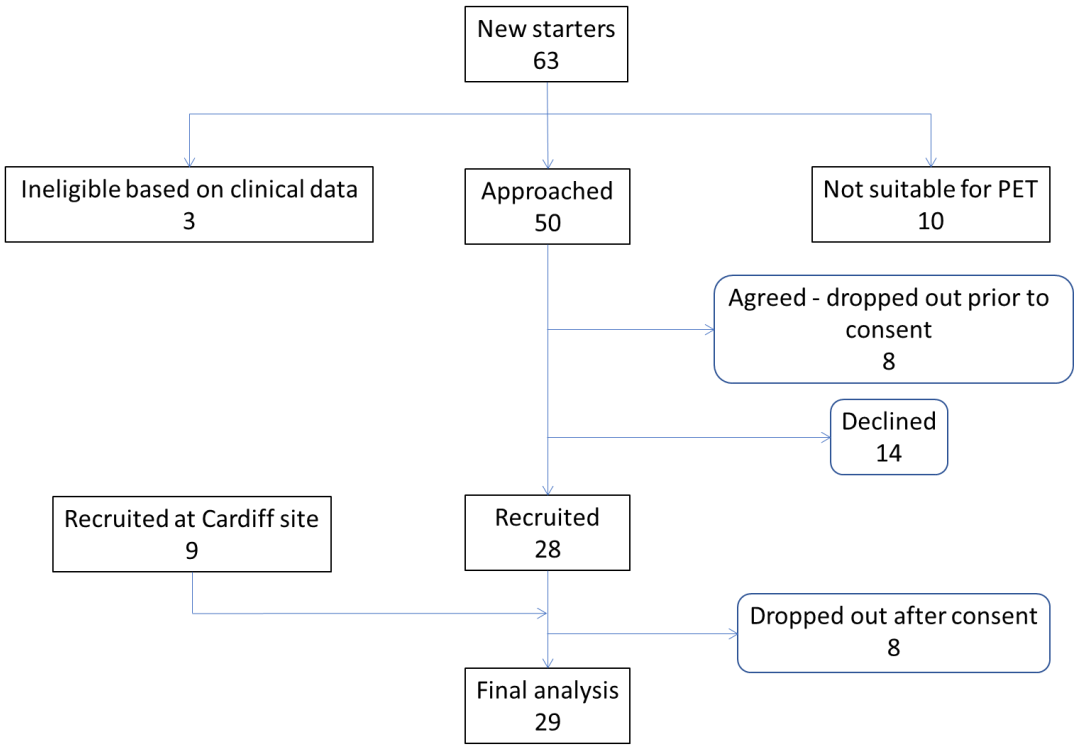

Figure 4.1 Flow diagram summarising the study recruitment process

29 participants were included in the final analysis. Primary diagnoses of renal failure were; hypertensive nephropathy 27.6%, glomerular nephritis 17.2%, small vessel vasculitis 10.3%, polycystic renal disease 10.3%, small kidneys/unknown cause 10.3%, diabetic nephropathy 6.9%, reflux nephropathy 6.9% and other 10.3%. Demographic data for the cohort are detailed in Table 4.1.

Small solute transport as defined by dialysate to plasma ratio of creatinine at 4 hours ($D/P_{Cr}4H$) was significantly negatively skewed and therefore all analyses using this variable were done using non-parametric tests. As would be anticipated there was a highly significant correlation between $D/P_{Cr}4H$ and the

ratio of dialysate glucose at 4 hours compared with T0 (D_{4H}/D_0) (Spearman's r = -0.92 p=<0.0001). Therefore for the purposes of analysis, small solute transport will be presented here as it relates to $D/P_{Cr}4H$.

There was no significant relationship between age and $D/P_{Cr}4H$ in this cohort (Spearman's r = 0.269 p=0.159). $D/P_{Cr}4H$ trended towards being higher in male than female participants but didn't reach statistical significance (female median 0.75[0.69-0.85], male median 0.87[0.77-0.94] p=0.08). Comorbidity was documented using the Davies Comorbidity Index (DCI) [368]. This score was developed in PD cohorts using seven disease domains chosen to reflect the dominance of cardiovascular morbidity in end stage renal disease. Patients are then categorised into low-risk (score 0), intermediate-risk (score 1-2) and high-risk (score >2). There was no association between small solute transport rate and DCI. There was no significant difference between rate of small solute transport in patients with diabetes compared with non-diabetics (diabetes (n=9) $D/P_{Cr}4H$ 0.8[0.77-0.93], no diabetes (n=20) $D/P_{Cr}4H$ 0.84[0.655-0.915] p=0.759).

Table 4.1 Characteristics of study participants in the analysis

Patients (n)	29	Previous HD n(%)	8(27.6)
Age (yrs)	68 [58-77]	AVF n(%)	2(6.9)
Male n(%)	19 (65.5)	Serum	
Caucasian n(%)	26(89.7)	Haemoglobin (g/L)	116.5[108.5-127.5]
Systolic bp (mmHg)	140±21	Calcium (mmol/L)	2.35±0.15
BMI	27.6±5.7	Phosphate (mmol/L)	1.48±0.22
Davies Comorbidity Index		PTH (pmol/L)	19.35[11.2-42.9]
Low/intermediate/high(%)	34.5/62.1/3.4	CRP (mg/L)	6[2-10]
Diabetes n(%)	9(31)	Interleukin-6 (pg/ml)	1.48[0.72-2.69]
Medications n(%)		Albumin (g/L)	37.48±5
RAAS blockers	11(37.9)	Dialysate	
Beta blockers	13(44.8)	Interleukin-6 (pg/ml)	31.25[16.68-71.54]
Active vitamin D	18(62)	Albumin (mg/L)	404.3[321.2-510]
EPO	18(62)	Residual CrCl (L/wk)	62.68[47.79-97.18]
Phosphate binder	12(41.4)	D/P_{Cr} at 4 hours	0.83[0.71-0.93]
CAPD n(%)	13(44.8)	D/D_0 glucose at 4 hours	0.25[0.23-0.34]
Time on PD (wks)	15.4±5	Peritoneal albumin clearance (ml/4hourdwell)	20.4[15.6-25]

Data is presented as mean ± SD or median [IQR]. BMI=body mass index, RAAS=renin angiotensin aldosterone system, EPO=erythropoietin, CAPD=continuous ambulatory peritoneal dialysis, AVF=arteriovenous fistula, PTH=parathyroid hormone, CRP=C reactive protein, CrCl=creatinine clearance, D/P_{Cr}=dialysate to plasma ratio of creatinine, $D/D_{0\ glucose}$=ratio of dialysate glucose compared with time 0.

Relationship between small solute transport and systemic microvascular structure and function

The results of the microvascular tests in this cohort are summarised in Table 4.2. The results for small solute transport ($D/P_{Cr}4H$) were significantly negatively skewed and multiple transformations failed to significantly improve the distribution of data, consequently all correlations have been conducted using non-parametric tests (Spearman's correlation coefficient r_s).

In this cohort there was a significant relationship between age and the peak and AUC of the post occlusive reactive hyperaemic curve (r_s=0.425 p=0.033, r_s=0.432 p=0.031 respectively). There was no significant relationship between age and any of the other microvascular parameters. There was no significant relationship between any of the microvascular parameters and sex or systolic blood pressure. In this cohort we did not find any relationship between the microvascular parameters studied and residual renal function.

Table 4.2 Summary of the results of the tests of cutaneous and sublingual microvascular structure and function

Parameter	PD patients
Post Occlusive Reactive Hyperaemia (n=25)	
Peak response (AU)	57.4 [45.2 - 85.2]
Total response (AUC)	3907.84 ± 2098.23
Response in 1st minute (AUC)	2390 [1875 - 3660]
Iontophoretic application of vasodilators (n=23)	
Peak response to ACh (AU)	473.7 ± 179.7
Total response to ACh (AUC)	89175.2 ± 55021.2
Peak response to SNP (AU)	402.3 ± 141.7
Total response to SNP (AUC)	58329.2 ± 31915.2
SDF imaging of the sublingual circulation – vessels <20µm (n=25)	
TVD (mm/mm^2)	24.76 [23.63 – 28.07]
De Backer score (1/mm)	16.7 ± 2.34
PVD (mm/mm^2)	23.85 [21.88 – 26.91]
PPV (%)	95.06 [91.95 – 97.95]
MFI	2.91 [2.83 – 3]
Glycocheck (n=26)	
PBR (µm)	2.2 ± 0.22
RBC filling (%)	65.3 [59.8 – 68.5]

Data is presented as mean ± SD or median [IQR]. AU = arbitrary units, AUC = area under the curve, ACh = acetylcholine, SNP = sodium nitroprusside, SDF = sidestream dark field, TVD = total vessel density, PVD = perfused vessel density, PPV = proportion of perfused vessels, MFI = microvascular flow index, PBR = perfused boundary region, RBC = red blood cell

Post occlusive reactive hyperaemia (PORH)

25 of the participants had data adequate for analysis. The primary effect parameters for PORH were absolute peak flux measured in arbitrary units (AU) and area under the curve (AUC) for the total response and the first minute of the response. No relationship was found between $D/P_{Cr}4H$ and the absolute peak of the post reactive hyperaemic curve (Spearman's r = 0.159 p= 0.448) see Figure 4.2, or the area under the response curve (Spearman's r for AUC whole reaction = 0.073 p = 0.728, Spearman's r for AUC for 1st minute of reaction = 0.076 p 0.716). There was no significant relationship between time taken to reach maximum flux or time taken to return to baseline flux and small solute transport (p=0.328 and p= 0.885 respectively).

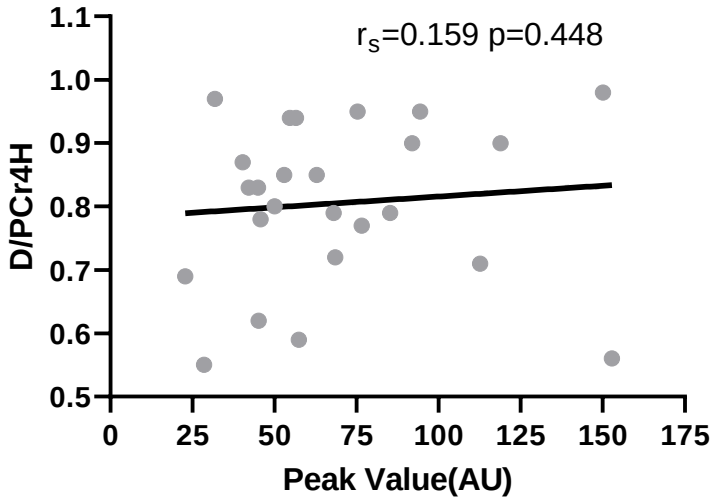

Figure 4.2 Graph of relationship between peak post-occlusive hyperaemia and small solute transport. $D/P_{Cr}4H$ = dialysate/plasma ratio of creatinine after a 4 hour dwell.

Iontophoretic application of acetylcholine and sodium nitroprusside

23 participants had data deemed adequate for analysis. The primary effect parameters for iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) were peak flux value measured in arbitrary units and AUC for the total monitoring period with baseline subtracted.

No statistically significant relationship was seen between $D/P_{Cr}4H$ and response to ACh or SNP application, Figure 4.3.

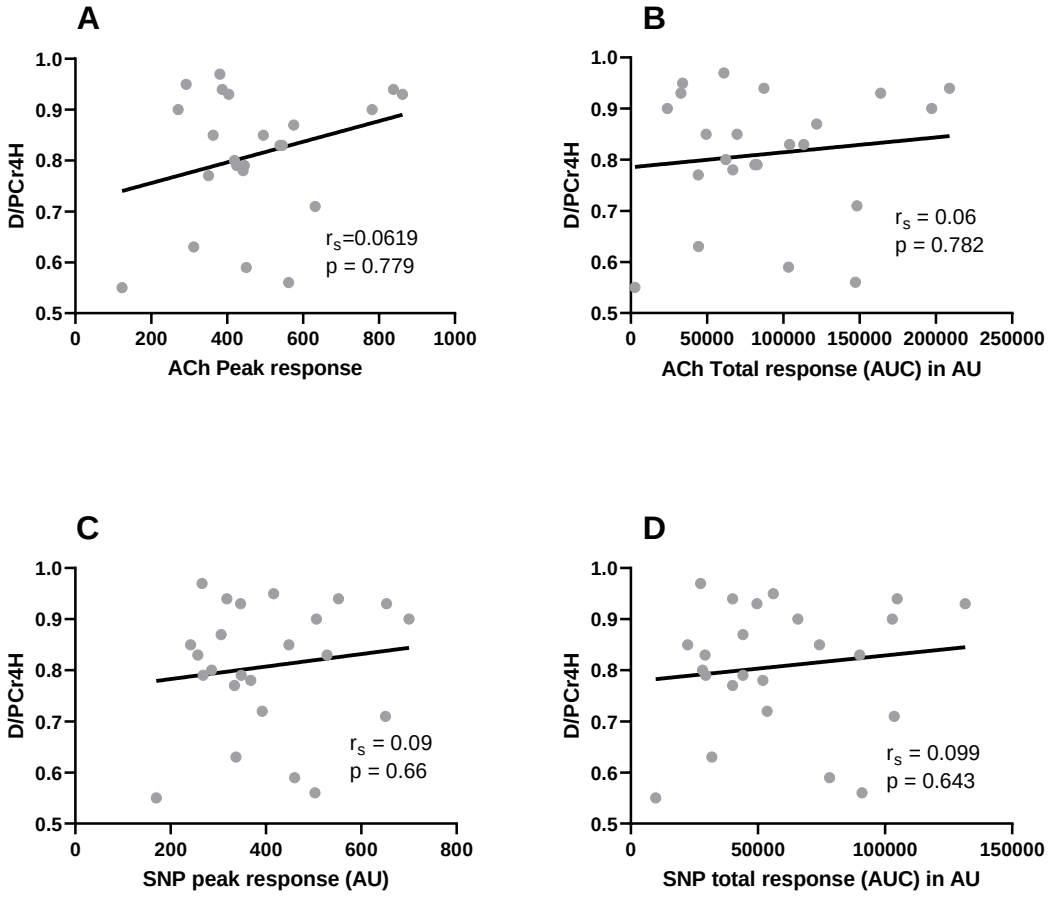

Figure 4.3 Graphs showing the relationship between small solute transport and A) peak response to ACh B) total response to ACh C) peak response to SNP and D) total response to SNP. P = >0.05 for all correlations. $D/P_{Cr}4H$ = dialysate to plasma ratio of creatinine after 4 hours, ACh = acetylcholine, SNP = sodium nitroprusside, AU = arbitrary units, AUC = area under the curve

Sidestream Dark Field (SDF) Imaging of the Sublingual Circulation

25 participants had data deemed adequate for analysis.

The primary effect parameters for SDF imaging of the sublingual circulation were; vessel density measured as total vessel density (mm/mm^2) and DeBacker score (1/mm) and vessel perfusion measured as perfused vessel density (mm/mm^2), proportion of perfused vessels (%) and microcirculatory flow index (MFI). The nature of these parameters is outlined in detail in Chapter 2. These parameters were measured for each of the 3 groups of vessels; small (<20µm), medium (20-50µm) and large (50-100µm) diameter.

No statistically significant relationship was seen between D/P_{Cr}4H and any of the SDF parameters for the smallest vessels (<20µm), Figure 4.4 and Figure 4.5.

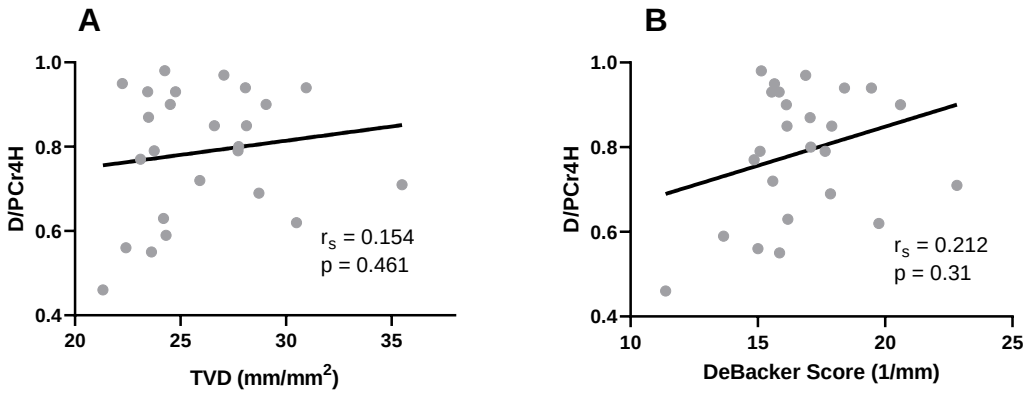

Figure 4.4 Graphs showing the relationship between small solute transport and A) total vessel density and B) DeBacker score in vessels <20µm, measured using SDF. P=>0.05 for both correlations. D/P_{Cr}4H = dialysate to plasma ratio of creatinine after 4 hours, TVD = total vessel density

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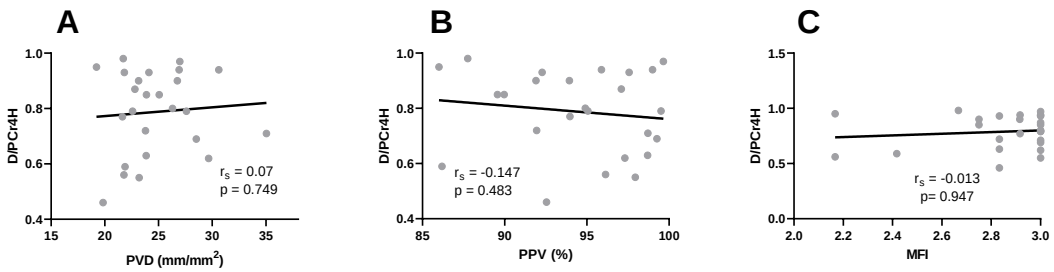

Figure 4.5 Graphs showing the relationship between small solute transport and A) perfused vessel density B) proportion of perfused vessels and C) microvascular flow index in vessels <20µm, measured using SDF. P=>0.05 for all correlations. $D/P_{Cr}4H$ = dialysate to plasma ratio of creatinine after 4 hours, PVD = perfused vessel density, PPV = proportion of perfused vessels, MFI = microvascular flow index

No significant correlations were found when analysis was extended to include all vessel sizes (up to 100µm).

Estimation of glycocalyx properties measured using SDF imaging and Glycocheck© software

26 participants had complete Glycocheck© data.

The primary effect parameters generated by the Glycocheck software are perfused boundary region (PBR) in µm and red blood cell filling (%) in vessels measuring 5-25µm. The measurements used to calculate these parameters are outlined in detail in Chapter 2.

No statistically significant relationship was seen between small solute transport and either of the Glycocheck parameters, Figure 4.6.

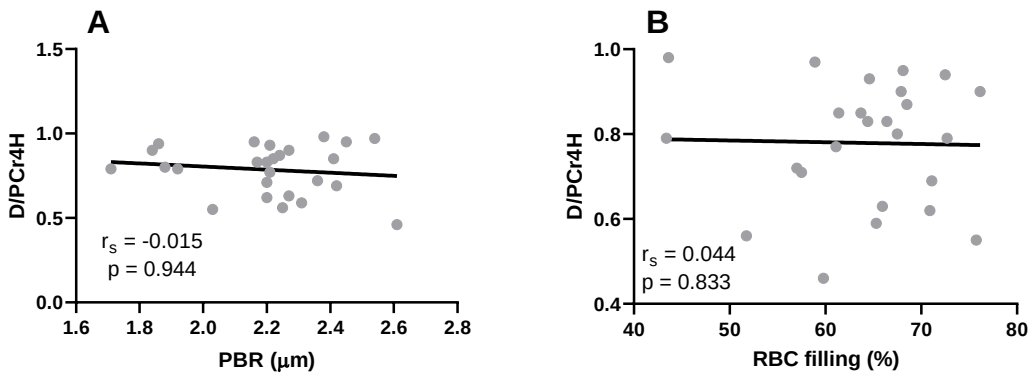

Figure 4.6 Graphs showing the relationship between small solute transport and A) perfused boundary region and B) red blood cell filling in vessels 5-25µm. p = >0.05 for both correlations. $D/P_{Cr}4H$ = dialysate to plasma ratio of creatinine after 4 hours, PBR = perfused boundary region, RBC = red blood cell.

Participants were grouped into quartiles according to $D/P_{Cr}4H$. There was no statistically significant difference between participants in the top and bottom quartiles for any of the microvascular parameters.

This study included a high number of fast transporters. It has previously been suggested that patients in the highest transport category early in their PD career are qualitatively different to the rest of the cohort[169]. I therefore repeated all correlation analyses excluding the top quartile of participants($D/P_{Cr}4H$ >0.93) . This did not reveal any significant associations.

Relationship between peritoneal albumin clearance and systemic microvascular structure and function

Albumin concentration was measured in the 4 hour dialysate sample and peritoneal albumin clearance was calculated as described above. Both were non-normally distributed and therefore non-parametric tests were used. There was a strong and significant correlation between peritoneal albumin excretion (mg/L) and peritoneal albumin clearance (ml/4 hour dwell) r_s=0.94 p=<0.0001.

There was a strong correlation between peritoneal albumin clearance and dialysate IL-6 concentrations (r_s=0.66 p=0.004). There was also a significant correlation between peritoneal albumin clearance and serum IL-6 (r_s=0.73 p = 0.0009).

There was no significant correlation between peritoneal albumin excretion or peritoneal albumin clearance and the measures of cutaneous and sublingual microvascular structure and function.

Relationship between inflammation, small solute transport and systemic microvascular structure and function

Systemic inflammation was measured using C reactive protein (CRP) and Interleukin-6 (IL-6) in blood samples taken at the time of the PET. Intraperitoneal inflammation was measured using IL-6 in the dialysate samples taken from the overnight dwell the night prior to the PET. Results for systemic and intraperitoneal inflammation were both non-normally distributed and therefore correlations were assessed using non-parametric tests.

There was a significant correlation between serum CRP and serum IL-6 (Spearman's r = 0.59 p = 0.0012). There was also a significant correlation between serum IL-6 and dialysate IL-6 (Spearman's r = 0.513 p = 0.0087). There was a strong, significant correlation between dialysate IL-6 and D/P_{Cr}4H (Spearman's r = 0.758 p = <0.0001), Figure 4.7. There was also a weaker but significant correlation between serum IL-6 and D/P_{Cr}4H (Spearman's r = 0.432 p = 0.0245), Figure 4.8. In a multiple regression analysis including systemic and intraperitoneal inflammation the association between D/P_{Cr}4H and systemic inflammation was no longer significant (p = 0.28).

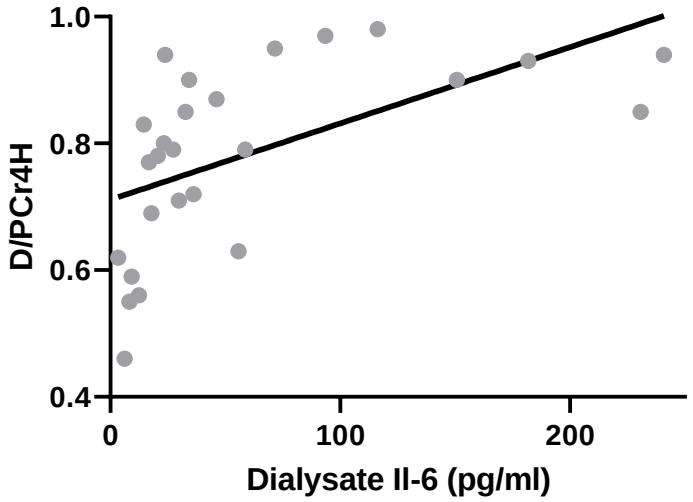

Figure 4.7 Graph showing the relationship between small solute transport and intraperitoneal inflammation. r_s = 0.758 p = <0.0001 D/PCr4H = dialysate to plasma ratio of creatinine at 4 hours, IL-6 = Interleukin-6

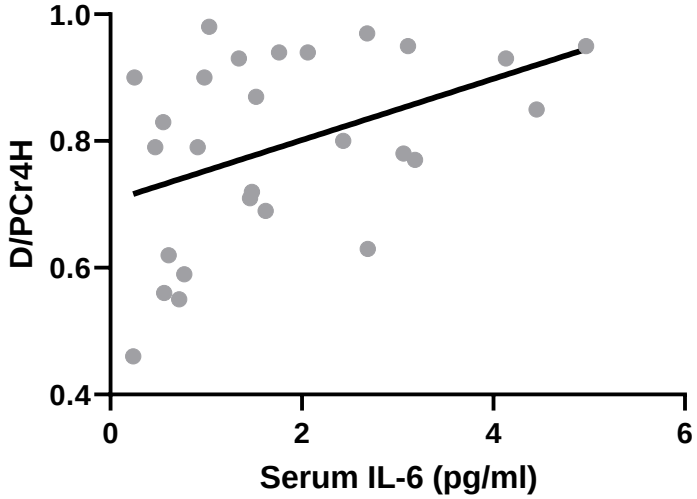

Figure 4.8 Graph showing the relationship between small solute transport and systemic inflammation. r_s = 0.432 p = 0.0245 D/PCr4H = dialysate to plasma ratio of creatinine at 4 hours, IL-6 = Interleukin-6

There was no significant correlation between dialysate IL-6 or serum IL-6 and the measures of cutaneous and sublingual microvascular structure and function.

Relationship between systemic microvascular structure and function and CKD mineral bone disease

CKD mineral bone disease was assessed in participants by measuring serum phosphate, calcium (corrected for albumin) and intact parathyroid hormone (PTH).

A significant negative correlation was observed between serum phosphate and peak response to SNP (r = -0.4589 p = 0.0276), Figure 4.9. There was a trend towards a negative correlation between phosphate and total response to SNP (AUC) however this did not reach statistical significance (r = -0.392 p = 0.0643). No significant correlation was observed between phosphate and any of the other microvascular parameters.

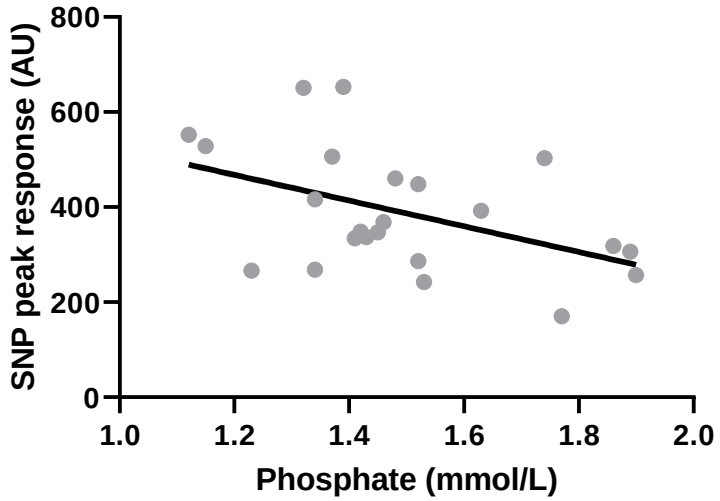

Figure 4.9 Graph showing the relationship between serum phosphate and peak response to iontophoresis of SNP, r = -0.4589 p = 0.0276. SNP = sodium nitroprusside, AU = arbitrary units

A significant negative correlation was also demonstrated between PTH and both peak and total response to SNP (r = -0.5957 p = 0.0027 and r = -0.5274 p = 0.0097 respectively), Figure 4.10. In addition a negative relationship was demonstrated between PTH and maximum post occlusive hyperaemia (r_s = -0.5723 p = 0.0067), see Figure 4.11. There was also a significant relationship between the area under the response curve during the first minute of the post occlusive response (r_s = -0.541 p = 0.0113) but not for the area under the total response curve (r = -0.3338 p = 0.1392), see Figure 4.12, or any of the other parameters of PORH. There was no significant relationship between PTH and the SDF measured parameters.

No correlation was demonstrated for calcium (corrected for albumin) and any of the microvascular parameters.

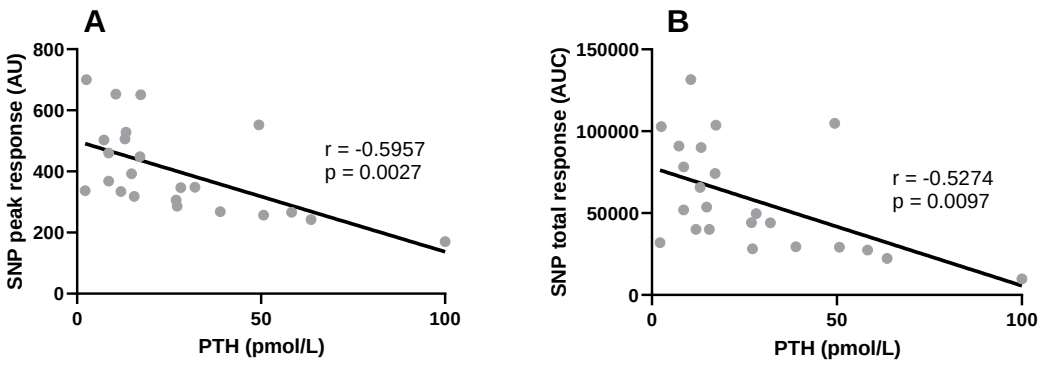

Figure 4.10 Graphs showing the relationship between serum PTH and A) peak response to SNP and B) total response to SNP. p = <0.05 for both correlations.
PTH = parathyroid hormone, SNP = sodium nitroprusside, AU = arbitrary units, AUC = area under the curve.

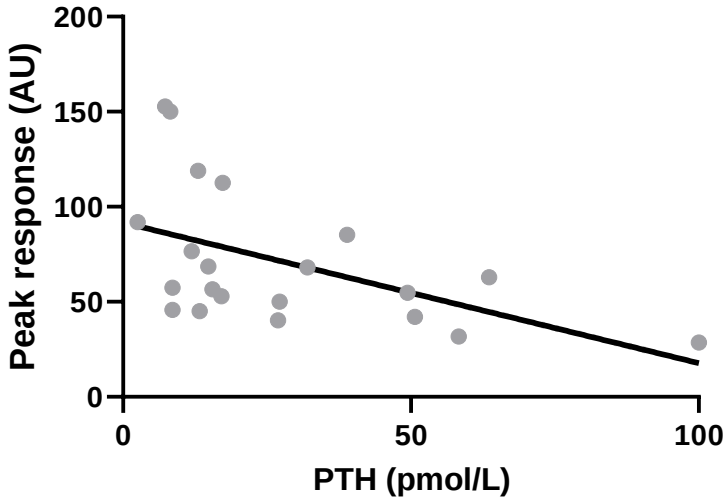

Figure 4.11 Graph showing the relationship between serum PTH and peak post occlusive reactive hyperaemic response, r_s = -0.5723, p = 0.0067. PTH = parathyroid hormone, AU = arbitrary units.

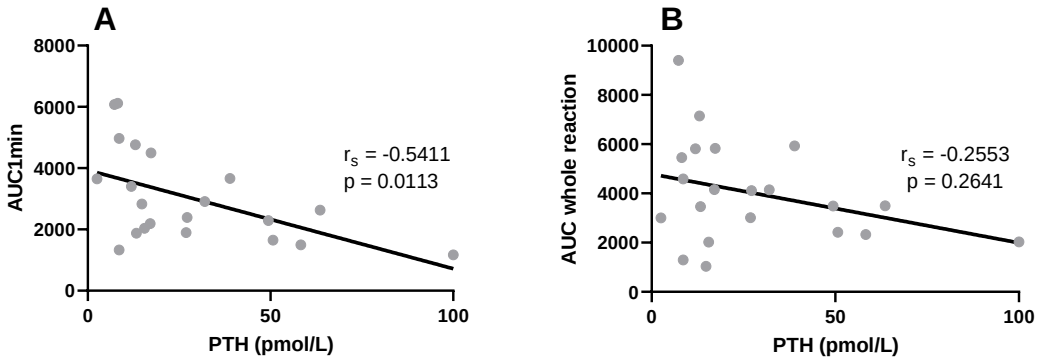

Figure 4.12 Graphs showing the relationship between serum PTH and area under the post occlusive reactive hyperaemic curve for A) the first min and B) the total reactive phase. PTH = parathyroid hormone, AUC1min = area under the curve for first minute.

4.5 **Discussion**

This is the first study to investigate the relationship between in vivo markers of systemic microvascular function and peritoneal solute transport in incident PD patients. The results of this study indicate no moderate or strong relationship between systemic microvascular function, measured in the cutaneous and sublingual circulations, and rate of small solute transport in incident PD patients. This study has however demonstrated a moderate-to-strong relationship between measures of mineral bone disease and cutaneous microvascular structure and function in PD patients. This study has also confirmed the previously reported strong association between small solute transport and intraperitoneal inflammation and demonstrated that this relationship exists even in the early stages of PD.

Multiple studies have demonstrated alterations in systemic microvascular structure and function in various vascular beds in patients receiving haemodialysis therapy. These include; thickening of the basement membrane and endothelial cell activation in skin biopsies[95], reduced capillary number in nail-fold and sublingual beds [99, 100, 115], impairments in both endothelium-dependant and independent skin reactivity [91, 107] as well as reduced maximum vasodilatory capacity in the cutaneous [104, 108] and coronary[119, 120] circulations. There is also some evidence for increased shedding of endothelial glycocalyx components and a reduction in the barrier properties of the vascular endothelial glycocalyx[90]. As reported in Chapter 3, work included in this thesis has demonstrated impairments in multiple aspects of cutaneous microvascular reactivity also exist in patients receiving PD.

The rate of small solute transport across the peritoneum is widely thought to be primarily dictated by factors pertaining to the peritoneal microvascular capillary wall. If the above systemic microvascular changes were reflected in the peritoneal microcirculation then theoretically they would result in alterations in the rate of small solute transport. The ‘Pore-Matrix Theory’ proposes that the anatomical correlate for the ‘small’ and ‘large’ pores is the interendothelial cleft with greater or lesser degrees of endothelial glycocalyx which act as the size discriminant factor[62]. According to traditional pore theory changes in vessel density would alter the small pore area and consequently small solute transport. Peritoneal microvascular density, in biopsy specimens taken prior to commencing PD, has been shown to correlate with baseline small solute transport [33, 34](r = 0.377 p = 0.014 and r = 0.506 p = 0.004 respectively).

Albumin has a molecular radius of 36 angstrom, the majority of it’s transport is predicted to be via large pores but when there is a high degree of convective transport it can also be cleared to a limited extent by small pores. According to pore theory protein clearance via the large pores should be proportional to small solute transport as large and small pores are located within the same capillaries. However pore-matrix theory would seem to dictate that loss of glycocalyx may have a proportionately greater impact on the transport of albumin than on small solutes, due to increased leakiness of small pores or creation of more large pores. This is an attractive hypothesis as peritoneal dialysis patients have previously been shown to have increased systemic albumin leak compared with non-uraemic controls[369] and peritoneal protein clearance/peritoneal albumin excretion has been linked to comorbidity in several studies[370, 371]. These authors suggested that increased flow of proteins through the large pore pathway reflects systemic endothelial barrier

dysfunction. No-one has tested whether peritoneal albumin excretion rate is directly related to systemic endothelial dysfunction.

In this study I did not find any relationship between small solute transport, measured as D/P_{Cr} at 4 hours, or peritoneal albumin clearance and any aspect of microvascular function or structure, studied in the cutaneous and sublingual circulations.

Before concluding that there is no relationship between the systemic and peritoneal microcirculation in these patients there are several factors pertinent to this study that need to be considered.

Comparison with previous studies reporting relationships between small solute transport and clinical variables

Participants were recruited into this study across two geographically distinct sites from consecutive patients starting PD who met the eligibility criteria. This resulted in a wide range of participants both in terms of age (range = 26-87 years), demographics and underlying renal pathology (Table 4.1). Compared with other large reported cohorts in which the relationship between small solute transport and other clinical variables was considered, participants in this study tended to be older, but otherwise were relatively comparable to other cohorts with regards sex, diabetes and level of co-morbidity, Table 4.3. In other studies higher transport in males has been reported[70, 168], in this study there was a trend towards increased small solute transport in men compared with women but this did not reach statistical significance (female median 0.75[0.69-0.85], male median 0.87[0.77-0.94] p=0.08).

The 29 completed participants in this study would allow detection of a correlation coefficient of 0.5. A positive correlation between age and small solute transport has previously been reported[168, 296, 372]. In this study small solute transport and age were both significantly negatively skewed however there was no significant correlation between age and $D/P_{Cr}4H$. The correlation with age reported in the Stoke study[296] was r = 0.15 and this study was not powered to detect this level of correlation. However a correlation with age has not been reported in all studies[70, 373]. The size of other reported correlations in the Stoke PD study[296] were also small (SST v urine volume r = 0.11, SST v level of co-morbidity r = 0.1). If this degree of correlation exists between D/PCr4H and the tests of systemic microvascular structure and function I performed, this study would be underpowered to detect them.

Table 4.3 Previous studies examining the relationship between peritoneal small solute transport (PSST) and clinical variables.

	Age (yrs)	**Sex (%male)**	**Davies co-morbidity index (DCI)[368] Low/intermediate/high (%)**	**Diabetes mellitus (DM)(%)**	**Significant correlation with PSST**
This study N = 29	68	65	34.5/62.1/3.4	31	
Stoke PD[296] N = 574	58.8		50/39/11	14.8	Age ↑ Male sex ↑ DCI ↑
Global Fluid[70] N = 959	55.6	61.6	35.6/56.8/7.6	40	Male sex ↑ DM ↑ (incident pts only)
ANZDATA[373] N = 3,188	58	55.9	NA	38.9	Age ↑
CANUSA[168] n = 606	54.8	60	NA	30	Age ↑ Male sex ↑ DM ↑

One previous study has attempted to assess the relationship between peritoneal small solute transport and sublingual endothelial glycocalyx properties[169]. Several others have compared peritoneal transport rate with flow mediated dilatation (FMD) of the brachial artery[359, 374]. FMD is considered the 'gold standard' measure of in vivo endothelial function in conduit vessels, however the response is to some degree dependant on the vasodilatory ability of the downstream microvessels[375]. Vlahu and colleagues reported no relationship between sublingual endothelial glycocalyx and peritoneal transport[169]. Similarly no association was reported between small solute transport and FMD[374]. Both of these studies were relatively small (n=15 and n=31 respectively) and therefore a weak to moderate correlation could not be excluded (< r=0.6). In a larger study of 72 participants, Han and colleagues[359] did report a correlation of -0.238 ($p = 0.044$) between small solute transport and FMD using Pearsons correlation analysis. However when this was included in a multivariate analysis, adjusted for residual renal function, peritoneal small solute clearance, duration of PD, age, hs-CRP and fibrinogen, this association was lost. None of these three studies were in incident patients, strong correlations between systemic and peritoneal microcirculation in this context would be unlikely in my opinion.

Reported correlations between small solute transport and intraperitoneal factors, specifically inflammation, are much stronger, see below.

Size of the cohort

The original target recruitment number of 46 would have allowed detection of a correlation coefficient of 0.4 with power of 80% and 2-sided $\alpha = 0.05$. During the study period 29 participants were recruited and undertook both study visits. I

faced a number of challenges with regards recruitment and retention of patients in this study. The majority of these challenges were related to the burden of ill-health and therapy experienced by these patients. 15% of new starters at the Exeter site during the study period were deemed by the clinical team to be inappropriate for PET, as a result of frailty or issues with PD functioning. A further 8 patients who expressed an interest in taking part in the study were prevented from doing so by intervening health issues. Although there was no way of me mitigating against these 'losses' it will be something I will take into consideration when planning for future studies. At the Exeter recruitment site 28% of patients who were approached declined to take part. The main reason given for declining was burden of existing hospital appointments coupled with the burden of their therapy. An awareness of the treatment burden already experienced by these patients was a major factor in the design of this study and I attempted to limit the additional burden of participation in this study. It was impossible for the microvascular tests to be conducted on the day of the PET therefore they were combined into only one additional study visit. The number of microvascular tests was limited to the number that could be reasonably performed in half a day, reducing not only the number but also the length of the visit. Compared with similar studies in different patient populations conducted by our group I rationalised the number of blood tests included in the protocol based on an awareness that dialysis patients have regular blood tests and difficult venous access. Feedback from the steering group on previous studies was that participants found parking at the hospital site stressful, therefore parking permits were provided to all participants. Participants were reimbursed for all monetary expenses that arose from their involvement in the study. However throughout the course of the study I also received feedback from

participants regarding their perceived benefits from participating including; time to speak with a renal doctor at length about their renal disease and treatment and feeling good about contributing to local research that may help people in the future.

All the studies outlined in Table 4.3 are larger than this study. Their analyses are based on routinely collected data or data/samples that can be easily collected during routine clinical visits thus significantly reducing the burden to patients of participating in the study. Additional burden associated with participation in a study such as mine, where the investigations cannot be conducted as part of routine clinical care, cannot be completely negated and this is likely to have impacted on the number of patients recruited.

Heterogeneity of the cohort

All *in vivo* studies of the microcirculation have to contend with significant inter-subject variability and intra-subject temporal and spatial heterogeneity[110]. In this study, inter-subject variability was reduced by standardising the pre-test meal and by 'peritoneal fasting' of all participants. Additionally all microvascular tests were conducted by myself thus eliminating any inter-observer factors. Despite this, as this was a cross-sectional study, in an inherently heterogeneous cohort, variability in the outcome measures may have reduced my ability to detect 'small' correlations. In common with many renal cohorts in this study there was significant heterogeneity of age, primary renal disease, co-morbidities and medications. Many of these factors will have direct relevance to the microcirculation and introduce significant variability into the outcome variables. Heterogeneity in the cohort coupled with small sample size may explain why we did not reproduce correlations seen in other cohorts between

peak post-occlusive hyperaemia and age[376] and sex[377]. These studies[376, 377] were conducted in much younger, healthier cohorts thus reducing the outcome variability. Most studies of renal cohorts will be faced with significant heterogeneity as end stage renal disease is a phenotype and not a specific pathology. Like most studies recruiting at a small number of sites restricting recruitment to defined pathologies, for example polycystic kidney disease, would compromise ability to recruit sufficient numbers of patients. Such studies would also limit the applicability of findings.

Intra-subject spatial and temporal variability is more difficult to control for. Even when examined contemporaneously, different vascular beds can exhibit varying responses to the same insult. For reasons of practicality the microvascular tests and the PET test were conducted at separate visits. Criteria were set limiting the time between the study visits to less than 1 month and participants were not studied if they developed a significant illness in the intervening period, however unmeasured differences in the microcirculation between the two visits cannot be excluded.

Relevance of the cutaneous and sublingual circulations to the peritoneal circulation

The majority of studies examining structural changes in the peritoneal microcirculation have focused on changes over time on PD and not on incident patients[71], consequently they can only comment on the effect of exposure to dialysate on the microvasculature. As discussed previously, with time on PD the peritoneum is exposed to a completely nonphysiological environment independent of the rest of the circulation, therefore in all but new PD patients no attempt should be made to compare the peritoneal circulation with systemic

vascular beds. In a study of paediatric patients (mean age 6.7 years)[378], pre-dialysis omental biopsies from children with advanced CKD showed significantly reduced microvascular density compared with healthy age-matched controls. In contrast in a study of peritoneal biopsies taken from adult patients at the time of catheter insertion[34] i.e. prior to exposure to dialysate, they found no difference in microvessel density in the uraemic patients compared with healthy controls. Although participants in the paediatric[379] study were matched with controls for age, CKD at a young age is likely to cause significant growth retardation, body dimensions also influence microvessel density and due to small patient numbers they were unable to fully match for this. Comparison of these two studies is further complicated by the observation that total peritoneal microvessel density is strongly age dependant[13], following a U-shaped curve with bimodal peaks in infancy and adulthood. Reduced cutaneous microvascular density has been demonstrated in the nail-folds of pre-dialysis CKD patients[99, 100]. One study of the sublingual circulation[115] and two in the capillary nail-folds[98, 99] have also indicated reduced microvascular density in dialysis(mixed cohorts including PD and HD) patients compared with healthy controls, dialysis patients in these studies had a mean duration of treatment ranging from 2 to 5 years. However there are no studies directly comparing peritoneal biopsy findings with systemic microvascular findings in incident/predialysis individuals. In uraemic patients reductions in capillary number have been reported in the systemic and peritoneal microcirculation independently however any relationship between the two is purely speculative.

The sublingual circulation and the mesenteric circulation share a common embryological origin. In animal models of sepsis and cardiac arrest, there has been reported significant correlation between microcirculatory changes in the

sublingual bed and the gut, measured using SDF[273, 274]. However in human studies the relationship is less clear. In patients with abdominal sepsis on ICU, there was no correlation between sublingual flow indices and those measured in the intestine via an abdominal stoma[380, 381]. The majority of clinically applicable studies imaging the sublingual circulation using SDF have been conducted from an acute care/intensive care perspective. Consequently all the above animal and human SDF studies are in subjects with significant haemodynamic instability. How these findings relate to stable patients is unclear. Of note one of the ICU studies of intraabdominal sepsis[381] was conducted over the first 3 days of the patients care and they noted that by day 3, when flow in the sublingual circulation and the intestinal circulation was starting to normalise, there was a significant correlation between flow in these beds (r_s = 0.74 p = 0.006). To my knowledge there are no comparable studies in stable patients, directly comparing the peritoneal microcirculation with other vascular beds. Therefore it remains unclear how well the cutaneous and sublingual circulations reflect the peritoneal microcirculation in stable PD patients.

Despite the limitations of this study discussed above, the lack of a strong correlation between the systemic microcirculation and peritoneal solute clearance indicates a disconnect between the two and advances our knowledge of the factors most influential in this process. In pre-dialysis patients, correlations between peritoneal transport and intraperitoneal factors such as microvascular density and inflammation and markers of endothelial dysfunction have been shown to be much stronger than systemic factors[33, 34].

Recently published work in a large cohort has confirmed that intraperitoneal inflammation plays a significant role in rate of peritoneal transport and suggested that peritoneal and systemic inflammation are uncoupled in incident and prevalent PD patients[70]. Dissociated levels of inflammation between the peritoneum and other vascular beds may account for a lack of strong correlation in this study.

The role of inflammation

There is increasing evidence that in any study attempting to examine the relationships of measured variables with solute transport, the most important covariate is intraperitoneal inflammation.

Multiple studies have reported significant associations between intraperitoneal inflammation (IL-6) and small solute transport [382, 383] in prevalent PD cohorts. Using a multivariate linear regression model, the Global Fluid investigators confirmed that dialysate IL-6 concentrations were the most significant predictor of small solute transport[70] not only in prevalent patients but also in incident patients. This relationship was independent of all patient related factors and dialysis prescription.

In patients yet to start PD levels of intraperitoneal inflammation, measured in biopsy samples taken at the time of PD catheter insertion have been shown to be higher than non-uraemic controls and correlated with subsequent small solute transport[33, 34]. This confirms that there are varying degrees of intraperitoneal inflammation present even in the dialysate naïve peritoneum, this variation may account for the inter-patient variation in transport status observed in incident patients. In these studies[33, 34] systemic inflammation measured at

the time of catheter insertion did not correlate with subsequent transport rate. Additionally there was no correlation between intraperitoneal inflammation and diabetes, a well recognised cause of systemic inflammation[34]. In the incident patients of the Global fluid study there was an association between diabetes and plasma IL-6 concentration but not dialysate IL-6 concentrations[70]. This dissociation between the inflammatory response of the peritoneum and the wider circulation to a systemic insult is not yet explained.

A more recent subgroup analysis of the incident patients in the Global cohort has demonstrated that peritoneal protein clearance (PPCl) is also a consequence of local inflammation not systemic inflammation or comorbidity[384]. These authors suggest that the previously noted association between PPCl and co-morbidity is a consequence of the inverse correlation to plasma albumin. This is partially as a result of albumin's reverse acute phase response associated with systemic inflammation but primarily a result of mathematical coupling, the calculation of PPCl has albumin as its denominator which is depressed in systemic inflammation. In a multilevel analysis including systemic and local inflammation PPCl was not found to be an independent predictor of survival[384]. In this study we found significant relationships between both systemic and intraperitoneal inflammation and peritoneal albumin clearance. Based on the lack of relationship in larger cohorts and the strong relationship between serum albumin and systemic inflammation reported in our cohort it is plausible that a proportion of this relationship may be due to the use of albumin.

In this study there was a strong and significant relationship between intraperitoneal inflammation and small solute transport (r_s = 0.75 p = <0.0001) in

keeping with the above published literature. We also report a significant association between systemic inflammation and small solute transport (r_s = 0.432 p = 0.0245). However in a multiple regression analysis including systemic and intraperitoneal inflammation the association between D/P_{Cr}4H and systemic inflammation was no longer significant (p = 0.28). In this study, as in Global[70] the dialysate concentration of IL-6 was >20 times that in plasma despite the diluting effects of 2L of instilled solution. Using mathematical models based on the 3 pore model these high levels have been shown to be the result of local production of IL-6[70], in the Global cohort 87% of patients had dialysate IL-6 concentrations higher than predicted by diffusion across the peritoneal membrane. Therefore the relationship between systemic and intraperitoneal inflammation noted here and in Global may be the result of intraperitoneal IL-6 spilling over into plasma[70].

The impact of mineral bone disease on the systemic microcirculation

In this study there was a significant negative correlation between serum phosphate levels and hyperaemic response to SNP application. There were also significant negative correlations between PTH levels and response to SNP and also the post occlusive hyperaemic response.

A reduction in functional renal mass results in phosphate retention, decreased synthesis of calcitriol, hypocalcaemia and consequent hyperparathyroidism[385], collectively termed CKD mineral bone disease. There is a well-established relationship between progressive CKD mineral bone disease and increased rates of cardiovascular morbidity and mortality[386, 387], the result of arterial calcification and stiffness[388]. There is increasing experimental evidence of the negative effects of disturbed mineral homeostasis

on the microcirculation, comprised not only of vessel stiffening but direct effects on the endothelium and endothelial dependant vasodilatation as described below.

In vitro endothelial cell-based assays have demonstrated impairments in; angiogenesis, vessel wall morphology, endothelial cell migration and endothelial survival associated with a hyperphosphataemic milieu[389]. Exposure of bovine vascular endothelial cells to increased phosphate levels resulted in increased production of reactive oxygen species and decreased NO production, the result of inhibitory phosphorylation of eNOS[136]. Inhibition of eNOS was reversed by a low-phosphate diet, resulting in improved aortic vasodilatation in a rat model of CKD[390]. Thus there are multiple mechanisms by which phosphate may cause microvascular dysfunction. In cultured endothelial cells from larger vessels, PTH promotes osteoblastic differentiation of endothelial cells[391] and increases the expression of endothelial to mesenchymal transition markers which promote ectopic calcification of vessels[392]. Potential mechanisms by which PTH causes impairment of normal endothelial function in the microcirculation are not fully elucidated.

Even in healthy individuals phosphate has been shown to acutely impair *in vivo* endothelial function, short term exposure to high a dietary phosphorus load resulted in significantly reduced endothelial function measured by FMD[136]. In non-CKD cohorts strong negative correlations have been shown between phosphate levels and microvascular/endothelial dysfunction in the form of postocclusive capillary recruitment (-5% per 1mg/dl increase in phosphate 95%CI -10% to -0.1%)[135], retinal venular dilatation (-0.23% per 1mg/dl increase in phosphate)[135] and FMD (r_s = -0.42 p = 0.006)[136]. 75% of

patients in my study had phosphate levels within the normal measurement range. However these associations have been shown for phosphate levels in the normal range[135] and pathological ranges[136].

In hyperphosphataemic CKD4 patients, a reduction in phosphate using sevelamer was associated with an improvement in FMD compared with patients on a calcium based binder that did not reduce phosphate levels appreciably [393]. In HD patients, maximal capillary density measured using nailfold capillaroscopy has been shown to correlate with intact PTH in children ($r = -0.45$ $p= 0.005$)[98] and phosphorus in adults ($r = -0.4$ $p = 0.002$)[99].

In this study there was a strong correlation between PTH and global skin microvascular dysfunction, demonstrated by post occlusive reactive hyperaemia. There were also strong correlations between endothelium-independent skin vasodilatation, measured as the increase in blood flux in response to SNP, and both PTH and phosphate, indicating that mineral bone disease is affecting the function of the smooth muscle cells. We found no association between endothelium-dependant vasodilation, measured as the response to ACh and any markers of mineral bone disease. One possible explanation for this finding could be that an NO independent component of the vasodilatory response to ACh e.g. prostaglandins or EDHF has been upregulated to compensate for the reduced smooth muscle response.

There was no correlation between any of the markers of mineral bone disease and vessel density measured in the sublingual circulation, this is in contrast to the above studies reporting capillary rarefaction in the nailfolds of dialysis patients associated with mineral bone disease[98, 99]. Defects in angiogenesis

sufficient to cause measureable reductions in capillary density may reflect the chronic consequences of exposure to high levels of phosphate and PTH, compared with the acute effects on the endothelium measured by the above reactive tests. Patients in this study had a shorter duration of dialysis therapy (15 weeks compared around with 2 years) and lower average phosphate (median 1.44 mmol/L compared with 1.65mmol/l[99] and 2.2mmol/l[98]) and intact PTH (median 16.3pmol/l compared with 51.2pmol/l[98] and 20pmol/l[99]) than the patients in the nail-fold studies. Alternative explanations include; systemic insults such as hyperphosphatemia differentially affect different vascular beds or capillary rarefaction is specific to capillaries and the vessels examined in the sublingual circulation include vessels that were larger than capillaries (all those >20um) and that this reduced the ability of the sublingual techniques to assess capillaries directly.

This is the first study in CKD patients, showing correlations between markers of CKD-mineral bone disease and functional impairments of the skin microcirculation. The exact mechanisms by which PTH and phosphate negatively affect microvascular function are yet to be fully elucidated.

In conclusion this is the first study to robustly study the relationship between *in vivo* systemic microvascular structure and function and baseline solute transport across the peritoneum in incident PD patients. The study population is well described and rigorously standardised with regards factors that influence the microcirculation including those specifically relating to PD. In this heterogeneous but generally representative PD cohort there was no significant relationship between cutaneous or sublingual microvascular parameters and peritoneal small solute transport. Due to the heterogeneity of the cohort and

relatively small patient numbers a small correlation cannot be excluded. This study adds to the increasingly compelling evidence for the role of intraperitoneal factors, specifically intraperitoneal inflammation, in small solute transport. It seems likely that the significant influence of intraperitoneal inflammation would compound any relationship between systemic microvascular function and peritoneal transport that is too small to have been appreciated in this study.

Chapter 5 24 hour glycaemic profiles of non-diabetic peritoneal dialysis patients

5.1 Introduction

Cardiovascular morbidity and mortality in dialysis cohorts is well in excess of the general population. Although the majority of this risk is attributable to traditional cardiovascular risk factors and the consequences of chronic uraemia, the process of dialysis itself may contribute. Glucose in peritoneal dialysate is freely absorbed from the peritoneal cavity at levels in excess of 100g per 24 hours. Measuring the metabolic burden of this absorbed glucose is complicated by the fact that traditional glycaemic indices such as HbA1c are less reliable in dialysis cohorts. As reviewed in detail in Chapter 1, continuous glucose monitoring(CGM) has been successfully used to delineate the response to glucose based dialysate in patients with diabetes. The systemic consequences of peritoneal glucose absorption have not been studied in non-diabetic cohorts.

5.2 Aims and objectives

Primary Aim

Using continuous interstitial glucose monitors to compare the 72 hour glycaemic profiles of stable, non-diabetic PD patients with non-dialysis controls

Secondary Aim

To compare the metabolic profiles of non-diabetic PD patients with non-dialysis controls

Objectives

To recruit 20 stable PD patients without a diagnosis of diabetes

To recruit 20 control patients who have CKD 5 (eGFR ≤15ml/min) without a diagnosis of diabetes

To measure standard metabolic indices in both groups – HbA1c, fasting plasma glucose, insulin resistance (HOMA-IR)

To measure the response to a standard oral carbohydrate load in both groups – 75g oral glucose tolerance test

To use 72 hour CGM traces to describe the glycaemic profiles of non-diabetic PD patients

To use 72 hours CGM traces to compare average interstitial glucose levels and glycaemic variability in PD patients with non-dialysis controls

To assess the relationship between dialysate glucose exposure and interstitial glucose levels in non-diabetic PD patients.

5.3 **Methods**

This was a cross-sectional, observational study conducted in an outpatient setting. The study was approved by South Central – Hampshire A Research Ethics Committee (REC ref 17/SC/0266) and was conducted according to the declaration of Helsinki. The aims, objectives and conduct of the study were reviewed by the local lay steering committee (Peninsula Research Bank steering committee). All patient facing material was reviewed by the general steering committee and additionally by patients with an interest in renal research, feedback was incorporated into the final drafts of these documents.

PD patients were recruited from patients attending the Royal Devon and Exeter Hospital for their dialysis care. Potential participants were identified by the

clinical care teams, offered a participant information sheet and with their agreement they were referred to the research team.

Inclusion criteria:

Stable on PD for >2 months

Aged 18 years or older

Able to give informed consent

Able and willing to wear a continuous glucose monitor

Exclusion criteria:

Established diagnosis of diabetes mellitus

Any infection requiring treatment with antibiotics within the last 30 days

Use of variable dose of medication known to influence glucose metabolism e.g. glucocorticoids

Chronic or active pancreatitis

Having a serious disease or condition considered by the investigators to interfere with the results of the study

Control patients were recruited from patients attending the ‘renal low clearance clinic’ at the Royal Devon and Exeter Hospital. Patients were eligible for inclusion if their estimated glomerular filtration rate was less than or equal to 15ml/min. Other inclusion and exclusion criteria were as for the PD group.
The control group was defined as patients with CKD 5 but not yet on dialysis. It was considered that these patients would be most comparable to the PD cohort both in terms of the effect of chronic uraemia on insulin resistance and being subject to similar dietary restrictions.

All participants gave written informed consent.

All participants attended for a single study visit at Exeter NIHR Clinical Research Facility. Participants were not studied within 30 days of an inflammatory illness. Participants attended for their study visit in the morning, orally fasted for at least 8 hours overnight. PD patients were asked to attend without any dialysate in situ, though their overnight dialysis regime the night before was conducted as normal. Data were recorded on; demographic details, past medical history, current medications, most recent PET test and dialysis regime. The daily dialysis glucose exposure (DDG) was calculated as the total grammes of unhydrated glucose within the 24 hour dialysate regime (e.g. 2 litres of 1.36% glucose based dialysate = 2 x 13.6 grammes = 27.2 grammes) as previously described[70].

Oral Glucose Tolerance Test

At the beginning of the study visit participants underwent a standard 75g oral glucose tolerance test.

A cannula was placed in a dorsal hand vein and fasting bloods were taken (T_0). Participants then drank 75g of anhydrous glucose (113mls of Polycal) diluted in water to a total volume of 250mls. Participants were asked to consume the drink within a 5 minute period. Once it was completely consumed a stop clock was started. Blood samples were taken at 15 minute intervals for 1 hour and 30 minute intervals for the following hour. Glucose levels were analysed at the bed side using a Yellow Springs Instrument, YSI 2300 STAT Plus (YSI UK Ltd, Hampshire). Additional samples from each of the time points were frozen at -80^{o}C for future analysis. After 2 hours (T_{120}) the cannula was removed.

The OGTT was used to compare participants responses to a standardised carbohydrate load and also to categorise them into 1 of 3 categories defined by the WHO[394];

- Normal glucose tolerance
    - FPG <6mmol/L
    - 2 hour glucose <7.8mmol/L
- Impaired glucose tolerance
    - FPG 6-7mmol/L
    - 2 hour glucose 7.9-11mmol/L
- Diabetes
    - FPG >7mmol/L
    - 2 hour glucose >11mmol/L

Meeting the diagnostic criteria for diabetes was an exclusion criteria for this study and therefore any participants with glucose levels indicative of diabetes were excluded at this point.

Fasting blood samples

In addition to the above, fasting bloods were used to measure HbA1c and fasting insulin levels. Insulin was measured using the Elecsys Insulin assay by Cobas, 801 module of the Cobas 8000 automated analyser and reported in pmol/L.

Fasting insulin levels were also used to calculate the homeostatic model assessment of insulin resistance (HOMA-IR) using the following equation;

HOMA-IR = Fasting insulin(µUnits/ml) x fasting glucose(mg/dL) / 405

HOMA-IR is a static test that gives an estimate of insulin sensitivity from fasting plasma glucose and insulin concentrations[395]. Higher values represent greater insulin resistance. 30-80% of insulin is excreted by the kidney[396], therefore the utility of HOMA-IR in renal cohorts has been questioned. However analysis of insulin kinetics and c-peptide levels in a large study of HD and PD patients[173] demonstrated that higher insulin levels in dialysis patients were the result of increased secretion in response to lower insulin sensitivity and not simply impaired insulin clearance. Good agreement between HOMA-IR and hyperinsulinaemic clamps (gold standard measure of insulin resistance) has been shown in non-dialysis CKD[397], HD[398] and PD cohorts[190]. HOMA-IR has also been shown to predict cardiovascular outcomes in PD patients [399, 400]. For these reasons and its ease of measurement compared with clamp tests it remains the most commonly quoted index of insulin resistance in the renal literature[401, 402].

Continuous Glucose Monitoring

Following the OGTT the FreeStyle Libre© (FSL) flash glucose monitor was applied, as described in Chapter 2.

Participants were asked to wear the FSL for a 72 hour monitoring period, during this period they were required to ‘scan’ the reader against the sensor at least 8 hourly to collect data. There is no current consensus on the duration of CGM

monitoring required to accurately reflect the glycaemic profile[306]. Studies with long monitoring periods have struggled to calculate some of the measures of glycaemic variability, frequent interruptions to the trace occur as the monitoring period progresses due to reduced compliance[403]. Normal reference ranges for CGM derived outcomes[404] and the minimum data set from which glycaemic variability can be consistently measured [306, 405] are derived from 72 hours of data. All comparable studies using CGMs in PD patients with diabetes derived their outcomes from 72 hours of continuous monitoring [212-214, 218].

During the monitoring period participants were asked to keep a record of their food intake in a food diary. No restrictions were placed on their oral intake or level of activity. They continued with their prescribed PD regime and usual medications. Each individuals PD regime remained constant during the monitoring period.

CGM derived outcomes

After 72 hours the FSL sensor was removed and data were uploaded on a PC using FreeStyle Libre Software. This software was used to produce a graphical representation of the traces for each individual participant (Figure 5.3).

Raw data were analysed using EasyGV 2011©. EasyGV is a macro-enabled Excel workbook that allows calculation of 10 different measures of glycaemic variability from CGM data. This software was developed by and used with the permission of Nathan Hill, University of Oxford.

All outcome measures were decided *a priori.* It was anticipated that all participants would be exposed to a different pattern of glycaemia in the day time

compared with night time. Additionally APD and CAPD result in different patterns of glycaemic exposure, reflected in differing glycaemic patterns in PD patients with diabetes [218]. Data were therefore analysed for the whole 72 hour monitoring period and as ‘day time’ readings and ‘night time’ readings. Day time was defined as 09.00 to 21.00 and night time as 21.00 to 09.00. There is no consensus in the literature with regards the definition of day time readings and night time readings when analysing CGM data [406, 407]. APD regimes typically last 8-9 hours and commence when the patient goes to bed, the 12 hour night time window (21.00-09.00) ensured that the 24 hour period wasn’t broken mid-way through an APD regime. Splitting a 24 hour period into shorter sections does not seem to have a significant negative impact on absolute or relative reliability of the CGM generated outcome measures[408].

The CGM data was used to derive the following outcome variable:

- Average interstitial glucose level
    - Over the whole 72 hour monitoring period
    - Of the ‘day time’ readings
    - Of the ‘night time’ readings
- Within participant standard deviation of interstitial glucose
    - Over the whole 72 hour monitoring period
    - Of the ‘day time’ readings
    - Of the ‘night time’ readings

Glycaemic variability was calculated as within patient standard deviation of glucose. Most measures of glycaemic variability, including MAGE and CONGA are highly correlated with overall standard deviation[208]. It is also the marker of glycaemic variability most readily understood by a non-specialist audience.

Analysis of data from traditional CGMs had to incorporate a degree of data cleaning, removing data points that did not adequately correlate with the capillary blood glucose calibration measurements. However as the FreeStyle Libre is factory calibrated and requires no additional calibration during the monitoring period all recorded data points were considered valid. The FSL is still susceptible to missing data as if the participants do not 'scan' the reader against the sensor at least 8 hourly some data is lost. There is considerable discussion in the literature[306, 405], but no consensus, regarding the frequency of data points required to accurately calculate glycaemic variability or the length of missing data that would invalidate a data set[307]. In patients with Type 1 diabetes glycaemic variability measured by standard deviation becomes less reliable (10% error compared with values collected every 5 minutes) if observations are more than 2-4 hours apart[306]. Methods have been proposed using linear interpolation to 'fill' data gaps[405]. However in this study of 72 hour traces from 47 participants[405], where 7.5% of data was missing, application of the linear interpolation process improved calculation of time-dependant measures such as CONGA but had no significant effect on average glucose or standard deviation of glucose.

Therefore, all CGM data points were considered valid, any 24 hour periods with continuous data gaps of more than 4 hours were excluded and interpolation of missing data was not attempted.

Statistics

Statistical analysis was performed using StataSE-16 (Stata Corporation, Texas, USA). Graphical analysis was performed using GraphPad Prism 8 software. All variables were tested for normality by visual inspection of a histogram plot and

by Shapiro-Wilks test. For normally distributed data, between group differences were assessed using an ANOVA and correlations were assessed using Pearson's correlation coefficient. For non-normally distributed data, between group differences were assessed using the Kruskal Wallis test and correlations were assessed using Spearman's rank correlation coefficient. A result was considered statistically significant if $p \leq 0.05$.

A formal power calculation was challenging due to lack of pre-existing data in this cohort. Additionally a clinically relevant change in glycaemic variability has yet to be established [409]. The aim of this study was to better describe the glycaemic profiles of non-diabetic PD patients, this data could be used to inform power calculations in future studies. The recruitment target was 20 participants in each of the two groups. Allowing for drop-outs, 17 completed participants in each group would allow detection of a 1SD difference in glycaemic markers between the groups with 80% power and a two sided α of 0.05.

5.4 **Results**

During the recruitment period 37 prevalent PD patients were identified as eligible to be included in the study. 4 patients were too frail to regularly attend outpatient appointments and therefore weren't approached. 2 patients were using only non-glucose based dialysate. 4 patients did not attend an outpatient appointment during the recruitment period. The remaining patients were approached by the clinical team and offered information about the study. 8 patients declined to take part. 4 patients had intervening medical issues precluding them from taking part. Reasons for declining to take part were primarily related to burden of pre-existing hospital appointments and travel

time/distance. 2 patients worked full time and 1 patient was full time carer for their spouse.

35 potential control participants were approached and given a participant information sheet. 10 declined to take part, reasons for declining included travel time/distance and lack of time. Of the remaining potential participants 16 contacted the research team and were recruited.

16 control participants and 15 PD patients were recruited into the study. No participants dropped-out of the study. All consented participants completed the study visit and the 72 hour monitoring period.

Primary renal diagnoses in the controls were; hypertensive nephropathy 31.25%, polycystic renal disease 18.75%, glomerular nephritis 18.75%, obstructive/reflux nephropathy 18.75% and other 12.5%. Primary renal diagnoses for the PD patients were; hypertensive nephropathy 46.7%, glomerular nephritis 26.7%, polycystic renal disease 13.3%, small kidneys/unknown cause 6.7% and small vessel vasculitis 6.7%. Demographic data for the cohort are detailed in Table 5.1.

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Table 5.1 Characteristics of study participants in control and PD groups and by PD modality

	Controls (n=16)	PD (n=15)	CAPD (n=6)	APD (n=9)	P value
Age (yrs)	65[54-70.5]	74[69-79]	76.5[70-79]	74[68-77]	**0.019**[a]
Male gender	11 (69)	10 (67)	3 (50)	7 (78)	0.55
Caucasian	16 (100)	15 (100)	6 (100)	9 (100)	
BMI	26.8±2.4	26.3±4.6	24.7±3.6	27.3±5.1	0.402
eGFR (ml/min)	11[10-14]				
Cr/Cl (L/wk)		54.81±27.5	68.86±30.2	42.77±19.5	0.087[b]
Duration of PD (mths)		12[9-32]	13[9-23]	12[10-32]	0.968
$D/P_{Cr}4H$		0.82±0.11	0.79±0.1	0.84±0.12	0.427
DDG (g)		153.88±79	74.48±38.5	206.81±46.03	**<0.0001**[b]
Icodextrin usage %		13 (86)	5 (83)	8 (88)	0.777

Data is presented as mean±SD or median[IQR]. [a] controls v PD patients [b] CAPD v APD. PD = peritoneal dialysis, CAPD = continuous ambulatory peritoneal dialysis, APD = automated peritoneal dialysis, BMI = body mass index, eGFR = estimated glomerular filtration rate, $D/P_{Cr}4H$ = dialysis to plasma ratio of creatinine at 4 hours, DDG = daily dialysate glucose

Metabolic profiles of study participants

No participants met diagnostic criteria for a diagnosis of diabetes.

2 study participants met the criteria for impaired glucose tolerance based on fasting plasma glucose (1control, 1 CAPD). 8 study participants met the criteria

for impaired glucose tolerance based on the oral glucose tolerance test 2 hour glucose (4 controls, 1 CAPD, 3 APD). 6 participants met the criteria for impaired glucose tolerance based on HbA1c (4 controls, 1 CAPD, 1APD). There was no cross-over between participants with impaired glucose tolerance by OGTT and those with impaired glucose tolerance by HbA1c. In healthy populations insulin resistance has been previously defined as HOMA-IR >2.6. 8 participants had HOMA-IR values greater than 2.6 (6 controls, 2APD).

A summary of the glycaemic indices in the study participants is presented in Table 5.2. There were no significant differences between the control and PD groups for HbA1c, fasting plasma glucose, plasma glucose 2 hours post oral glucose tolerance test or markers of insulin resistance.

Table 5.2 Glycaemic indices in study participants by group and by PD modality

	Controls (n=16)	PD (n=15)	CAPD (n=6)	APD (n=9)	P value
HbA1c(mmol/mol)	38.25±3.8	38.9±3	38.2±4.1	39.5±2.1	0.68
FPG (mmol/L)	5.05±0.51	4.86±0.63	4.64±0.89	5.01±0.38	0.30
2hr OGTT (mmol/L)	6.9±1.5	7.3±1.6	6.8±1.1	7.7±1.8	0.39
Fasting insulin (pmol/L)	84.2±35.9	79.5±33.9	62±12.3	85.3±37.3	0.578
HOMA-IR	2.3[1.8-3.8]	2.1[1.7-3.3]	1.8[1.5-2]	2.6[1.9-3.6]	0.36

P value for ANOVA across all groups, FPG = fasting plasma glucose, 2hr OGTT = plasma glucose 2 hours post 75g oral glucose tolerance test, HOMA-IR = homeostatic model assessment for insulin resistance, PD = peritoneal dialysis, CAPD = continuous ambulatory peritoneal dialysis, APD = automated peritoneal dialysis

CGM generated outcomes in PD patients versus controls

All participants completed the 72 hour CGM monitoring period. 16 participants had no missing data points. The total number of missing data points were 154 which represents 1.72% of the total data. Only 1 participant had a period of missing data greater than 4 hours, this 24 hour period was excluded from the analysis. No participants reported any difficulties in using the flash glucose monitoring system or any discomfort associated with its use.

There was no significant difference in the average interstitial glucose levels (mmol/L) between the PD patients and the controls; over the whole monitoring period (PD 5.1±0.63 v controls 5.08±0.69 p = 0.935), for the day time periods (PD 5.31±0.72 v controls 5.44±0.75 p = 0.618) or the night time periods (PD 4.86±0.76 v controls 4.69±0.7 p = 0.512) see Figure 5.1

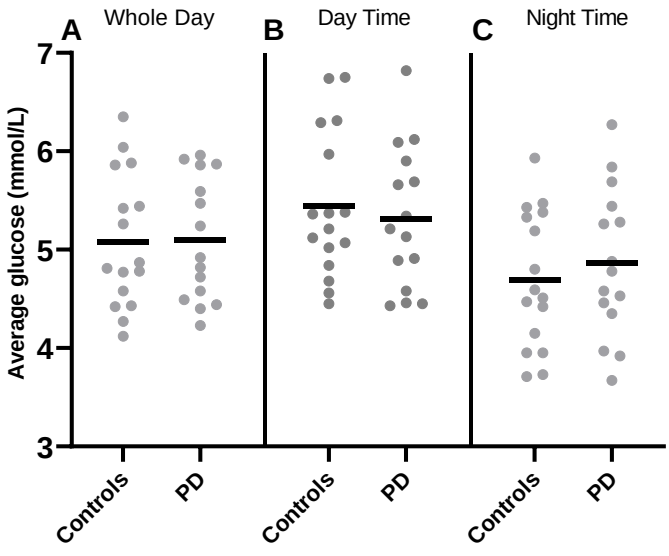

Figure 5.1 Average interstitial glucose levels (mmol/L) in controls versus PD patients. Data is presented for A) whole study period, B) daytime recordings and C) night time recordings. Line drawn at mean value. P = >0.05 for all 3 periods. PD = peritoneal dialysis

The was no significant difference in the standard deviation of interstitial glucose (mmol/L) between the PD patients and the controls; over the whole monitoring period (PD 1.19±0.24 v controls 1.26±0.25 p = 0.47), for the daytime periods (PD 1.19±0.26 v controls 1.32±0.37 p = 0.286) or the night time periods (PD 1.03±0.25 v controls 0.99±0.27 p = 0.754) Figure 5.2

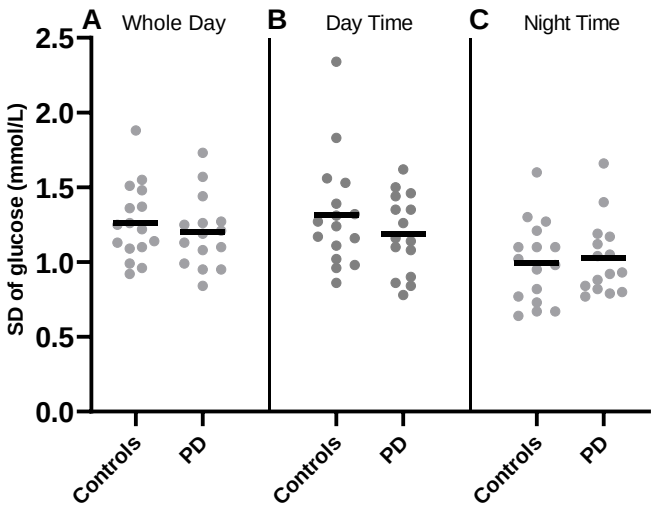

Figure 5.2 Standard deviation of interstitial glucose (mmol/L) in controls versus PD patients. Data is presented for A) whole study period, B) day time recordings and C) night time recordings. Line drawn at mean value. P=>0.05 for all 3 periods. SD = standard deviation, PD = peritoneal dialysis

CGM generated outcomes by PD modality

Early in the study period very different patterns of glycaemia were evident in APD patients compared with CAPD patients. The aim of the study was to characterise the glycaemic profiles of PD patients and therefore it seemed most appropriate to analyse them as separate groups with an understanding that this would reduce the power and ability to detect small differences. Example traces from each of the 3 groups; controls (Figure 5.3), CAPD (Figure 5.4) and APD (Figure 5.5) are shown below. CAPD patients followed a similar overall pattern to the control patients with variation in interstitial glucose during the day

associated with meals and lower, less variable interstitial glucose at night (see below). However APD patients demonstrated a day time pattern similar to the controls but night time interstitial glucose levels were higher than fasting levels for the duration of the APD program and dropped to fasting levels once therapy with glucose containing fluid stopped (Figure 5.6). This is analysed in greater detail below.

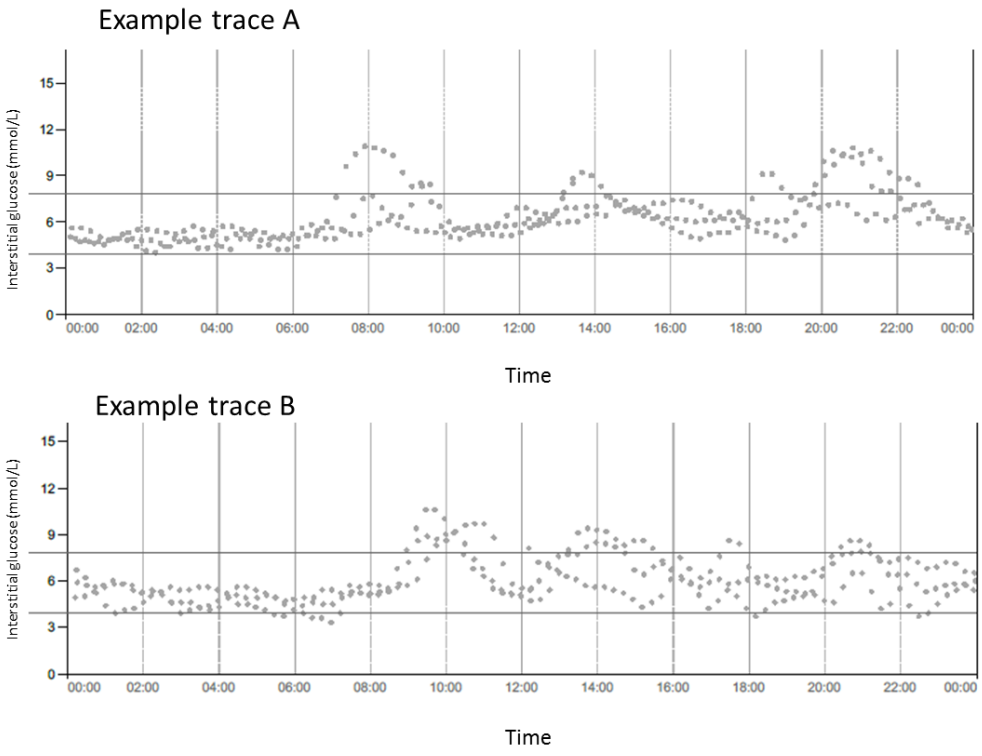

Figure 5.3 Example continuous glucose monitoring traces from 2 individuals from the control group. The 3 days of monitoring are superimposed over a 24 hour period

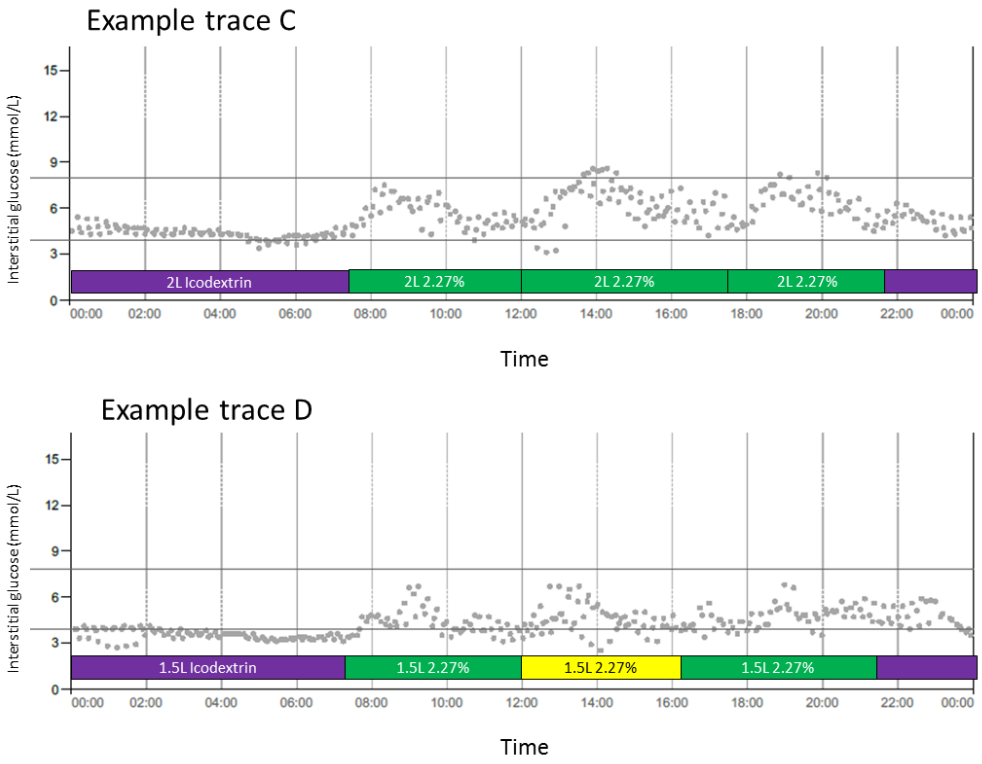

Figure 5.4 Example continuous glucose monitoring traces from 2 individuals from the CAPD group. The 3 days of monitoring are superimposed over a 24 hour period. Dialysis fluid in situ is shown across the bottom

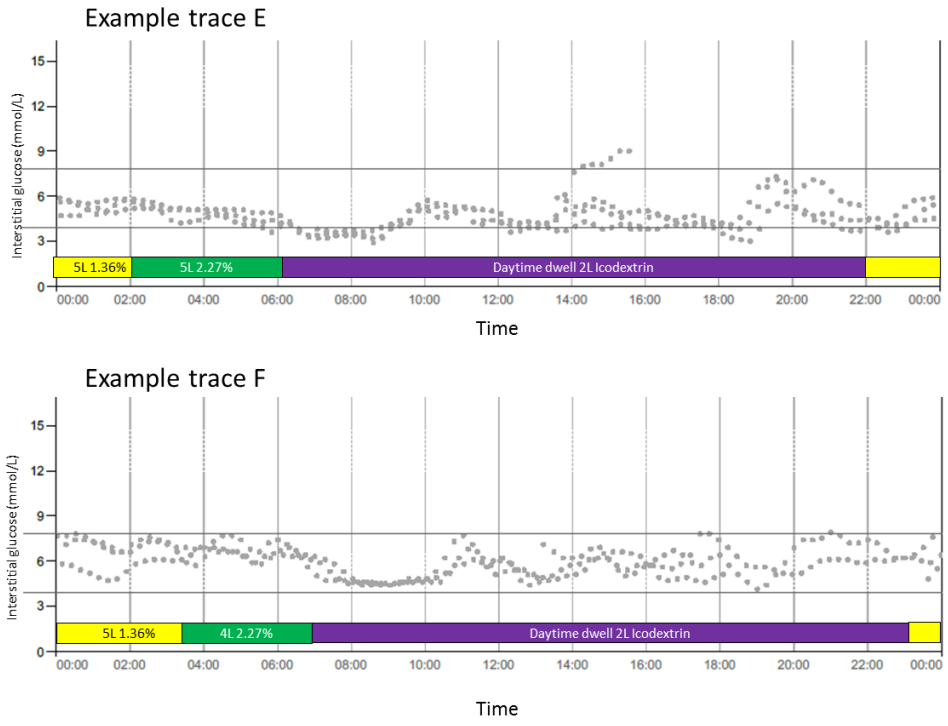

Figure 5.5 Example continuous glucose monitoring traces from 2 individuals from the APD group. The 3 days of monitoring are superimposed over a 24 hour period. Dialysis fluid in situ is shown across the bottom.

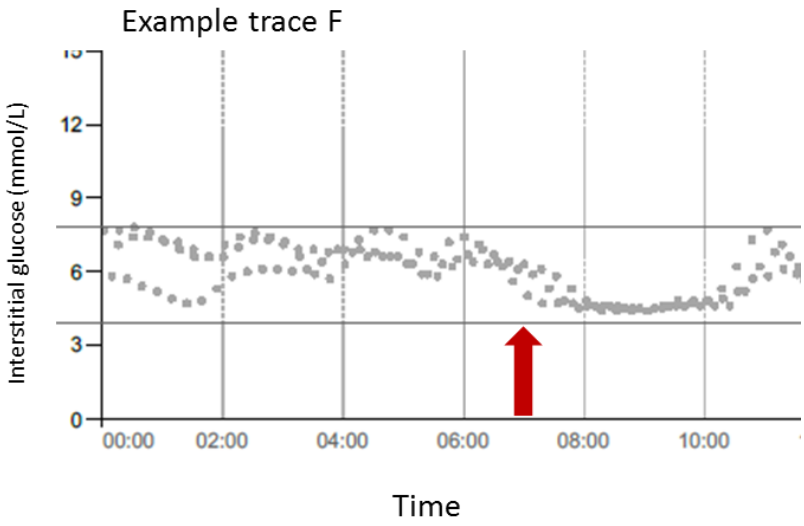

Figure 5.6 Zoomed in section of CGM trace from a participant in the APD group. The trace shows interstitial glucose readings over the 3 day monitoring period between midnight and 11.00am. The red arrow indicates cessation of the glucose containing APD regime and instillation of the day time fill of Icodextrin.

There was no difference in the average glucose (mmol/L) value between the three groups over the whole monitoring period (controls 5.08±0.69 CAPD 4.9±0.64 APD 5.23±0.62 p = 0.637) and during the day time (controls 5.45±0.75 CAPD 5.46±0.86 APD 5.21±0.65 p = 0.729). The average night time glucose (mmol/L) in APD patients was significantly higher than CAPD patients (APD 5.25±0.65 v CAPD 4.28±0.5 p = 0.026). There was no statistically significant difference in average night time glucose between the controls (4.69±0.7) and either APD (p=0.145) or CAPD (p = 0.605).

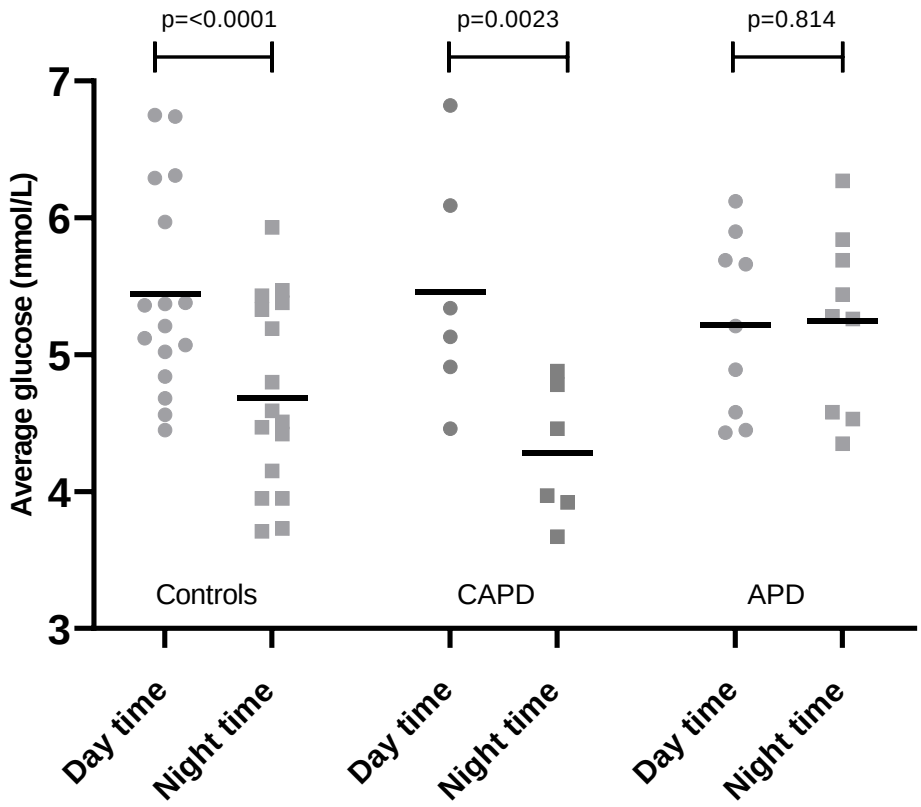

Figure 5.7 Differences between average day time and average night time interstitial glucose levels by group. Line drawn at mean. P values are shown. CAPD = continuous ambulatory peritoneal dialysis, APD = automated peritoneal dialysis

The control and CAPD groups demonstrated a statistically significant drop in their average night time glucose compared with day time values which was not seen in the APD group. In control patients the average night time glucose was 0.76mmol/L lower than the average daytime glucose (p=<0.0001). In the CAPD patients this value was larger at 1.18mmol/L (p=0.0023). In APD patients the average night time glucose was 0.03mmol/L higher than the average day time glucose (p = 0.814), Figure 5.7. This difference in the day time to night time interstitial glucose levels was significantly different in the APD patients compared with both controls and CAPD patients (p= 0.001 v controls, p=<0.0001 v CAPD) Figure 5.8. There was no statistically significant difference between the pattern seen in controls and CAPD patients (p=0.168). This pattern

of night time ‘dipping’ was seen in the majority of control patients and all the CAPD patients Figure 5.9.

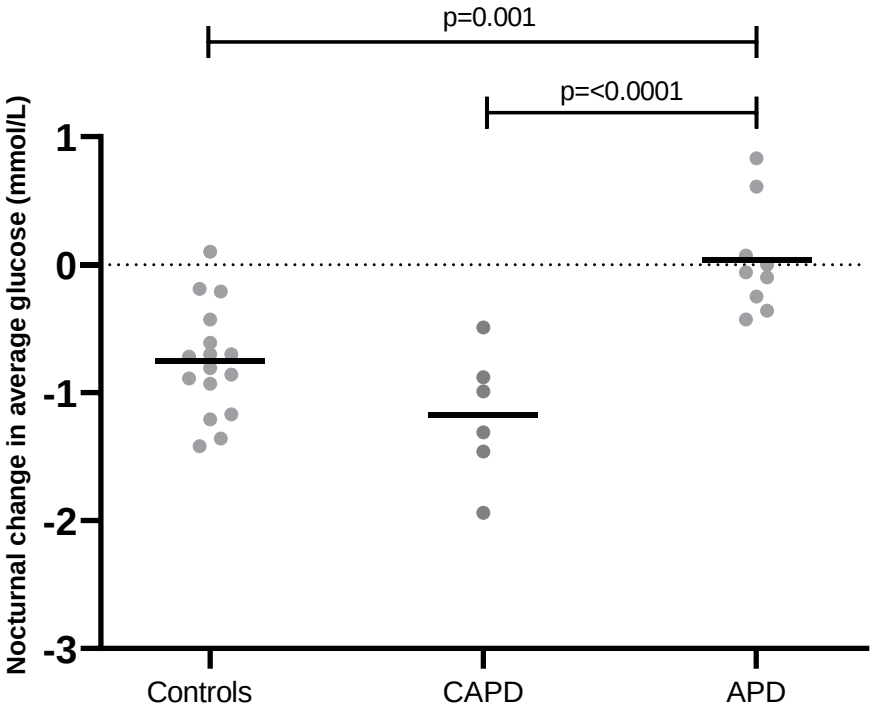

Figure 5.8 Change in night time average glucose compared with day time average glucose by group (value plotted = night time average – day time average therefore negative values represent a lower value at night time than during the day time). Line drawn at the mean. Significant p values (<0.05) are shown. CAPD = continuous ambulatory peritoneal dialysis, APD = automated peritoneal dialysis

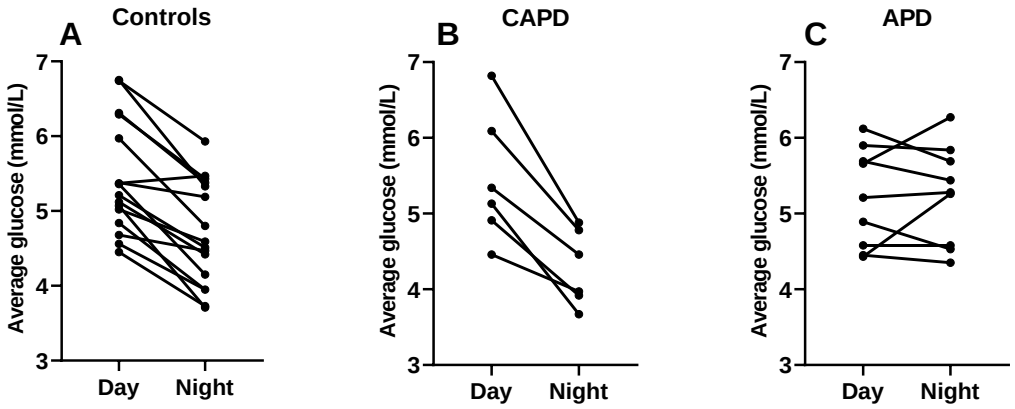

Figure 5.9 Nocturnal dipping of interstitial glucose. Change in average day to night glucose for individual participants in A) control group, B) CAPD group and C) APD group

There was no difference in the SD of glucose (mmol/L) across the 3 groups over the whole monitoring period (controls 1.26±0.25 CAPD 1.27±0.33 APD 1.15±0.17 p = 0.5), day time periods (controls 1.31±0.37 CAPD 1.16±0.29 APD 1.21±0.26 p = 0.541) or night time periods (controls 0.99±0.27 CAPD 1.05±0.34 APD 1.01±0.19 p = 0.923). Figure 5.10

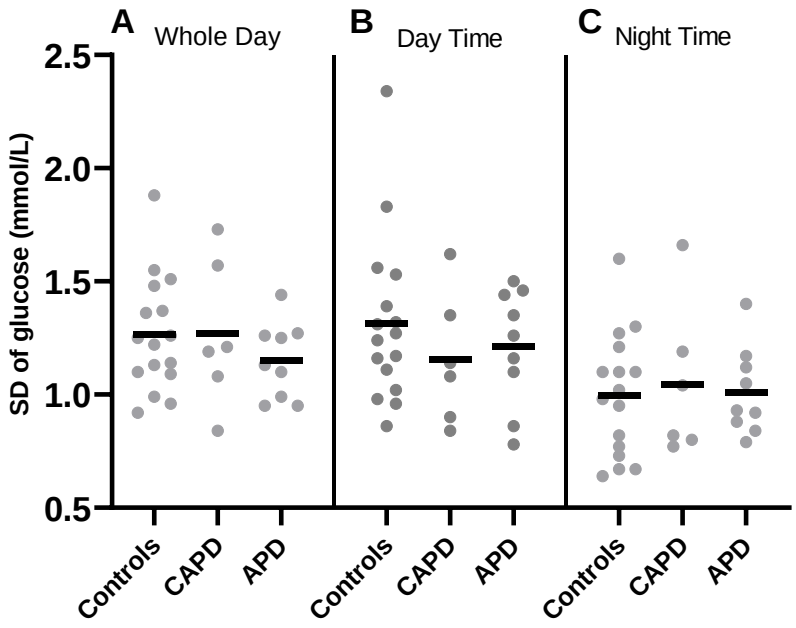

Figure 5.10 Standard deviation of interstitial glucose (mmol/L) by group. Data is presented for A) whole study period, B) day time recordings and C) night time recordings. Line drawn at mean value. P=>0.05 for all 3 periods. SD = standard deviation, CAPD = continuous ambulatory peritoneal dialysis, APD = automated peritoneal dialysis.

Relationship between standard diagnostic markers of diabetes and CGM outcomes

In the study cohort as a whole, there was no correlation between HbA1c and average interstitial glucose (r = 0.0077 p = 0.968) or SD of interstitial glucose (r= -0.1 p = 0.599). There was no correlation between fasting plasma glucose and average interstitial glucose (r = 0.31 p = 0.09) or SD of glucose (r=0.25 p=0.18). There was no correlation between 2 hour post OGTT glucose and average interstitial glucose (r = 0.18 p = 0.32) or SD of glucose (r=0.26 p=0.16).

In the study cohort as a whole, there was a strong and significant correlation between HOMA-IR and BMI (r_s = 0.716 p = 0.0001). There was no correlation between HOMA-IR and average interstitial glucose or SD of glucose (r_s = 0.123 p = 0.54, r_s = 0.124 p = 0.538 respectively).

Relationship between peritoneal glucose exposure and CGM outcomes

In the PD group as a whole there was no correlation between daily dialysate glucose exposure and average interstitial glucose or SD of glucose (r = 0.13 p = 0.65, r = -0.14 p = 0.61 respectively) Figure 5.11. There was no correlation between rate of peritoneal transport (D/P_{Cr} at 4 hours) and average interstitial glucose or SD of glucose (r = -0.13 p = 0.66, r = -0.22 p = 0.46 respectively). There was no correlation between length of time on PD and average interstitial glucose or SD of glucose (r_s=0.02 p = 0.94, r_s = 0.29 p = 0.294 respectively).

There was no correlation between HOMA-IR and rate of peritoneal transport, daily dialysate glucose exposure or length of time on PD.

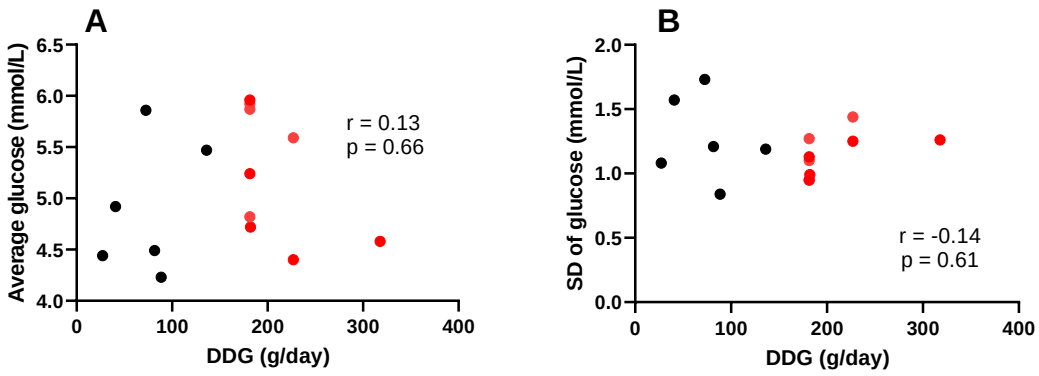

Figure 5.11 Graphs showing the relationship between daily dialysate glucose exposure (DDG) and A) average interstitial glucose levels and B) standard deviation of glucose. Black dots = CAPD patients, red dots = APD patients.

5.5 **Discussion**

This is the first study to use CGMs to examine real-life glycaemic profiles in non-diabetic patients with advanced CKD and on PD. Flash glucose monitoring which was primarily designed for use in people with diabetes proved to be easy to use and acceptable to a non-diabetic cohort. This resulted in comprehensive data sets with very little missing data.

There was no significant difference between the PD patients and CKD 5 controls with regards traditional markers of glycaemia or average glucose and standard deviation of glucose calculated using CGM data. In this study, the degree of peritoneal glucose exposure was not related to the CGM derived outcomes or traditional metrics. We did however see a significant difference in the pattern of interstitial glucose in APD patients compared with CAPD patients and controls. APD patients lacked the nocturnal ‘dip’ in interstitial glucose observed in the other two groups.

Is there a difference in interstitial glucose levels measured over 72 hours between PD patients and non-dialysis controls?

It has been suggested that commencing PD results in new onset hyperglycaemia[178]. In my study there was no statistically significant difference between the 72 hour average interstitial glucose and SD of interstitial glucose in the PD and control groups. Although overall duration of PD was relatively short in my study (12 months) previous studies have suggested that new onset hyperglycaemia could be expected in a median of 11.4 months[182].

Due to a lack of previously published work the degree of difference one might expect between the groups was difficult to estimate. Previous studies have compared two ‘non-diabetic’ cohorts using CGM data. In the Wang study[410]

of participants with normal glucose tolerance versus participants with impaired glucose tolerance they reported a difference in average blood glucose of 0.22mmol/L and a difference in SD of glucose of 0.26mmol/L. In Buscemi's study[411] of obese participants with and without metabolic syndrome they report a difference in average blood glucose of 1.8mmol/L. 16 controls and 15 PD patients in my final analysis would have allowed detection of a 1.05SD difference between groups. This equates to a difference in average blood glucose of 0.65mmol/L and difference in glycaemic variability (SD of glucose) of 0.25mmol/L. Therefore it is possible that there are differences in interstitial glucose between the PD patients and controls smaller than these values that this study was unable to detect.

The control and PD groups were well matched for gender, ethnicity and BMI. There was however a significant difference in age, with control patients on average 9 years younger than PD patients. PD patients in this study were marginally older than average for the UK as a whole (64.4 years) (UK Renal Registry (2019) UK Renal Registry 21st Annual Report – data to 31/12/2017, Bristol, UK.). However this should in theory increase glucose levels in my PD patients[179], enhancing any difference between the groups, yet none were seen in my data. It is unlikely that this small difference in age has altered my conclusions. This is also an entirely Caucasian cohort which as discussed below may limit the translatability of these results.

60% of the PD patients in this study were on APD. This is in contrast to larger studies such as The Global Fluid Study [179] wherein only 15.8% of prevalent patients were on APD. This probably reflects the fact that Global commenced recruitment in 2002 and was censored in 2011. The proportion of patients on

APD has increased in the last 10 years. The most recent data from the UK renal registry reports APD usage as 59.8% of total PD, therefore my cohort is a good reflection of national practice patterns.

It has been suggested that those most at risk of hyperglycaemia associated with PD are those with impaired fasting glucose prior to dialysis [178, 181]. As this was not a longitudinal study we have no data on PD patients' glucose tolerance prior to commencing dialysis. However only 1 of the PD patients met the criteria for impaired fasting glucose and therefore it is possible that in a cohort with higher levels of impaired fasting glucose a larger difference may have been observed.

Although there are potentially small differences in average interstitial glucose and glycaemic variability between PD patients and pre-dialysis patients that this study was not powered to detect the lack of a large and statistically significant difference in this essentially representative cohort is reassuring.

Does the mode of PD affect interstitial glucose levels measured over 72 hours?

In my PD study cohort CAPD and APD resulted in significantly different patterns of interstitial glucose measured using CGM.

Although overall average interstitial glucose and SD of glucose were not different between the 3 groups, subdivision of each 24 hour period into 'night time' (21.00 – 09.00) and 'day time' (09.00 – 21.00) revealed more subtle differences. The significant nocturnal 'dip' in interstitial glucose observed in control patients and CAPD patients was absent in APD patients. This increased nocturnal glucose was attributable to peritoneally absorbed glucose as when

the PD regime ceased interstitial glucose levels started to fall almost immediately (Figure 5.6).

Each 24 hour period was split into 12 hour periods that best reflect when APD and CAPD patients received the majority of their glucose containing dialysate. This could introduce bias into the results if for instance APD patients got up earlier as a result of their dialysis finishing, ate their breakfast earlier and consequently the glucose spike associated with breakfast was included in their 'night time' readings resulting in the higher average night time value observed. Analysis of the food diaries completed by all participants confirmed no systematic difference in the timing of meals between the CAPD and APD groups.

Systematically different patterns of interstitial glucose for APD and CAPD, have been previously noted in CGM studies in PD patients with diabetes [214, 218]. Okada et al[218] retrospectively studied 23 CGM traces from diabetic PD patients (12APD, 11 CAPD). They reported reduced SD of glucose and a reduction in the difference between minimum and maximum glucose levels in the APD traces compared with CAPD and concluded that CAPD causes increased glucose fluctuations. They don't elaborate further on the different patterns in the two groups, it is possible that the reduced fluctuations in the APD group are the result of reduced differences between day time and night time values as seen in my cohort.

CAPD patients are also subject to a peritoneal glucose load however there was no significant difference in the day time average interstitial glucose values between the CAPD patients and either APD patients or controls. There are

several potential explanations for this measureable increase in interstitial glucose associated with APD that is not seen in CAPD patients. CAPD exchanges often coincided with meal times. It is possible that the effect of the peritoneally absorbed glucose is small in comparison to that of the meal, insulin released in response to the oral carbohydrate load results in a more efficient reduction in glucose levels. APD patients are exposed to a greater daily dialysate glucose load as a consequence of the shorter dwell times. APD dwells are typically 2 hours compared with the 4 to 6 hour dwells in CAPD. Glucose is absorbed most rapidly during the early portion of dwells when the diffusion gradient is highest. In healthy humans, plasma glucose tolerance varies during each 24 hour period with glucose tolerance being higher in the morning than the evening[412]. This is thought to be the result of a stronger pancreatic β-cell response in the circadian morning[413]. Therefore the additional glucose load from APD may be less well tolerated during the circadian night.

In other studies using CGMs in healthy individuals under ‘everyday’ conditions a statistically significant drop in night time glucose compared with day time glucose, similar in magnitude to that seen in our control patients (between 0.4-0.7 mmol/L) [328, 414] has been reported. The physiological significance of a reduction in or complete lack of this ‘dipping’ is unclear.

Circadian rhythms are daily variations in behaviour and biological activity. These rhythms originate from an internal mammalian timing system composed of a central pacemaker in the hypothalamic suprachiasmic nucleus and multiple clocks in peripheral tissues[415]. Diurnal variations in β-cell response and insulin sensitivity of peripheral tissues results in the variable glucose tolerance discussed above[415]. However humans may also develop reduced glucose

tolerance when subjected to conditions of circadian misalignment[416]. According to the circadian disruption hypothesis metabolic health is optimal when the different daily rhythms oscillate in synchrony with each other. Food intake during the wrong circadian phase has been shown to cause obesity in mice [417] and there is increasing evidence from human studies that 'time-restricted feeding' including a prolonged fasting period overnight is beneficial for weight loss[418] and reducing insulin resistance[419]. Theoretically, the additional glucose load from APD that is asynchronous with natural circadian rhythms coupled with the absence of any significant period of fasting during the 24 hour period may contribute to circadian misalignment and further exacerbate insulin resistance. Inflammatory reactions also exhibit circadian variation and are susceptible to circadian desynchrony [420]. Peritoneal macrophages harvested from mice who had undergone circadian disruption exhibited a heightened cytokine response to an inflammatory challenge compared with control mice[421]. At a local level an increased inflammatory reaction to the dialysate could contribute to peritoneal membrane damage. On a systemic level increased intraperitoneal inflammation results in an increase in absorbed glucose.

To what degree does peritoneal glucose exposure affect interstitial glucose levels?

In this study there were no significant associations between the CGM derived outcomes (average interstitial glucose levels and SD of glucose) and proxy measures of peritoneally absorbed glucose (daily dialysate glucose and peritoneal transport rate).

Previous observational studies using CGMs in diabetic PD patients have reported conflicting results with regards the link between dialysate glucose and interstitial glucose levels. A study of 20 PD patients with diabetes [218] reported no correlation between peritoneal small solute transport rate, used as a surrogate marker of glucose absorption, and SD of glucose. There are several issues with this study that make their results difficult to interpret. It was a retrospective study of patients who had worn CGMs, the indication for CGM use is not documented. Those who were asked to wear CGMs are likely to have worse blood glucose control than those patients who were not. They didn't report on any association between glucose fluctuations and actual dialysate glucose exposure or absorption, they used solute transport rate as a surrogate marker of glucose exposure. Conversely Mori et al[214] in their study of 10 PD (9 CAPD, 1 APD) patients with diabetes reported strong correlations between SD of glucose and D/P_{Cr} and D/D_0 glucose (r=0.713 and r=0.735 respectively) again used as surrogate markers of glucose exposure. However they found no association between SD glucose and the total 24 hour peritoneal glucose absorbed (estimated by PET)[214]. This was a small study of 10 patients and therefore they lacked power to adjust for potential confounders. The relationship between glycaemic variability and solute transport rate (used here as a proxy for glucose absorption) has the potential to be confounded by multiple factors including age and inflammation.

Single dwell studies have consistently shown an association between instillation of a glucose based dialysate and a rise in blood glucose [204-206]. These studies are however conducted under experimental as opposed to real life conditions, participants are fasted prior to the exchange. CAPD exchanges often occur within close proximity of a meal, these studies are therefore unable

to account for how the glucose absorbed from dialysate is handled in the context of additional carbohydrate loads. These studies also often use concentrations of glucose (3.86% and 4.25%)[204, 205] that are no longer routinely used in clinical practice since the widespread uptake of Icodextrin thus reducing the relevance of these studies to current clinical care.

Interventional studies in patients with diabetes have shown improvement in long and short term glycaemic indices with glucose sparing PD regimes. The interventional arm of the IMPENDIA/EDEN study[192], which used icodextrin and an amino-acid based dialysate for 2 of their daily exchanges showed a reduction in HbA1c of 0.5% over 6 months compared with the control group using only glucose based dialysate. A much smaller study of 8 diabetic patients[213] showed a reduction in 24 hour average glucose and glycaemic variability, measured using CGMs, when patients used the above glucose sparing regime compared with 4 glucose based exchanges. When interpreting these studies it should be noted that there is very little APD representation (IMPENDIA was 90% CAPD) and the 'glucose sparing regimes' used would now be considered standard prescriptions and therefore these results may not be reflective of modern cohorts.

A large observational study (Global Fluid Study) of over 600 non-diabetic PD patients[179] across three countries reported in 2016 that daily dialysate glucose exposure (calculated as in my study) predicted random glucose levels in prevalent (>90 days of PD) patients. They found no such association in incident (<90 days of PD) patients. The dialysis regimes used by patients in the Global study poorly reflect modern regimes, only 15.8% of prevalent patients were on APD and Icodextrin usage was 23.8%. However the main challenge in

interpreting the results of their study is the use of random plasma glucose as the outcome measure. The reasons for using random plasma glucose, in a large multi-centre study using data collected from routine clinical measurements, are understandable. However as outlined in Table 5.3 there is significant intra-subject variability of blood glucose levels even in non-diabetic subjects. The present study demonstrates that PD modality also significantly influences the pattern of interstitial glucose levels. Given these findings the relevance of a single random plasma glucose is questionable.

A randomised control trial in non-diabetic APD patients randomized participants to icodextrin or 2.5% glucose in the long dwell for 90 days[194]. They reported no difference between fasting plasma glucose levels and HbA1c between the control and intervention group. This finding that dialysate glucose reduction has minimal impact on glycaemic indices in those with good glycaemic control is supported by a previous study in diabetic PD patients[422], wherein a significant reduction in HbA1c after a year on a glucose sparing regime was only seen in those participants with baseline HbA1c ≥6.5%.

If peritoneal glucose exposure does significantly affect blood glucose levels then that does not seem to translate into worse survival in the short term. A recently published large observational study of over 4000 diabetic and non-diabetic incident PD patients[423] reported that cumulative glucose exposure was not associated with patient survival over their 2 year follow up but was associated with technique failure. In a group of patients without diabetes such as those presented in this thesis, the average interstitial glucose levels and degree of glycaemic variability as measured by CGM that may result in increased mortality is unclear. CGM technology is relatively new and as such is

yet to be used in a large study with significant length of follow up to adequately determine mortality risk. The majority of large studies reporting increased mortality with increasing HbA1c include only patients with diabetes [424] and are therefore difficult to translate to a non-diabetic population. A large epidemiological study of community dwelling individuals in the United States demonstrated increased all-cause mortality in non-diabetic individuals with HbA1c <5% but found no significantly elevated risk at HbA1c levels >5.4%[425].

I found no evidence of a strong relationship between daily dialysate glucose and interstitial glucose levels in this mixed modality cohort of non-diabetic PD patients. The number of PD patients in my final analysis (15), would have allowed detection of a moderate to strong correlation of $r \geq 0.65$ between dialysate glucose exposure and interstitial glucose.

Although my cohort has high usage of the glucose sparing dialysate Icodextrin (86%), high prevalence of APD with it's associated shorter dwells and increased total volume of dialysate has resulted in a mean daily dialysate glucose exposure (153.8g) comparable to if not higher than other studies[179]. Daily dialysate glucose exposure in g/day is easy to calculate and has been used as a proxy for glucose absorption in other studies[70], however it remains a relatively crude measure of an individual's glucose absorption as it does not account for rate of small solute transport and length of dwell. Formulas for calculating absorbed glucose such as that published by Grodstein[188] were based on CAPD patients. Given the shorter dwell time in APD, rate of small solute transport in these patients may have a more significant impact on the proportion of glucose they are exposed to that is absorbed. The nuances of APD for example tidal APD (only a proportion of the initial fill volume is drained

and replaced with fresh dialysate) make estimation of absorbed glucose even more challenging. Analysis of APD and CAPD patients as a single group is therefore made more challenging. Although small numbers preclude statistical analysis of any relationship between peritoneal glucose and interstitial glucose within each modality visual inspection of the CGM traces implies a relationship between peritoneal glucose and interstitial glucose for the APD patients (Figure 5.6).

The glycaemic profiles obtained in this study suggest that in the future, studies of glycaemic profiles and the impact of absorbed glucose on PD patients, should analyse CAPD and APD patients as separate groups. Analysing them together risks missing potentially important but subtle relationships.

Glycaemic profiles in non-diabetic CKD5 and PD patients

The use of CGMs to study patterns of glycaemia is still relatively new. This is the first study to use them to examine patterns of glycaemia in renal patients without diabetes. Several studies have attempted to establish reference ranges for CGM generated outcomes in non-diabetic cohorts Table 5.3. Several of these studies incorporate a diabetic group for comparison[410, 411]. Lack of consensus in reporting outcomes and experimental difficulties in modulating glycaemic variability without also altering HBA1c mean that even in diabetic cohorts there is no guidance on the degree of glycaemic variability that impacts clinical outcomes.

Table 5.3 Studies using continuous glucose monitoring in non-diabetic cohorts

	Nature of participants (n)	Ethnicity	Age (yrs)	Average blood glucose (mmol/L)	Standard deviation of glucose (mmol/L)
Hill et al[404]	NGT	Asian (7) African American (6) Caucasian (44) Hispanic (13)	29.9 26.7 27.3 27.7	5.3±0.4 5.2±0.3 5.0±0.5 5.1±0.4	1.7±0.9 1.9±1.1 1.5±0.7 1.3±0.7
Freckmann et al[328]	NGT (21)	-	-	5.0±0.3	-
Borg et al[327]	NGT (80)	68% Caucasian	41	-	0.8±0.2
Zhou et al [414]	NGT (434)	Chinese	43	5.7±1.2	0.8±0.3
Wang et al[410]	 NGT (53) IGT (53) T2DM (52)	Chinese	 45.25 51.89 51.21	 5.93±0.61 6.15±0.72 9.1±2.79	 0.97±0.37 1.23±0.47 1.95±0.63
Buscemi et al[411]	 Obese no MS (5) Obese with MS (8) T2DM (5)	-	 32 33 44	 4.1±0.1 5.9±0.1 9.0±0.1	-

NGT = normal glucose tolerance, IGT = impaired glucose tolerance, T2DM – type 2 diabetes mellitus, MS = metabolic syndrome

The values for average interstitial glucose and SD of glucose vary between cohorts. There are also significant differences in the variability within the data sets, SD of mean glucose varies from 0.1 to 1.2mmol/L in these non-diabetic cohorts. Some of these differences may be attributable to ethnicity which has been shown as a strong risk factor for developing Type 2 diabetes [426]. For this reason, Hill et al [404] present their data by ethnic group although they do not present data on whether the differences between their groups are statistically significant. It is important to note that the two largest studies in Table 5.3 were conducted in Chinese populations and therefore we must be cautious when comparing their values with our Caucasian study population. Another consideration when comparing my study with those above is the positive association between blood glucose and age[179].The studies presented in Table 5.3 were all conducted in participants at least 15 years younger than the participants in my study.

Despite this, based on the above data the average interstitial glucose in my cohort as a whole is below those quoted in the Chinese studies and similar to other cohorts with normal glucose tolerance and normal renal function. My cohort represents a more heterogeneous and comorbid group than the 'healthy' subjects used in an attempt to generate normal values and therefore exhibit more inter-subject variability.

The intra subject SD of glucose is also within the range seen in my data. Although we lack a prescribed 'normal' SD or average glucose my cohort falls within the range of other studies in people with normal glucose tolerance. Importantly the average interstitial glucose and SD of glucose we have reported

are appreciably lower than that seen in non-renal, diabetic cohorts[410, 411, 427].

There are a small number of studies utilising CGMs in PD patients with diabetes. As would be expected they report higher average interstitial glucose and SD of glucose than the cohort of PD patients without diabetes described in this thesis. Using a standardised CAPD regime of 3 1.36% day time dwells and one 3.86% night time dwell Marshall et al [213] reported an average 24 hour glucose of 9.37mmol/L in PD patients with diabetes. In a Japanese study of PD patients with diabetes that included both APD and CAPD patients [218] they retrospectively studied the CGM traces of 20 PD patients. They reported an average 24 hour glucose of 9.1±1.9mmol/L and SD of glucose of 2.3±0.8mmol/L. In a larger study in Singapore, retrospectively analysing CGM traces from 60 PD patients with diabetes (40% on APD), the average interstitial glucose was 11.3±3.2mmol/L.

Are traditional markers of glycaemia reflective of glucose handling as measured by CGM?

In this study traditional (fasting plasma glucose and OGTT) and time averaged measures (HbA1c) of glycaemia did not reflect 72 hour interstitial glucose profiles in control patients or patients on PD. Mean values for traditional markers of glycaemia in my cohort were comparable to previous studies in non-diabetic ESRD cohorts [173, 428]. Estimates of the prevalence of insulin resistance in non-diabetic CKD cohorts vary widely between 19-50%, dependant on measurement method and threshold value applied[429]. In my study 25% of participants were classified as insulin resistant, with HOMA-IR >2.6.

In contrast to the concerns surrounding the negative impact of absorbed dialysate glucose, it has also been suggested that insulin resistance may improve upon commencing dialysis[175]. Although in this study I did not collect longitudinal data, by comparing PD patients with non-dialysis controls we found no evidence of a negative or positive effective of PD therapy on metabolic parameters. There were no statistically significant differences between insulin resistance (HOMA-IR) or any of the standard markers of glycaemia (HbA1c, fasting plasma glucose, OGTT) between the PD patients and their CKD 5 controls. Although the duration of PD in the PD group was relatively short (12 months) a previous study assessing insulin resistance by using the gold standard hyperinsulinaemic clamps, reported an improvement in insulin resistance after only 5 weeks[175].

A lack of deterioration in metabolic parameters in the PD group is on the whole reassuring however I also found that these traditional metrics did not reflect patients' actual glycaemic exposure over 72 hours measured using CGMs. In the cohort as a whole there was no correlation between average interstitial glucose levels and either HbA1c, fasting plasma glucose, 2 hour post OGTT glucose or HOMA-IR. This has also been reported in other studies using CGMs in PD patients with diabetes [214, 216, 218]. With 31 completed participants my study would have been able to detect a moderate correlation of r= 0.45-0.5. Although there may be a weaker correlation present in this cohort that we were not powered to detect the clinical significance of such a weak correlation is doubtful.

Although this study was not designed to examine how reliable standard glycaemic markers are it still gives some insight into their utility as research

outcome variables. The lack of correlation between 2 hour post OGTT glucose and CGM outcomes coupled with the poor reproducibility of OGTT and its relatively onerous methodology make it an unattractive means of defining glucose tolerance in PD patients or monitoring changes in glycaemia in response to treatment regimes.

The effect of overnight peritoneal glucose load on fasting plasma glucose is speculative. This would be best examined by paired experiments with and without overnight peritoneal fasting. In this study there were no systematic differences between fasting plasma glucose levels in controls (orally and peritoneally fasted overnight), CAPD patients (orally fasted, non glucose based dialysate overnight) and APD patients (orally fasted but glucose based dialysate overnight). Small numbers in each of the 3 groups limit the ability to detect subtle differences in fasting plasma glucose between groups. However examination of the raw traces from APD patients indicate that once the APD cycle is complete interstitial glucose returns to fasting levels within a maximum time of 2 hours and typically within 30 minutes (Figure 5.6). Although this requires more thorough investigation it may indicate that although peritoneal and oral fasting would be considered gold standard for measuring fasting plasma glucose, a measurement without dialysate in situ and at least 2 hours after the over night dialysis has finished may be an acceptable and more practical alternative.

Participants in this study had comparable levels of metabolic derangement to other published cohorts. Although we should exercise caution in comparing HbA1c which is said to reflect glycaemia over 2 to 3 months and the much shorter period of CGM monitoring undertaken by the participants in my study,

my data adds to the growing body of evidence suggesting that in patients with end stage renal disease HbA1c correlates poorly with actual glycaemic exposure measured using CGMs.

Potential limitations of this study

This is the first study to prospectively examine glycaemic patterns in non-diabetic PD patients using CGMs. To analyse any potential effect of peritoneally absorbed glucose they are compared to a control group whose insulin sensitivity will also be influenced by the effects of chronic uraemia and who are subject to the same dietary restrictions. Both cohorts are well characterised with regards their metabolic profiles. The FreeStyle Libre flash glucose monitors proved easy to use and acceptable to the participants and this resulted in data sets with minimal missing data points.

There are some important limitations to this study. As discussed above this is a relatively small observational study which lacks power to detect small differences between groups. Subdivision of the PD patients by PD modality further reduced statistical power. I aimed to recruit 20 participants into each of the groups but it was not possible to meet this target for the reasons outlined in the results section.

The PD group had a relatively short duration of therapy of 12 months however as discussed above previous studies have indicated that significant metabolic alterations have been documented in shorter time frames.

The flash glucose monitors were not masked, consequently participants could see real-time interstitial glucose levels and their historic values. In theory this

could have influenced participants' behaviour reducing the generalisability of data obtained. However unlike studies in diabetic populations these participants were not educated on 'normal' glucose levels and therefore had no frame of reference for what was 'good' or 'bad'. The only behaviour they could change would be food intake as they weren't on any glucose lowering treatments and they were asked not to alter their dialysis regime during the monitoring period. In conclusion I do not believe that the unmasked nature of the CGMs negatively affected the generalisability of the results obtained in this particular study.

The goal of the study was to obtain 'real-life' glycaemic profiles and therefore participants' food intake and PD regimes were not standardised. Standardising food intake may have reduced variability in the data but may have negatively impacted recruitment and in practice would have been very difficult to implement. Altering dialysis regimes without obvious benefit to the patient is ethically difficult to justify. There were a number of potential advantages to this 'real-world' setting. As can be seen by comparing our results with those from experimental single dwell studies the impact of absorbed glucose appears to be influenced by concurrent food intake. As this is the first study of its kind it was important to gain an understanding of the degree of variability that could be expected in real world settings and the impact of 'normal' food intake. For these reasons, this data will be useful for those designing future studies aimed at measuring the impact of glucose sparing regimes.

In conclusion, in this study PD therapy was not associated with any significant change in parameters of glycaemic control measured using CGMs and traditional glucose metrics compared with CKD 5 non-dialysis controls. This study adds to the growing body of evidence that HbA1c does not reflect

glycaemic exposure in this patient group. In this cohort comprising APD and CAPD patients, utilising contemporary glucose sparing regimes, we found no relationship between dialysate glucose exposure and CGM derived outcomes. The distinctly different patterns of glycaemia noted in CAPD and APD patients require further investigation and suggest that in future studies of glycaemia in PD, patients should be analysed by modality.

Chapter 6 Overall Discussion

Cardiovascular disease is the major cause of morbidity and mortality in peritoneal dialysis patients[430]. This increased morbidity and mortality is the result of multiple insults which cannot be fully abrogated. The overall aim of this thesis was to investigate potential contributory factors. Dysfunction of the systemic microcirculation is a known cardiovascular risk factor [431, 432]. This thesis thus addressed the following questions. Is the systemic microcirculation adversely affected in patients with end stage renal disease who are on PD? What association, if any is there between dysfunction in the systemic microcirculation and movement of small solutes across the peritoneal membrane? How may PD therapy itself contribute to cardiovascular risk in the form of peritoneally absorbed glucose?

The microcirculation in end stage renal disease

In this thesis I used minimally invasive, *in vivo* tests of microvascular structure and function to examine the cutaneous and sublingual microvasculature of 28 PD patients. I found a greater degree of cutaneous microvascular dysfunction in end stage renal disease patients on peritoneal dialysis compared with age and sex matched healthy controls and also controls matched for major cardiovascular risk factors including diabetes and previous cardiovascular events.

This is an observational study and cannot ascribe causality. However my finding that the PD patients exhibited a reduced peak post occlusive hyperaemia and reduced response to application of sodium nitroprusside compared with controls, implies an additional microvascular burden that is attributable to the effects of uraemia plus or minus renal replacement therapy. Systemic

microvascular dysfunction has also been demonstrated in other renal cohorts[99, 100, 108], composed of predominantly haemodialysis patients. My results are the first to report evidence of systemic microvascular dysfunction in a purely PD cohort and in particular in PD patients early in their treatment. The underlying mechanisms of dysfunction and affected pathways are likely to be multifactorial. My results indicate impairments in multiple vasodilatory pathways. In the PD group there was a statistically significant reduction in the reaction to a period of arterial occlusion (a test of global microvascular function) compared with both healthy and cardiovascular risk matched controls. The PD group also exhibited a reduced response to application of SNP (an endothelium independent reaction) but not application of ACh (an endothelium dependent reaction) compared with healthy controls. The mean response to ACh was 0.5 SD lower in the PD patients than in controls. Given this study was powered to detect a difference of 0.75 SD or more I am unable to exclude the possibility that the ACh response was actually impaired in the PD patients but the study was not sufficiently powered to detect the difference. Additionally there are multiple potential confounding factors, such as medications, which might have affected the endothelial response but that it was not possible or appropriate to statistically adjust for given the size of the cohort.

Patients with end stage renal disease have many risk factors for microvascular dysfunction. This is the first study to report that functional impairments of the cutaneous microcirculation are more marked in PD patients with greater degrees of mineral bone disease, as evidenced by the negative association between parathyroid hormone, phosphate and cutaneous microvascular. This is an interesting potential mechanistic route.

The underlying pathophysiology of CKD mineral bone disease is complex with numerous factors closely interrelated and different factors predominating at different stages of kidney disease. Retention of phosphate, decreased levels of calcitriol, intrinsic alterations within the parathyroid gland, skeletal resistance to the actions of PTH and hypocalcaemia all contribute to increased PTH secretion[385]. The relationship between mineral bone disease and vascular dysfunction, or the bone-vascular axis, is likely to be equally complex. There is increasing evidence that the concentrations of newer biomarkers of mineral bone disease such as fibroblast growth factor-23 (FGF23) and Klotho are disturbed earlier in the course of CKD than more traditional measures such as phosphate and PTH[433]. FGF23 is a hormone produced by osteoblasts and osteocytes. It acts to inhibit phosphate reabsorption in the kidneys however in CKD this action is lost due to loss of renal Klotho-FGF receptor 1 complex, resulting in high phosphate and FGF-23 levels[434]. In animal models it has also been shown to have a more direct effect on the vasculature via increased production of inflammatory markers such as transforming growth factor-β and tumor necrosis factor-α[435], increased superoxide formation and decreased NO bioavailability[436]. How this relates to humans is yet to be explored. My findings from these first investigations in patients with PD are in keeping with larger studies in humans without renal disease which demonstrated a relationship between serum phosphate concentrations and microvascular dysfunction[135] along with improvement in endothelial function associated with reduction in serum phosphate concentrations[393]. Modulation of phosphate concentrations has also been associated with better large vessel cardiovascular outcomes in animal models[437], but this has not been replicated in human studies[438].

Increased phosphate, even within the reference range, has been associated with increased cardiovascular risk[439]. The exact mechanisms of this remain poorly understood. I*n vitro* experiments and animal models have demonstrated that high phosphate and PTH concentrations have several effects on vasculature, both endothelial and non-endothelial, as discussed in detail in Chapter 4. *In vitro*, osteoblastic differentiation of smooth muscle cells has been demonstrated after only 72 hours of exposure to high PTH concentrations [391]. *In vitro, h*igh phosphate concentrations result in increased production of reactive oxygen species and reduced NO availability [136], this is likely to impact endothelial functioning. *In vivo,* microvascular dysfunction in patients with chronically high phosphate and PTH concentrations, as reported in this study and by others[98, 99], is likely to be the result of smooth muscle cell dysfunction or increased vessel stiffness as seen in larger vessels along with direct actions on the endothelium as described above. In addressing this question further it would be important to take skin biopsies, comparison of the functional findings with histological observations in the cutaneous vessels, for example changes in the medial layer or evidence of endothelial activation, would help when drawing conclusions about the most influential mechanisms.

The control groups in this study were chosen for pragmatic reasons. Our research group had recently collected a large data set using the same techniques in patients with normal excretory renal function. From this data set I was able to compile well matched control groups that allowed exploration of the differences between renal and non-renal cohorts without additional participant burden i.e. recruiting new participants. The nature of the control groups in this study and its cross-sectional design meant that I was able to investigate the combined effect of PD and long term uraemia on systemic microvascular

function while correcting for co-morbidities, age and sex. Another interesting aspect to explore would be to assess the potential impact of the PD therapy itself (separate to the effects of uraemia) on the microcirculation. To further demarcate the impact of PD on the microcirculation from those of long term uraemia would require separate control groups comprised of patients with ESRD not on dialysis and ideally a group on haemodialysis. Controlling for confounding factors in these relationships, such as length of CKD and medications would require relatively large patient numbers and would be difficult to conduct at a single site. To be cost effective such an investigation would most likely need to be nested into another study in which such patients were being recruited.

Many of the issues discussed above could be explored further in a longitudinal study, with repeated measures of microvascular function, following a well characterised cohort of patients through the stages of CKD to describe the natural history of microvascular dysfunction in these patients. This type of longitudinal study would also address most robustly the impact of different modes of renal replacement therapy on the microcirculation. Retention of dialysis patients in longitudinal studies, has been demonstrated to be difficult, risking attrition bias. The ongoing INTHEMO [141] study lost nearly 30% of participants within the first 6 months. The number of patients needed for such a study would be large and necessitate a multi-site and probably multi-national group effort as demonstrated by the SUMMIT consortium (https://www.imi-summit.eu/).

The conclusions we can draw from this study are limited by relatively small numbers and the heterogeneity of the cohort. However it is the first study to

demonstrate a statistically significant difference in cutaneous microvascular function between end stage renal disease patients on PD and controls with normal renal function. This data will be useful in the planning of future studies of the microcirculation in this understudied patient group.

The relationship between the systemic and peritoneal microcirculations

This is the first study to prospectively investigate the hypothesis that dysfunction of the systemic microcirculation will be reflected in dysfunction of the peritoneal microcirculation and consequent faster transport of small solutes. I found no evidence of a moderate or strong relationship between the structure or function of the cutaneous and sublingual microcirculations and the rate at which small solutes are transported across the peritoneum during peritoneal dialysis in a cohort of patients with duration of PD less than 6 months. Although this was a small study it was robustly designed and conducted. Conclusions are limited by the relatively small numbers and heterogeneity of the cohort. However, the strong correlation between intraperitoneal inflammation and transport rate in this study and in others [70] indicates that intraperitoneal factors are likely to be much more influential than systemic factors. This study would have been further enhanced by having peritoneal tissue from each participant, ideally in the form of a peritoneal biopsy taken at the time of catheter insertion. This would have allowed direct comparison between the structure of the systemic and peritoneal microcirculations, at least prior to PD introduction. To my knowledge no previous studies have attempted to correlate peritoneal and systemic microvascular structure in this way. In the context of this doctoral work, the complexity and costs of sample processing at the point of collection along with subsequent analysis proved prohibitive to the inclusion of this potential study component.

One previous study aimed to investigate the relationship between the systemic and peritoneal microcirculation and found no association between sublingual microcirculatory parameters and peritoneal transport[169]. As discussed previously their study had several limitations including the long duration of PD therapy in their cohort and the fact that it was a retrospective analysis of a subset of patients recruited to answer a different question. This therefore left unanswered questions and made exploring this potential relationship in my study important. I believe, based on the data from my study, that further investigation of this relationship even with larger patient numbers is unlikely to reveal a strong and therefore clinically meaningful relationship between the two.

Glycaemic profiles in peritoneal dialysis patients

In this thesis I present 'real-world' 72 hour glycaemic profiles from PD patients and CKD 5 non-dialysis patients. This is the first study to collect data from these patient groups using continuous glucose monitors. The monitors were well tolerated and resulted in good quality data. While this was a small, preliminary study of patients with a relatively short duration of PD, the negative impact of absorbed glucose on overall glucose handling reported in PD patients with diabetes [192] was not observed in this non-diabetic cohort. Mean interstitial glucose levels over the whole study period were very similar across the 3 groups. Larger numbers may have rendered the differences statistically significant but it is unlikely that this degree of difference (between 0.15 and 0.3mmol/L over 24 hours) would be clinically significant.

Data from this study provides novel insight into the differential effects of CAPD and APD on interstitial glucose levels in non-diabetic patients. I was able to demonstrate a statistically significant difference between mean night time

glucose in the APD group versus CAPD group. The difference between the controls and the APD patients was not statistically significant although this may be the result of Type II statistical error due to the small sample size. There was no difference in the mean daytime glucose across the three groups.

The different effect of peritoneally absorbed glucose on interstitial glucose concentrations in day versus night is a very interesting observation. The measurable effect of absorbed glucose on interstitial glucose concentrations during night time may be a result of absence of concurrent food intake. One could theorise that insulin released in response to oral carbohydrates is sufficient to metabolise the proportionally smaller extra intraperitoneal glucose load and therefore it's overall effect is not appreciated. This study was not designed to comment on the exact relationship between absorbed glucose and interstitial glucose as this could and has been done more effectively by examining blood glucose fluctuations during individual dwells [204-206]. However future studies of this kind could build on my findings of the potential impact of meals on the glycaemic response to PD exchanges, investigating the effects of individual dwells with and without a preceding standardised meal. Adjusting for the overall effects of dietary intake in any study especially one such as mine is very challenging. I found that dietary intake was extremely variable both within and between participants. To my knowledge none of the previous studies attempting to link peritoneally absorbed glucose with markers of glucose metabolism have attempted to adjust for or control for dietary intake.

An alternative theory to explain the measurable effect of absorbed glucose on interstitial glucose concentrations during night time would be that patients are less well adapted to metabolise the extra glucose load at night. As a result of

natural circadian rhythms the circadian night is associated with poorer glucose tolerance and augmented inflammatory responses compared with circadian day. I have shown that intraperitoneal inflammation has a significant effect on peritoneal transport of small solutes and therefore it is conceivable that relatively more glucose is absorbed during the night as a result of increased local inflammation and this is augmented by poorer night time glucose tolerance.

A further avenue that warrants exploration is the impact of the additional carbohydrate load of APD during circadian night, this disruption of normal circadian rhythms could theoretically negatively impact overall glucose metabolism. In my data there is a suggestion that the APD patients had more impaired insulin sensitivity than CAPD with higher fasting insulins and HOMA-IR. This was not statistically significant and clearly splitting the PD patients into APD and CAPD means this study was only powered to detect large differences between groups but it is conceivable that the mode of glucose exposure impacts overall glucose metabolism. The impact of different types of PD could be investigated by repeated periods of CGM monitoring in the same patient prior to commencing PD, after a period of CAPD and then again after a switch to APD , with complementary inflammatory markers(systemic and intraperitoneal) and biomarkers of peripheral clock disturbances (for example melatonin, cortisol and clock genes).

The different patterns of glycaemia in APD and CAPD patients demonstrated in this thesis warrant further investigation. It indicates that where possible, in studies of the relationship between peritoneal glucose and systemic glucose handling, CAPD and APD patients should be analysed as separate groups. This

is particularly important in large multi-national studies, PDOPPs[440] has demonstrated considerable international variations in PD modality use, resulting in different levels of solute clearance and therefore a high likelihood of different patterns of glucose absorption.

Data collected as part of this thesis has provided further evidence supporting intraperitoneal inflammation as the major determinant of peritoneal transport rate [70]. According to pore theory inflammation should cause an increase in bi-directional transport and therefore may affect the degree of glucose absorbed [213]. Continuous glucose monitoring could be used to further investigate the effect of different levels of intraperitoneal inflammation on systemic glucose parameters. Over recent years several dialysate solutions have been developed that are more physiological and therefore theoretically result in less local inflammation[441]. Decreased glucose absorption has been associated with these solutions in single dwell studies[213]. Using a variety of regimes in the same patient it would be possible to use the CGMs to investigate the effect of different dialysate formulations on interstitial glucose.

In common with all previous studies of continuous glucose profiles in PD patients this study was essentially exploratory in nature. Despite this it gives an important and previously undescribed insight into the patterns of glycaemia that would be expected in non-diabetic patients on PD and also those patients with advanced renal disease who are not yet on dialysis. This data can be used in future studies to help inform best and most appropriate outcome measures and aid in power calculations for larger interventional studies.

Recruitment to renal studies

There are several issues highlighted by the cohorts in this thesis that are common to the majority of studies of patients with renal disease particularly the dialysis population [442]. End stage renal disease is a phenotype, not a specific pathology and therefore dialysis cohorts are heterogeneous by nature. In addition to variation in primary renal diagnosis the majority of patients will have multiple co-morbid conditions and prescribed medications which may also influence outcome variables in any given research setting. The cohorts presented in this thesis are generally representative of PD cohorts however as anticipated they display high levels of heterogeneity. Limiting participants to a single renal diagnosis would reduce heterogeneity but significantly limit relatability of findings to the wider dialysis population. It would also impact ability to recruit the required number of participants.

For HD patients there is the additional challenge of studying individuals on an intermittent therapy where timing of investigations may have a significant impact on measured outcomes. As I have demonstrated in this thesis, the timing of investigations in PD patients is similarly important. Despite the 'continuous' nature of their therapy they are systematically exposed to varying concentrations of dialysate which seem to differentially impact glycaemic handling dependant on modality, concurrent food intake and time of day. A lack of appreciation of these significant variations may lead to inappropriate conclusions. Additionally, without consensus on timing of investigations comparison of results between studies is limited.

The studies in Chapter 4 and 5 both did not meet recruitment targets. As a consequence of their multi-morbidity and the intensity of renal replacement

therapy, dialysis patients are at high risk of intervening illnesses which preclude their involvement in a study or necessitate dropping out, this had a significant impact on recruitment to my studies. Although the percentage of patients approached who agreed to take part was higher than anticipated, recruitment was negatively impacted by intervening illness or other clinical factors outside the study (e.g. problems with PD catheter function) which prevented patients who would be eligible from taking part. Fragile physical health coupled with a high pre-existing burden of medical interventions means recruitment and retention of patients in renal studies remains a challenge.

These issues explain in part why despite the high relative burden of kidney disease the amount of reliable clinical study data available to guide care of dialysis patients is limited compared with other conditions [443]. It has been suggested that a focus on biochemical markers and a disregard for patient centred outcomes is also contributory. The SONG-PD initiative [444] aims to establish a set of core outcomes for trials in patients on PD based on the shared priorities of healthcare professionals, patients and carers. It is hoped that in the future studies can be prioritised using these outcomes of common importance. This should give patients and caregivers a greater stake in PD research going forward and aid patients and clinicians in informed decision making.

The work presented in this thesis contributes to our knowledge of the degree of systemic microcirculatory dysfunction exhibited by patients with end stage renal disease. Correlations reported here and by others, between CKD mineral bone disease and systemic microvascular function indicate potential mechanisms of dysfunction which require further investigation. This work adds to a growing

body of evidence that intraperitoneal rather than systemic factors, specifically inflammation, are most influential in peritoneal small solute transport. The patterns of interstitial glucose concentrations in non-diabetic patients on PD I have reported, will inform future studies aimed at minimising the metabolic consequences of PD. Modality specific differences in the systemic effects of peritoneally absorbed glucose and their effects on natural circadian rhythms require further investigation.

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