Investigation of the risk factors for development of infection by continuous ambulatory peritoneal dialysis patients

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By

Noura Hamad Abdullah Al-Dayan, Msc

Department of Infection, Immunity and Inflammation

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Abstract

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Noura Hamad Abdullah Al-Dayan

Chronic kidney disease is a progressive condition resulting in morbidity and mortality. Lost kidney function can be replaced by transplantation or dialysis (haemodialysis or peritoneal dialysis). Although peritoneal dialysis is simple and cheap, there are two major problems: protein loss and peritonitis. Despite protein supplements and aseptic technique, little is known about the correlation between protein loss and infection. The overall aim of this project was to characterise dialysate protein profiles of patients and investigate factors that might increase infection e.g. catecholamines.

Protein profiles of daytime and overnight dwells were investigated using proteomic techniques: the total number of proteins in the dialysates was similar. Sequence analysis showed these were plasma proteins: e.g. albumin, fibrin-beta, IgG, complement C3 and transferrin. Three proteins not reported previously were detected: ceruloplasmin, albumin-myristate-azapropazone complex, and albumin-myristate-tri-iodobenzoate complex. Protein concentrations in overnight dwells were higher than in daytime samples. Differences were also found in other parameters, e.g. pH, glucose, and total iron.

Direct measurements of ability of *S. epidermidis* to grow in peritoneal dialysates showed variation between patients. Addition of iron or catecholamines significantly increased growth, indicating dialysates were iron-limited. Analysis of dialysate exposure on *S. epidermidis* virulence showed that biofilm formation and haemolytic toxin production were significantly stimulated, but that stimulation varied between patients. One-year follow-up peritoneal dialysates showed that higher protein concentration occurred compared with initial dialysates. This may explain the apparent tendency of the one-year dialysate to sustain greater *S.epidermidis* growth. A correlation was found between dialysate protein concentration, and degree of growth stimulation/virulence. This suggests that a biomarker for infection risk in these patients could be protein concentration in their dialysate. Suggestions are made as to how this might be implemented into patient care.

To the spirit of my dad (God mercy be upon him)

To my mum (big heart)

To my little 3 stars (Yara, Abdullah and Rana)

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"Gratitude is not only the greatest of virtues, but the parent of all others" ***Mahatma Gandhi***

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Love,

Noura

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ABBREVIATIONS

AMPs	Antimicrobial peptide
APD	Automated Peritoneal Dialysis
APS	ammonium persulphate
BSA	Bovine serum Albumin
CA	Catecholamine
CAPD	Continuous Ambulatory Peritoneal Dialysis
CCPD	Continuous Cycling Peritoneal dialysis
CFU	Colony-forming units
C-NS	Coagulase-negative staphylococci
CO_2	carbon dioxide
D.T	Daytime
DO	Dopamine
DTT	dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
EPI	Epinephrine
Fe	Iron
Fg	fibrinogen
Fn	fibronectin
GDP	Glucose Degradation Products
GFR	Glomerular Filtration Rate
HCl	hydrochloride
HD	Hemodialysis
HK	Hexokiness
HPD	Human Peritoneal Dialysate
HPLC	High Performenace Liquid Chromatography
IgG	Immunoglobulin G
KDa	kilodalton
LA	Lauria agar
LB	Lauria broth

Na Cl	Sodium Chloride
NE	Norepinephrine
NIPD	nocturnal intermittent Peritoneal dialysis
O.D	Optical density
O.N	Overnight
PBS	Phosphate buffer saline
PD	Peritoneal dialysis
PDC	Personal Dialysis Capacity
PDS	Peritoneal Dialysis Solution
PET	peritoneal equilibration test
S. epidermidis	Staphylococcus.epidermidis
SB	Protein sample loading buffer
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodiun dodecyl sylphate polyacrylamide gel electrophoresis
SPA	standard permeability analysis
TEMED	N,N,N',N'-Tetramethyleneamine
Tf	Transferrin
TPD	tidal peritoneal dialysis
UFF	Ultrafiltration failure

Chapter 1.Introduction

1.1Kidney function and renal failure

The kidneys are a major organ which has multiple functions including: excretion of metabolic waste products, excretion of hormones, regulation of body fluids (acid-base balance, electrolyte concentration,), water and inorganic electrolytes re-absorption, gluconeogenesis and regulation of blood pressure. Nephrons are the basic functional units of the kidney and are long segmented tubes consisting of a glomerulus and a series of tubules. The human kidney is made up of approximately 1×10^6 nephrons (Guytone and Hall, 2006).

Nephrons are divided into a number of different segments based on the reabsorption and secretion characteristics of the tubule (Figure 1.1). Blood enters the glomerulus through the afferent arteriole, is filtered across the glomerular basement membrane with early urine entering Bowman's space. Blood leaves the glomerulus in the efferent arteriole and then into the peritubular capillaries. The early filtrate flows through the proximal tubule, the descending and ascending limbs of the loop of Henle, the distal tubule and finally into the collecting ducts. The loop of Henle acts as a counter current exchange mechanism that increases the solute concentration in the interstitial fluid as the loop descends. The gradient between the top and bottom of the loop can be as large as 1200 mmol L^{-1} in human kidneys (Stephenson , 1992) and over 2000 mmol L^{-1} in other species (Hervy & Thomas, 2003) .

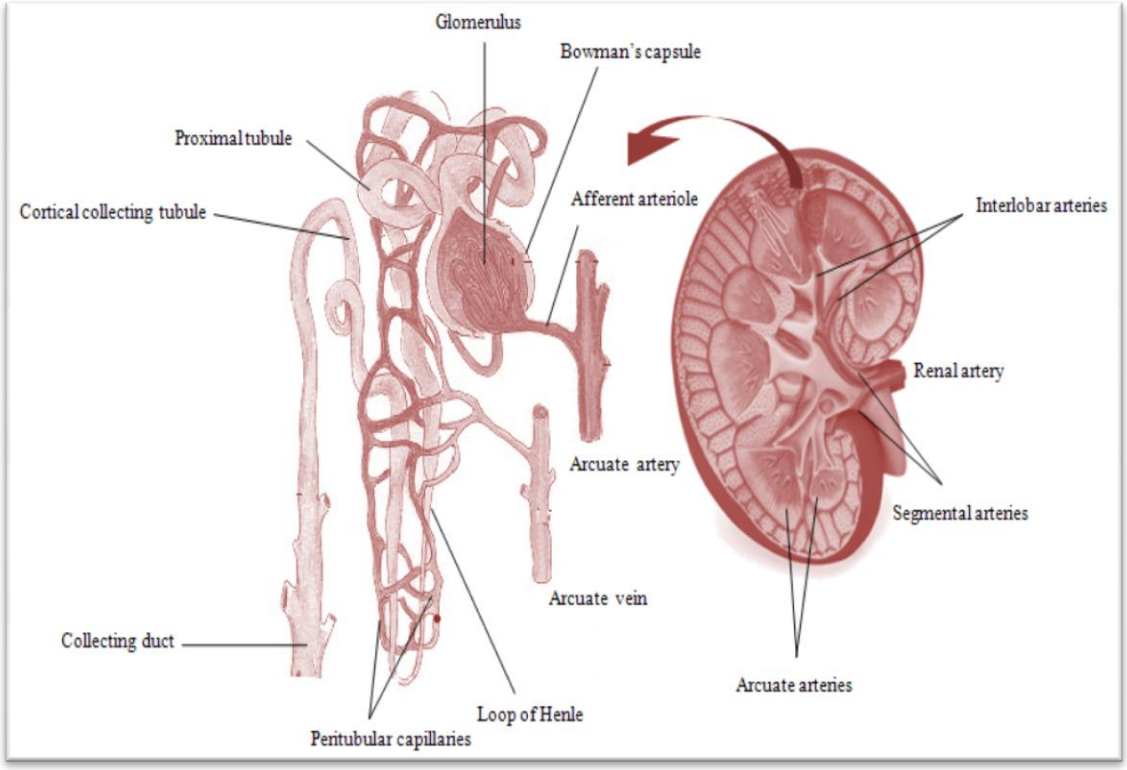

Figure 1.1 Sections of the human kidney and nephron

Chronic kidney disease is characterized by a progressive fall in glomerular filtration rate and clinically sustained rise in urea and creatinine (Hilton, 2006). It is a long - term medical condition which is progressive and results in significant morbidity and mortality. It is associated with complications including fluid overload, hypertension, anemia, bone disease and metabolic acidosis. Loss of nephrons leads to a progressive decline in glomerular filtration rate (GFR), and a value of less than 60 ml/min/1.73 m2 for 3 or more months consecutively is considered to be indicative of renal failure (Bash et al., 2009; Popovich et al., 1976; Walter & Emile, 2005). The causes of kidney disease are traditionally grouped into three categories: decrease in renal blood flow (pre-renal) (Kaufman et al., 1991; Hou et al., 1983); direct renal parenchymal damage (intrinsic renal causes) (Kaufman et al., 1991; Liano & Pascual, 1996) and urinary tract obstruction (post-renal) (Liano & Pascual, 1996).

1.2 Renal Replacement Therapy

There are two types of treatment for patients with kidney failure, transplantation and dialysis. There are two types of dialysis, hemodialysis and peritoneal dialysis. Choice of renal replacement therapy is made collaboratively between hospital team and patient, with the priority being to ensure that patients maintain their independence (Conway et al., 2009; Hou et al., 1983)

1.2.1 Transplantation

Kidney transplantation is the most successful form of renal replacement therapy; it is associated with higher patient survivals, lower hospitalisation rates and a superior quality of life (Vathsala, 2005). However, the number of donors is insufficient for the number of dialysis patients and therefore there is a large population of patients with endstage renal disease who are living on dialysis (Brown et al., 2010; Dempsetr, 1963).

1.2.2 Dialysis

Dialysis is the movement of solutes down a concentration gradient across a semipermeable membrane. In haemodialysis (HD) the blood is exposed to an artificial machine enclosed membrane, and occurs outside the body. In the case of peritoneal dialysis (PD) there is a movement and exchange of molecules across the peritoneal membrane, occurring inside the body. With both techniques small solute and water

removal occurs by dialysis, i.e down a concentration gradient, and ultrafiltration, i. e down a hydrostatic pressure gradient (see Figure 1.2).

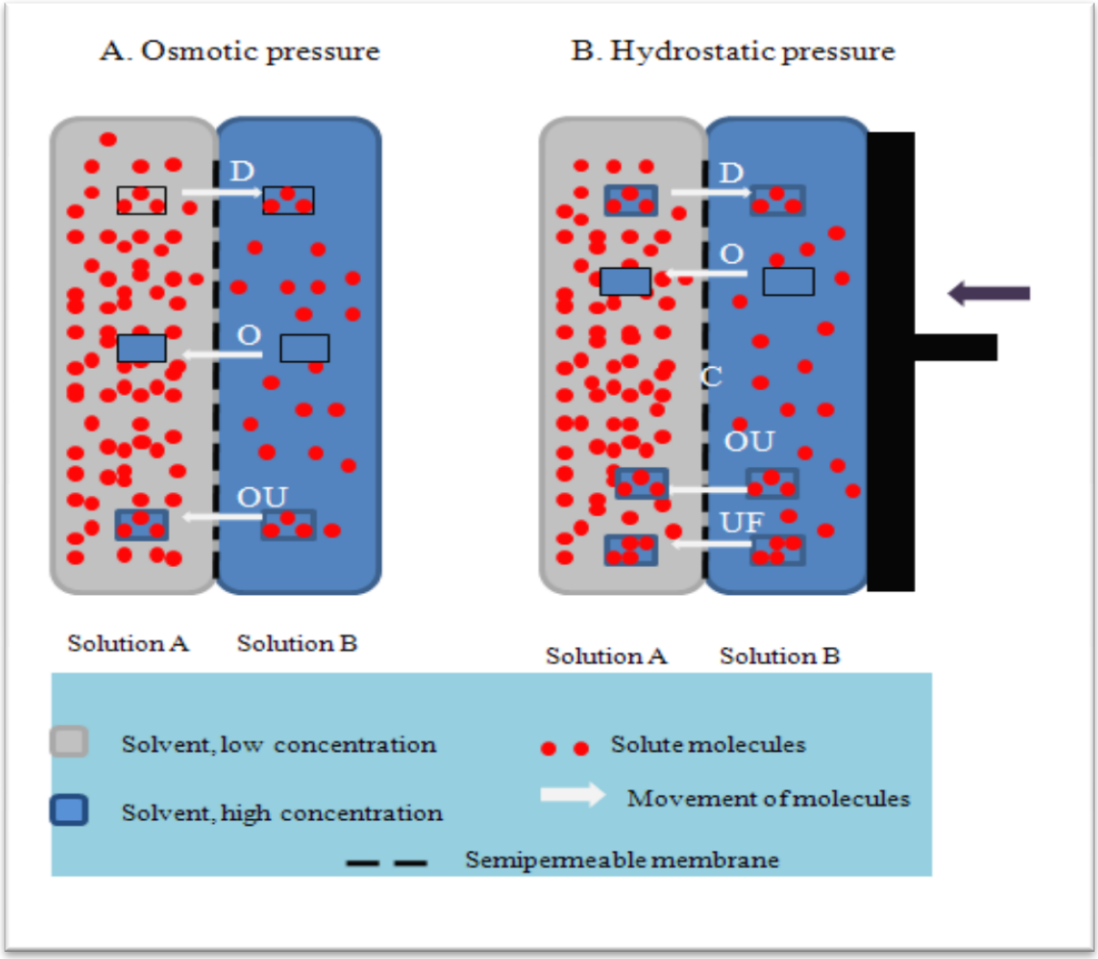

Figure 1.2: Principle of Dialysis, A. osmotic pressure or B. hydrostatic pressure. D=Diffusion, O=osmosis and OU= osmotic ultrafiltration, UF = ultrafiltration by hydrostatic pressure, C=Convection.

There are two processes central to dialysis and they are summarized in Table 1.1

Process	Definition
Diffusion	Solute moves randomly from the side of higher to the side of lower concentration depending on 1. Physical size of the molecule relative to the size of the membrane pores, 2. The electrical charge and the shape of the molecule
Convection	Solute molecules are dragged by the solvent depending on porosity of the membrane- this occurs during ultrafiltration and the movement of water down either an osmotic or hydrostatic pressure gradient

Table 1.1: A summary of the different processes which drive dialysis.

1.2.2.1 Haemodialysis (HD)

Blood is removed from the patient usually via an arteriovenous fistula and enters a dialyzer where the blood is subjected to dialysis against a predetermined dialysate. Waste products cross the dialysis membrane, and the dialysate enters the waste drain. Blood is returned to the patient via a venous line. Haemodialysis is not without its complications which include complications of forming vascular access, blood clotting in the extracorporeal circuit, and perhaps most importantly bacterial infection, particularly in patients with indwelling dialysis catheters (Ahmed, 2009; Daugirdas et al., 2006).

1.2.2.2 Peritoneal Dialysis (PD)

1.2.2.2.1 Principle of peritoneal dialysis

The peritoneal cavity is an internal space lined by a semi-permeable membrane known as the peritoneal membrane (Flessner, 1991; Davies and Williams, 2003). The permeability and solute transport properties of the membranes that separate the blood vessels distributed within the tissue underlying the peritoneum are so efficient that the peritoneal membranes can be used to perform dialysis (Nolph et al., 1980) (Flessner, 1991). The peritoneal membrane has several size pores for the movement of various molecules which are differentiated into 3 types: ultra-small pores (0.8 nm in radius) that can transport water; intercellular small pores (4-6 nm) that transport small solutes and a few large pores > 20nm that transport large molecules such as peptides and protein (Ahmed, 2009). Ultrafiltration is achieved principally through the use of glucose in peritoneal dialysis fluids. The glucose creates an osmotic gradient within the peritoneal cavity drawing water in which can then be removed duringa dialysis exchange. Metabolite molecules such as urea, creatinine, vitamin B12, and phosphate diffuse from the blood vessels close to the peritoneal membrane into the peritoneal fluid, while the glucose and lactate, which are present in higher concentration in the dialysate, in turn diffuse into the blood (Figure 1.4).

Access to the peritoneal cavity in CAPD is achieved by using a catheter that has been inserted surgically (either under local or general anaesthetic) through the abdominal wall into the peritoneal cavity. There are various types of peritoneal access catheters but the most widely used are the straight or curled Tenckhoff catheters (Figure 1.3). Peritoneal dialysis occurs by filling the peritoneal cavity with a suitable peritoneal

dialysis solution (PDS), and over time allowing the diffusion of solutes (metabolites) into the PDS. Removal of the peritoneal dialysate (HPD) during a PD exchange allows the dialysate to be aseptically delivered into an empty sterile bag. This is followed by instillation of fresh replacement PDS allowing dialysis and ultrafiltration to begin again. The waste HPD is disposed of via the toilet.

The Tenckhoff catheter is a widely used design in peritoneal dialysis systems and was designed by Tenckhoff over 30 years ago (Tenckhoff, 1974). Long-term indwelling catheters are made of silicone rubber or polyurethane on which Dacron cuffs are placed (Tenckhoff et al., 1968, Tenckoff, 1974; Negoi, et al., 2006). The standard catheter is made up of 3 parts; an intra abdominal part, a subcutaneous tunnel part and an external part. The John Walls Renal Unit uses exclusively double-cuffed curled Tenckhoff catheters. Figure 1.3 shows 2 types of peritoneal catheters.

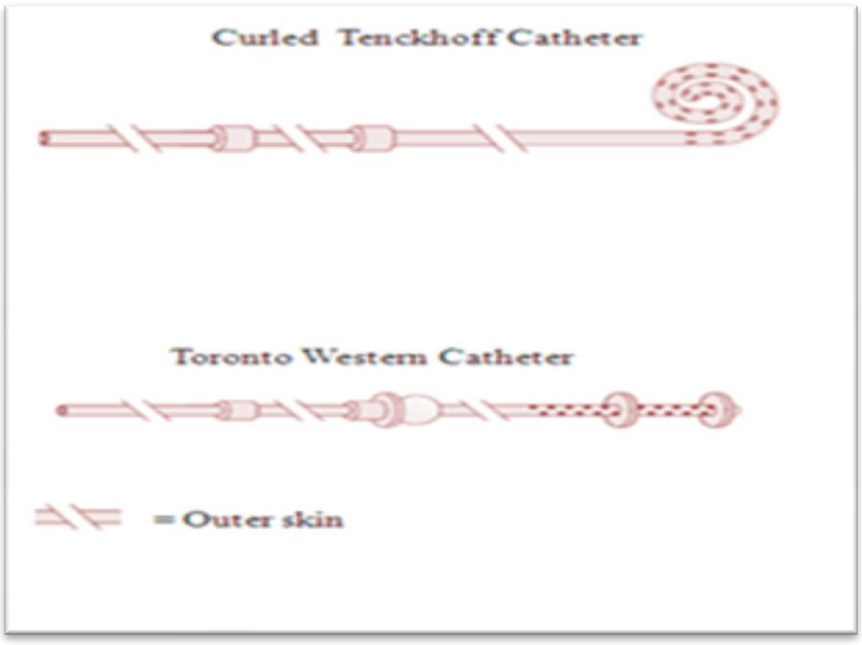

Figure 1.3: Shows 2 types of peritoneal catheters

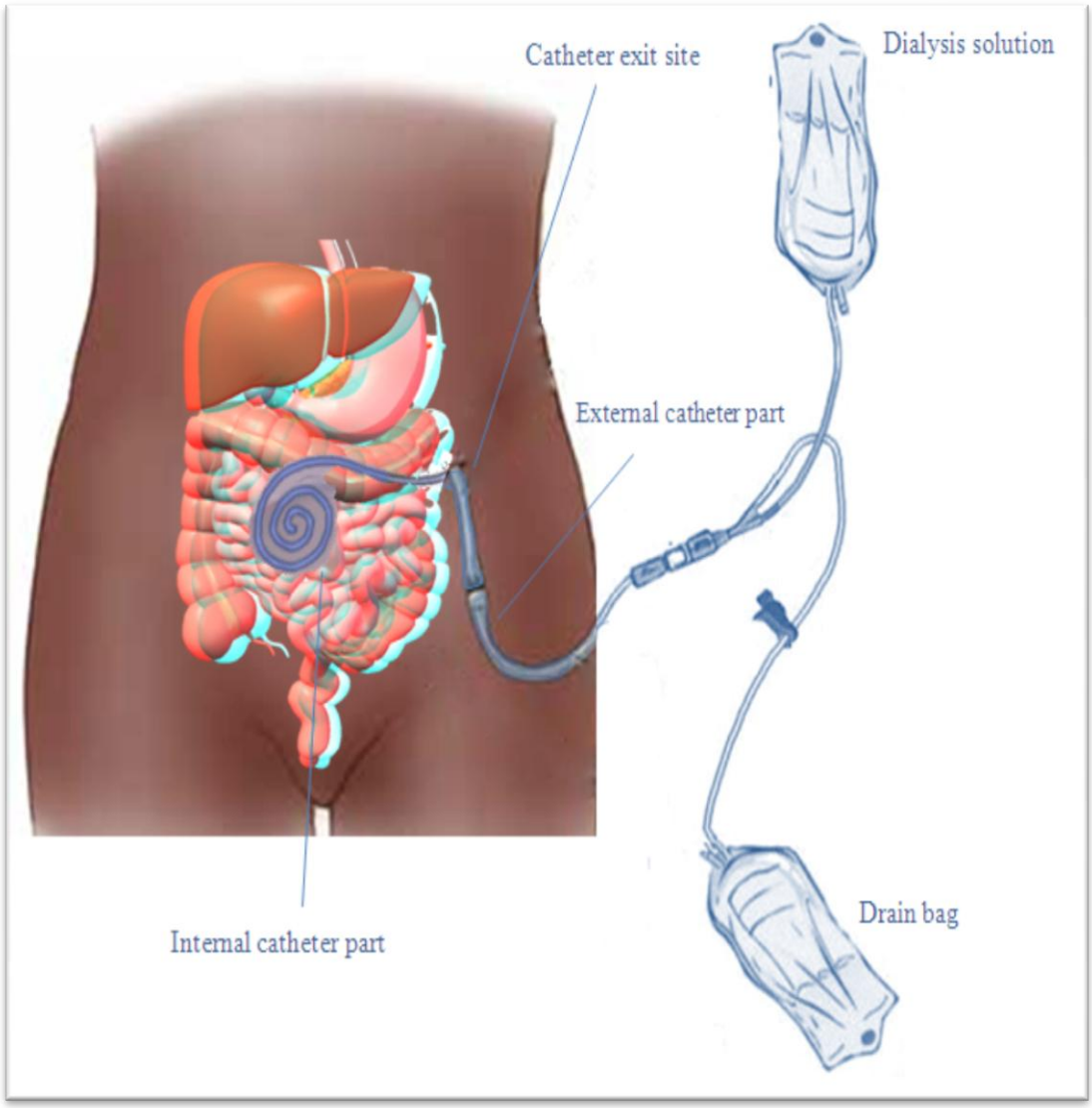

Figure 1.4: Peritoneal Dialysis

1.2.2.2.2 Peritoneal Dialysis Techniques

There are two main PD techniques: one uses a cycler machine and is called automated peritoneal dialysis (APD) while the other is called continuous ambulatory peritoneal dialysis (CAPD) and requires the patient to perform all the exchanges manually (Twardowski et al., 1995. These two types of dialysis are shown diagrammatically in Figure 1.5.

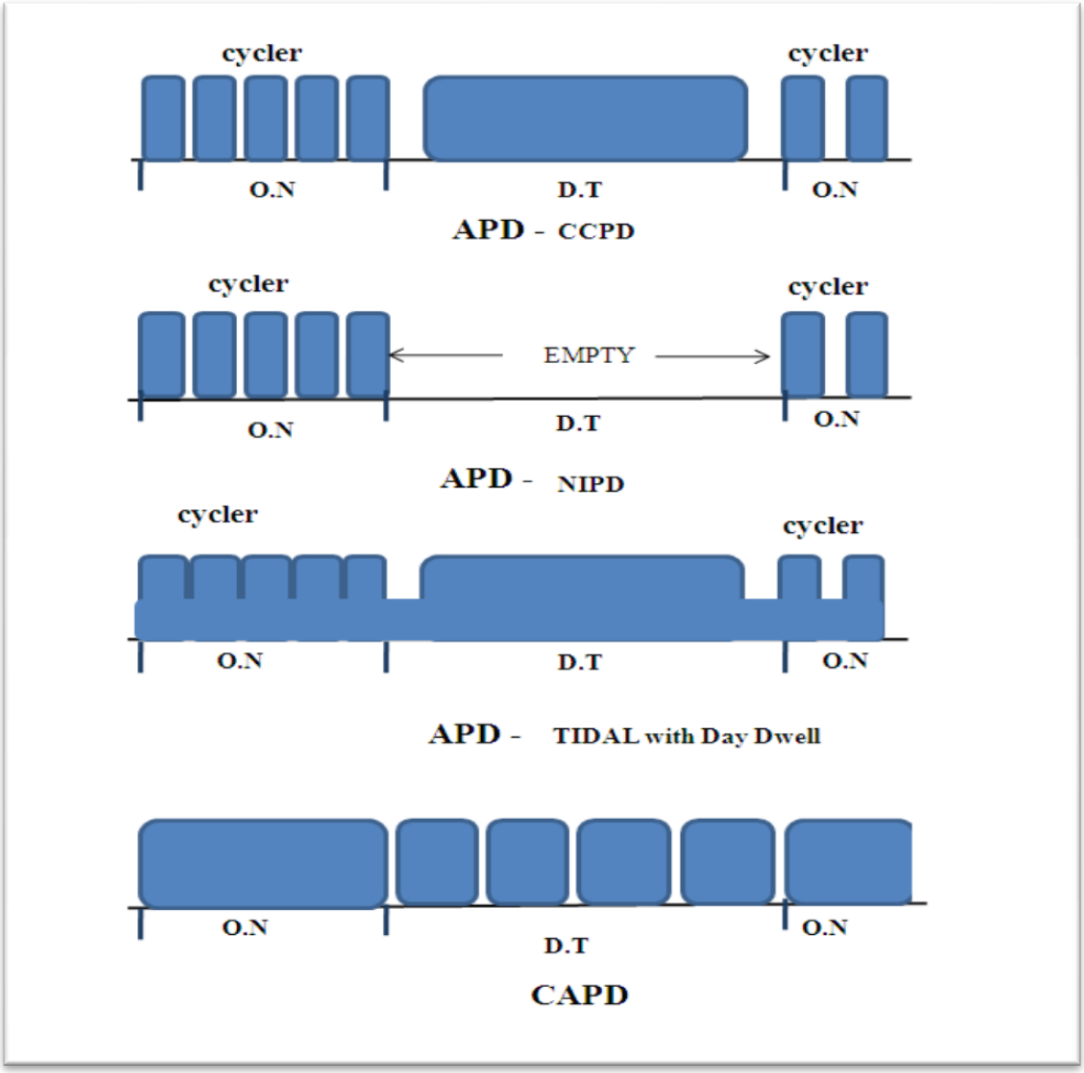

Figure 1.5: Peritoneal Dialysis Technique type: APD (CCPD and NIPD), CAPD. O.N= overnight, D.T= day time. Cycler = machine.

1.2.2.2.2.1 Automated Peritoneal Dialysis (APD)

The APD technique uses a machine cycler with or without manual PDS exchanges and exists in a variety of forms: Continuous Cycling PD (CCPD), nocturnal intermittent PD (NIPD) and tidal peritoneal dialysis (TPD). The APD patient connects the bags of dialysis solution to the cycler and connects the catheter to the cycler just before going to sleep. The cycler then circulates the dialysate solution in and out of the peritoneal cavity continuously for eight to ten hours. At the end of the cycle the fluid is drained out and the patient disconnects themselves from the machine. TPD is performed by filling the peritoneal cavity to the maximum capacity tolerable, and after short dwell time (e.g 20 min), half of the PDS volume is drained out and replaced with fresh solution. This exchange is repeated at equal intervals, ensuring continuous dialysis, meaning the consumption of a large amount of dialysis fluid (Munib, 2006).

1.2.2.2.2.2 Continuous Ambulatory Peritoneal Dialysis (CAPD)

CAPD consists of four to five PDS exchanges distributed over 24 hours. At each changeover usually 2 litres of dialysis solution is exchanged, which takes about 30 minutes to complete (Popovich et al., 1978). During a CAPD exchange the PDS inside the peritoneal cavity is drained out and replaced immediately with new solution. PDS remains in the peritoneal cavity for four to six hours (referred to in this thesis as the D.T dwell). However, the last exchange of the day remains in the peritoneal cavity overnight (referred to as the O.N dwell).

1.2.2.2.3 Peritoneal Dialysis Solutions (PDS)

Peritoneal dialysis solutions are composed of sodium, calcium, magnesium, chloride and lactate or bicarbonate as buffering agent. The pH of commercially produced glucose PDS is about 5.5 to prevent caramelization of glucose during heat sterilization. Glucose is used as an osmotic agent in most PDS. However, high concentrations of glucose and glucose degradation products (GDP) have been shown to reduce biocompatibility of glucose-based PDS, because they can cause alterations in peritoneal function (Liberek et al., 1993a; Liberek et al., 1993b; Witowski et al., 1995; Ha et al., 2000; Witowski et al., 2001; Mortier et al., 2004a; Mortier et al., 2004b).

Several studies have suggested that using a more neutral pH and ensuring a lower incidence of GDP can enhance the biocompatibility of the PDS (Topley et al., 1996, Jorres et al., 1998, Fusshoeller et al., 2004, Williams et al., 2004). Some newer commercial dialysis solutions have therefore used a neutral pH, lower GPD level, and bicarbonate or combination of both lactate and bicarbonate as buffering agents.

1.3.2 Osmotic agents (glucose, amino acids and icodextrin)

Glucose or dextrose at variable concentrations is the most often used osmotic agent in PDS. This is because glucose is inexpensive and relatively non toxic. However, a downside is that a considerable amount of glucose is absorbed from the peritoneal cavity leading to unwanted metabolic effects such as obesity and insulin resistance (Holmes and Shockley 2000). An extra glucose load is also problematic for diabetics; diabetes is not uncommon amongst dialysis patients. Also, after long term use glucose

may cause alterations in peritoneal membrane integrity and so compromise the effectiveness of the dialysis exchange functions (Davies et al., 2001; Daugirdas et al., 2001; Nakamoto et al., 2002).

Some studies have suggested using osmotic agents such as glycerol to substitute for glucose in PDS. Glycerol however has a lower molecular weight than glucose and is therefore more easily systemically absorbed, which can in some patients cause lower utrafiltration rates compared to glucose (Smit et al., 2000). In contrast, Van Biesen *et al.* demonstrated that using glycerol is safe and capable of achieving the same ultrafiltration as 2.27 % glucose (Van Biesen et al., 2004).

Amino acids have been used in PDS both as an osmotic agent and to improve the nutritional status in malnourished patients and reduce the metabolic burden of the glucose load. Amino acid solutions do not contain glucose and have a more physiological pH. Chan *et al*. showed that amino acid-based PDS cause less peritoneal membrane irritation than glucose solutions, and that patient protein synthesis was better preserved (Chan et al., 2003)**.**

Icodextrin is a glucose polymer and is used as a colloid osmotic agent. It has proved to be a useful osmotic agent generating sustained ultrafiltration (Peers and Gokal, 1998; Plum et al., 2002). Although it is much more expensive than glucose, several studies have shown that icodextrin improves PD patient fluid status, reduces blood pressure and extracellular water, improves glycemic control and overall quality of PD patient life (Woodrow et al., 2000; Davies & Willams, 2003; Konings et al., 2003 ; Marshall et al., 2003; Guo et al., 2002).

1.2.2.3 Outcomes of Peritoneal Dialysis

Several studies have shown that solute transport and peritoneal surface area increase in parallel with the duration of PD which leads to more rapid glucose absorption and loss of the osmotic effect of the agent, resulting in decrease net ultrafiltration (Heimburger et al., 1999; Smit et al., 2004; Oreopoulos and Rao, 2001). High peritoneal permeability has been considered a risk factor for both technique failure and patient mortality (Davies et al., 1998a; Davies et al., 1998 b; Cueto- Manzano and Correa- Rotter, 2000). Continuous exposure to PDS with high glucose concentrations or high amounts of GDP, hyperosmolality and low pH of PDS may damage the peritoneal membrane (Liberek et al 1993a; Witowski et al., 1995; Witowski et al., 2001; Mortier et al., 2004 a; Mortier et al., 2004b ; Sayarlioglu et al., 2004), leading to loss of peritoneal function.

The low pH of PDS, especially with lactate buffer solutions, can in some cases lead to rapid intraperitoneal acidification of the instilled peritoneal dialysate and suppression of host defence activity (Witowski et al., 1995; Mortier et al., 2004b). Uraemia is associated with chronic low-grade peritoneal inflammation (Prichard, 1999; Jacobs et al., 2004) which may also contribute to the observed alterations in peritoneal membrane structure and function. Neutral pH and osmolality in newer dialysis solutions seem to be beneficial to the maintenance of host defences (Topley et al., 1996; Jorres et al., 1998; Jones et al., 2001; Fusshoeller et al., 2004; Mortier et al., 2004b). Multiple episodes of peritonitis may also damage the peritoneal membrane or gradually change peritoneal function, which can both lead to technique failure (Davies et al., 1996; Piraino et al., 2005).

1.2.2.4 Soluble factors in peritoneal dialysate

Soluble factors in peritoneal dialysate are mainly produced by the peritoneal membrane (Koomen et al., 1994; Zweers et al., 1999). Macrophages, mesothelial cells and fibroblasts released into dialysis fluid are capable of producing various cytokines and growth factors (Ruddle, 1992; Beavis et al., 1997; Lai et al., 1999; Yao et al., 2004a; Yao et al., 2004b). These factors are important to host defence within the peritoneal cavity, but may also reflect occurrence of inflammatory processes in the peritoneum. The include high molecular weight molecules such as hyaluronan and soluble inflammatory mediators such as interleukin-6; both are found at higher levels in the dialysate than in plasma (Koomen et al., 1994; Brauner et al., 1996; Zweers et al., 1999).

1.2.2.5 Small solute kinetics and clearance in peritoneal dialysate

The process of small solute removal is different between the kidneys and peritoneal dialysis. The kidneys work continuously resulting in constant solute clearance and mass removal rates are at steady state. In dialysis, toxins are removed through a single membrane based step. The solute kinetics in dialysis is governed by a relationship between solute clearance and mass removal that is particular to the type of dialysis. The clearance of a small solute like urea depends on the ratio of its mass removal rate and plasma concentration at steady state (Clark et al., 2001).

1.4 Complications associated with peritoneal dialysis

There are several common infection-based and non-infection based complications of peritoneal dialysis (Munib, 2006). These can be divided into three main categories: mechanical, medical and infectious (Swartz, 1985; Cloonan et al., 1990; Piraino., 1997). Mechanical complications include technical aspects of the dialysis such as leakage of dialysate, medical complications such as electrolyte abnormalities, protein-loss associated malnutrition and cardiovascular effects. Infectious complications include peritonitis, tunnel infection and exit site infections. Overall, the two major problems affecting PD are peritonitis and malnutrition secondary to loss of amino acids and protein into the dialysate.

1.4.1 Peritonitis

Peritonitis is the most common clinical problem for PD patients; recurrent episodes can seriously limit peritoneal dialysis effectiveness (Davenport et al., 2009; Keane et al., 2000). Peritonitis causes significant morbidity and occasional mortality (Fielding et al., 2002 Perez Fontan et al., 2005). Peritonitis causes characteristic symptoms of abdominal pain and fever and general malaise, and results in a change in the colour of HPD (cloudiness). It is defined by the presence of more than 100 white blood cell/mm^3 of dialysate (Fielding et al., 2002; Troidle et al., 2003; Chow et al., 2006), of which more than 50% are polymorphonuclear neutrophils (Keane et al., 2000; Vas SI, 1983). There may also be a positive Gram stain result. Peritonitis must be treated rapidly by institution of antimicrobial therapy; normally a 2 week antibiotic course is required (Keane et al., 2000; Peacock et al., 2000).

Staphylococcal species are the most common microorganisms causing peritonitis in PD patients. In particular, Gram-positive skin associated species such as the coagulase-negative *Staphylococcus.epidermidis* followed by coagulase positive staphylococci such as *S. aureus* are the most frequently isolated bacteria in PD peritonitis (Barretti et al., 2009; Finkelstein et al., 2002; Zelenitsky et al., 2002). It has been reported that there is a higher rate of occurrence of peritonitis among diabetics than non diabetics (Lamb et al., 1995)

A number of factors are associated with development of peritonitis, some of which are related to technical issues, others to correct prescription or patient use of antibiotics and nasal carriage of staphylococci (Al-Hweish et al., 2008; Vargemezis et al., 2001). Several studies have shown that staphylococcal nasal carriage rates are higher in hospitals than those in the community (Lowy, 1998; Akoua et al., 2004; Farzana et al., 2008). Interestingly, the presence of depression in the PD patient is also associated with increased risk of infections. Troidle et al. reported that patients with more than one episode of peritonitis were more depressed more than the others with less episode of peritonitis (Troidle et al., 2003; Troidle & Finkelstein., 2006). However, it is not clear whether the depression is a response to the infection, rather than a contribution.

A recent study has also shown that there are other, but more variable risk factors, associated with an increased risk of peritonitis. These include: older age, using a single bag connection system, female gender, smoking and underlying renal disease (Kotsanas et al., 2007).

Mechanically, it has been shown that using a drainage system with an Ultra Y-set and twin bag system has markedly reduced the incidence of peritonitis (Burkart et al., 1990; Kiernan et al., 1995; Huang et al., 2001). The CAPD technique has the highest peritonitis rate among the other PD types followed by CCPD (Diaz-Buxo et al., 2004), which may be a result of multi attach and de-attach activities during the dialysate dwell exchanges (Ahmed, 2009). Development of antimicrobial resistance of microbes in patients experiencing recurrent infectious is an increasing problem. For instance, resistance to vancomycin has shown to be transferable to *S. aureus* (Troidle et al., 2003; Noble et al., 1992).

1.4.2 Protein loss

In terms of metabolic complications of PD, there is strong evidence that a patients protein and nutritional status is a significant predictor of outcome in terms of both long term health and mortality (Ahmed, 2009). Therefore, dialysis patients with any indication of protein malnutrition such as low serum albumin (less than 3.7g/dl), (Kaysen et al., 1984), and low systemic urea concentrations, are monitored and managed carefully (Acchiardo et al., 1983). This suggests that potentially there is a nutritional component that influences patient outcome (Ahmed, 2009).

There are several factors thought to be responsible for a decline in the nutritional status and overall well being of the PD patient. Uraemia causes loss of appetite and is also associated with lower intestinal motility, gastritis and ulceration, all of which affect the food intake and absorption (Bergstrom et al., 1993). Particular to PD, some blood proteins, including metabolically important bound substances, are dialysed out,

potentially contributing to nutritional imbalance (Blumenkrantz et al., 1981; Tjiong et al., 2007; Ahmed, 2009). Also, some factors have been reported that decrease patient food intake such as depression resulting in anorexia (Ahmed, 2009). In addition, some factors that might also increase protein catabolism include effects of inflammatory factors, metabolic disorders or metabolic acidosis, and infections (Ahmed, 2009).

Protein loss has been reported in many PD patient studies with different ranges being recorded (Westra et al., 2007; Miller et al., 1984; Blumenkrantz et al., 1981), The factors responsible for this protein loss have been investigated using different analytical methods and correlated with general characteristics of patients such as age, sex and type of dialysis protocol (Miller et al., 1979; Miller et al., 1981; Miller et al., 1983; Blumenkrantz et al., 1981). In addition to patient related factors, the chemical nature of PDS has also been shown to influence protein loss (Miller et al., 1982; Hammerschmidt et al., 1981).

In one study CAPD patients using five PDS exchanges per day had protein losses that ranged from 7 to 14 g/day (Rubin et al., 1981). In another study the average protein loss was around 8g/day (Blumenkrantz et al., 1981). The major component in the protein lost in the dialysate is albumin, comprising nearly 75% of total protein loss (Rubin et al., 1979; Pereira et al., 2005). Immunoglobulin G (IgG) is next in abundance at nearly 15%, with amino acid losses reported at around 3 to 4 g/day (Pereira et al., 2005). Also, a few first line defence proteins have been reported as being released into HPD such as the iron binding protein transferrin, and the complement defence protein C3 (Holmes et al., 1991; Krediet et al., 1996; Young et al., 1990; Cueto-Manzano et al., 2001). To date,

there has not been a systematic analysis of protein loss in PD patients, and so this is an objective of this project.

1.5 Assessment of peritoneal membrane function

Long-term peritoneal dialysis requires the preservation of the transport function of the peritoneal membrane in order to be successful. Ultrafiltration failure (UFF) remains one of the most common problems responsible for patient drop-out from PD treatment (De Vriese et al., 2001). It has been found that the UFF increases with time spent on PD due to progressive damage of the peritoneal membrane from the PDS, and infection. Peritonitis can significantly affect peritoneal membrane function as it may result in scarring (Davies et al., 1996; Blake et al., 1989). However, several studies have suggested that there is no relationship between long-term peritoneal membrane performance and the incidence of peritonitis (Heimburger et al., 1990; Lo et al., 1994).

Peritoneal membrane function can be monitored (Davies, 2001). The three main approaches to assess membrane function are: the peritoneal equilibration test (PET) (Twardowski er al., 1987; Davies et al., 1993); the standard permeability analysis (SPA) (Pannekeet et al., 1995) and the personal dialysis capacity (PDC) test (Haraldsson, 1995). The PET and SPA are single-dwell procedures using direct measurements, whereas the PDC uses data from several exchanges, performed over a 24- hour period (Davies, 2001).

1.6 Staphylococcus.epidermidis (*S.epidermidis*): Role as Pathogens

S. epidermidis is one of the most frequent organisms causing nosocomial infection, in particular those associated with indwelling medical devices such as catheters (National Nosocomial Infection Surveillance, 2004). This comes from the fact that *S. epidermidis* colonizes the human skin, thereby increasing significantly the probability of catheter contamination during the insertion process (Uckay et al., 2009; Emmett et al., 1975). Biofilm formation by *S.epidermidis* protects them from attack by the host immune system, and from the action of antibiotics (Costerton et al., 1999). Although, *S. epidermidis* infections are not usually life threatening, their frequency and the resistance to eradication from colonised catheters means they represent a serious threat to human health. Understanding *S. epidermidis* behaviour will ultimately drive the development of strategies to reduce the risk of infection and guide the design of targeted therapies (Otto, 2009).

S.epidermidis is the most frequent isolated species from human epithelia, and predominantly colonizes the axillae (armpits), head and nares (nostrils) (Kloos et al., 1975). *S.epidermidis* belongs to the coagulase-negative (C-NS) group of staphylococci. It differs from coagulase-positive staphylococci such as *S. aureus*, due to the lack of the enzyme coagulase; it has also much reduced numbers of virulence factors compared with *S. aureus* (Huebner and Goldmann, 1999).

S. epidermidis infections and the mechanisms by which it promotes human disease have become increasingly studied (Otto, 2009). Once *in vivo S.epidermidis* has to cope with a

number of host immune defence mechanisms. Although, there are a limited subset of host defence mechanisms such as production of antimicrobial peptide (AMPs) and neutrophils (Faurschou and Borregaard, 2003) which are used to kill the bacteria, *S.epidermidis* has evolved several mechanism to evade these attack proteins, as well as evading being ingested and killed by neutrophils.

1.6.1 Virulence Factors

Because *S.epidermidis* and other C-NS were considered non pathogenic for a long time, few of their virulence factors have been determined. With the recent interest in their involvement in nosocomial infection this has now changed and recently there has been increased clarification of the pathogenic properties of *S.epidermidis* and its ability to act as an infectious agent (Longauerova, 2006). A study by Pouramd *et al.* has also shown that many of the cell wall associated proteins of *S.epidermidis* are antigenic and generate a strong antibody response *in* vivo; many of the proteins identified as recognised by the immune system are also present in *S. aureus* (Pourmand *et* al., 2006).

1.6.1.1 Biofilm Formation

Biofilm formation is the most important virulence factor of *S.epidermidis* infections on indwelling devices (Raad et al., 1998; Huebner & Goldmann., 1999; Frebourg et al., 2000; Hanlon et al., 2004). In general, biofilm formation enables persistence of the bacteria on foreign material (Piette & Verschraegen, 2009). Also, as already mentioned,

biofilms protect the bacteria from the action of antibiotics and the immune system (Costerton et al., 1999; Mack et al., 2006).

Two different steps for biofilm formation by *S. epidermidis* have been distinguished (Von Eiff et al., 2001; Vuong & Otto, 2002; Vogel et al., 2000). Primary attachment is the first step of staphylococcal colonization of the polymer material of the foreign body; it might be direct attachment to the plastic surface or binding to host–matrix proteins that have previously coated the polymer material. The second step is intercellular adhesion which means multicellular cell clusters will be formed on top of an initial monolayer of bacteria. Later steps in biofilm formation include cell growth and biomass increase, maturation and formation of structures, senescence, detachment and dispersal to set up new areas of colonisation. Figure 1.6 shows the stages in *S. epidermidis* biofilm formation.

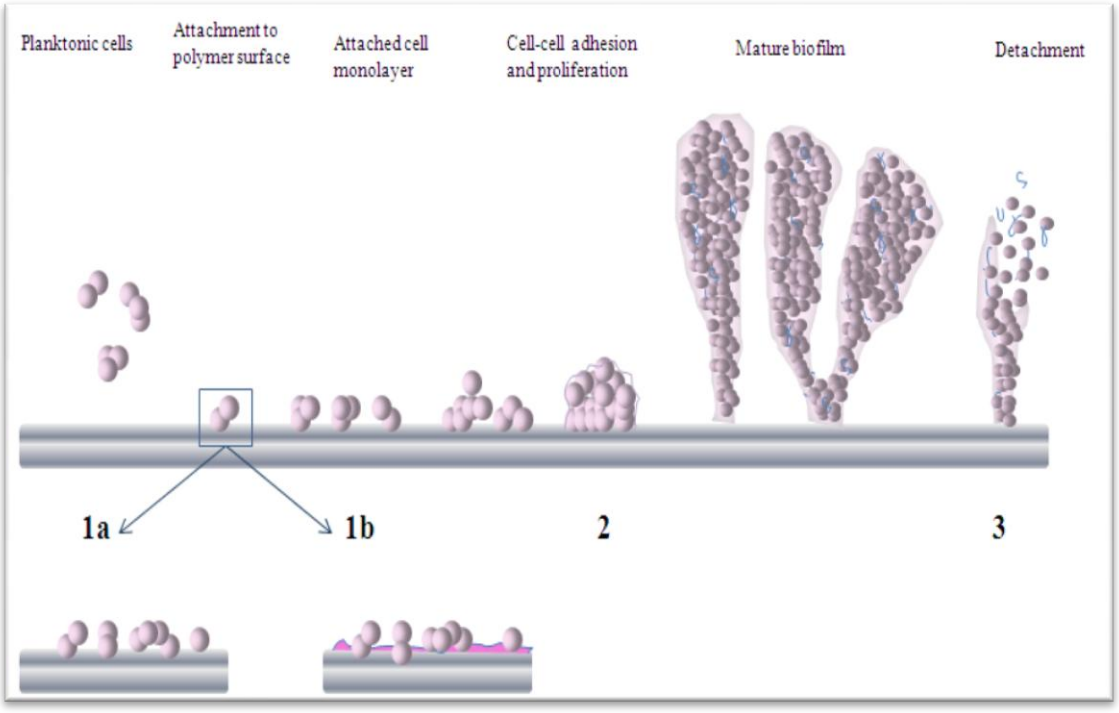

Figure 1.6: *S.epidermidis* biofilm formation. Panel 1a shows initial bacterial attachment to uncoated polymer-surface. Panel.1b shows attachment to a coated polymer surface Panel 2 shows cell-cell adhesion, growth and maturation which may involve formation of characteristic polyp like structures. Panel 3 shows senescence of the biofilm, detachment and dispersal.

Scanning electron microscopy has been used to visualize bacterial biofilm on peritoneal dialysis catheters (Dasgupta & Costerton, 1989). Clinical studies have demonstrated that biofilm formation is associated with peritonitis and catheter-related infection. This is though to be due to the power of biofilm to resist antibiotic treatment (Dasgupta et al., 1986; Dasgupta et al., 2002). Several studies have examined biofilm morphology on PD catheter surfaces (Dasgupta et al., 1986; Swartz et al., 1991; Dasgupta & Costerton, 1989; Verger et al., 1987; Kristinsson et al., 1986). Some researchers have argued that the presence of biofilm plays little role in the development of peritonitis, even if it is common on PD catheters. Dasgupta, 2002 looked at PD catheters removed from patients at various time intervals and was not able to show a clear relationship between the presence of biofilm and peritonitis (Dasgupta, 2002).

1.6.1.2 *S.epidermidis* production of exo-enzymes and toxins

S.epidermidis produces few host cell damaging exo-enzymes. A few toxins which are involved in pathogenicity have been identified and include a metalloprotease (Sloot et al., 1992) and a cysteine protease (Teufel & Gotz, 1993), both of which exhibit elastoyltic activity. Some studies have reported the ability of *S.epidermidis* cell wall proteins to attach to fibrinogen (Fg), fibronectin (Fn), vitronectin, collagen, transferrin and laminin (Bowden et al., 2005; Herrmann et al., 1988; Yu et al., 1994; Modun et al., 1998).

S.epidermidis has been shown to produce a haemolytic activity containing toxin called the detergent–like δ-toxin which causes lysis of erythrocytes and other cell types (Gemmell and Thelestam, 1981) and which has been implicated in the detachment of sessile cells from biofilm to facilitate dissemination of the bacterial community (Vuong et al., 2000). However, while haemolytic activity is possessed by *S. aureus* isolates,

relatively few of the C-NS have been shown to make haemolysins (Huebner & Goldmann., 1999). Although, the majority of known *S. epidermidis* virulence is associated with its ability to form biofilms, there are a few other important factors. Virulence-associated proteins produced by the C-NS are summarized in the Table 1.2 which has been adapted from Otto, 2009.

Virulence-associated proteins	**Function**
Biofilm	**Primary attachment to abiotic surfaces**
AtlE	Autolysin and adhesin, attachment to ploystrene, vitronectin binding
Aae	Bifunctional autolysin and adhesin
Teichoic acid	Affect attachment maybe through the binding of autolysins (Bowden et al., 2005)
Attachment to host matrix proteins	
SdrG (also known as Fbe)	Fibrinogen binding
SdrH	Putative binding function only
SdrF	Collagen binding
AtlE and Aae	Bind to various matrix proteins
Intercellular aggregation	**Intercellular aggregation**
PNAG(also known as PIA) and Bap	An intercellular polysaccharide adhesin
AAP	Accumulation
Exoenzymes	
Lipases	Persistence in host fatty secretions
Cysteine protease	Possibly tissue damage (Dubin et al., 2001)
Metalloprotease	Lipase maturation, AMPs resistance, tissue damage.
GluSE	Degradation of fibrinogen and complement factor C5
FAME	Detoxification of host-produced bactericidal fatty acid
Resistance to AMPs	
SepA protease	Involved in AMP degradation
Dlt,MprF,VraF and VraG	These proteins functions in the D-alanylation of teichoic acid , lysylation of phospholipids and are putative AMP exporters
Aps system	Regulates AMPs resistance mechanism
Toxins	
PSMs	Pro-inflammatory cytolysins
Other factors	
Staphyloferrins	Siderphores (iron acquisition)
SitA, SitB and SitC	An iron importer
FAME	Detoxication of bacteria fatty acid

Table 1.2 Virulence factors of *S.epidermidis*.

1.7 Aims and Objectives of the project

Development of a peritoneal infection in PD patients can have serious consequences, not only short term effects on health, but also the long term efficiency of the entire dialysis process. Despite long term research and active programmes to educate PD patients on the importance of good hygiene, most of those undergoing peritoneal dialysis can expect a peritonitis episode a year. One approach that has not been made is to systematically analyse the dialysate produced by the PD patient for any changes in its effects on the infectivity of the bacteria causing peritoneal infections.

The overall aim of this project is to therefore characterise the changes that occur in peritoneal dialysate that make it supportive of bacterial growth, as well as analysing the effects of hormonal and other factors in HPD on growth and virulence of bacteria most associated with PD patient infections. *S.epidermidis* will be the main microorganism studied as it is a natural member of the human skin microflora and is one of the most frequent causes of peritonitis. As a model organism, it is well understood at the molecular level. Correlation of the effects on *S. epidermidis* protein expression as a result of exposure to HPD fluid and other factors such as stress hormones will be undertaken. Since this will be the first time that a systematic analysis of human HPD has been undertaken, we will also characterise over time (from initial dialysis to the end point of the project -12-18 months) the protein profiles (using SDS-PAGE) of HPD from a selected range of patients. The aim is to determine if protein elimination profiles change with the duration of peritoneal dialysis, in order to determine if biomarkers that

may be prognostic for CAPD efficiency or infection susceptibility can be identified. This may then allow us to develop rapid tests for monitoring CAPD efficiency.

The specific aims of this project are therefore:

- To characterize the protein profile of peritoneal dialysate in patients new to CAPD
- To determine if the protein profile of peritoneal dialysates change over time.
- To study the HPD-associated factors that might increase the risk of infections
- To use proteomic strategies to identify *S. epidermidis* genes whose expression is modulated by stress hormones and HPD
- To study the effects of stress hormones and PDF on expression of virulence genes in *S. epidermidis* including production of biofilms.

Chapter 2.Material and Methods

2.1 Materials

2.1.1 Patients and clinical data

2.1.1.1 Patient HPD Samples

Patients with kidney failure who attended the Nephrology clinic at Leicester General Hospital, and who on clinical advice were receiving peritoneal dialysis, were involved in this study. I would like to thank them for their kindness in donating their peritoneal dialysis samples for this research. Human Peritoneal Dialysate (HPD), on average 100 ml sample volumes were obtained from these CAPD patients, and were used in the experiments in this thesis.

This project started initially with 44 HPD samples from 27 patients and foundation work was performed on these. However, consistent clinical data was only available for 14 of the 27 patients, and so for these patients only a more complete characterization of their HPD was possible. Of the 14 original patients (all of whom were at the start of their CAPD) 5 were involved in a follow up HPD after 12 months dialysis. Table 2.1 and Table 2.2 summarize the main clinical data of the original 14 CAPD patients and the 5 follow up patients. Most of the original 14 have supplied two different dwell; over night (O.N) and daytime (D.T) HPD. Additional clinical data for those patients is attached in the Appendix 3.

Patient ID	Type of Sample	Age	Sex	Diabetes	Taking oral iron
1	D.T , O.N	39	Female	N	N
2	D.T ,O.N	53	Female	Y	N
3	D.T, O.N	57	Female	N	N
4	O.N	67	Male	Y	N
5	D.T	69	Female	N	Y
6	D.T , O.N	60	Male	N	N
7	D.T , O.N	71	Male	N	N
8	D.T , O.N	58	Female	Y	N
9	D.T , O.N	61	Male	N	N
10	D.T , O.N	54	Female	N	N
11	D.T	67	Female	N	N
12	D.T , O.N	69	Female	N	Y
13	D.T , O.N	68	Male	Y	N
14	D.T , O.N	51	Male	N	N

Table 2.1 A summary of the clinical data for the 14 CAPD patients used in this study.

Patient ID	Type of sample	Age	sex	Diabetes	taking oral Fe
1	CCPD	67	Male	Y	N
2	CCPD	58	Female	Y	N
3	D.T , O.N	54	Female	N	N
5	CCPD	68	Male	Y	N
5	D.T , O.N	51	Male	N	N

Table 2.2 Summary of the clinical data for the five 18 month follow up CAPD patients who are the focus of the experiments in Chapter 6.

2.1.1.2 Peritoneal Dialysis samples

2.1.1.2.1 Peritoneal dialysis Solutions (PDS)

There were 3 different types artificial Peritoneal Dialysis solutions used in the majority of experiments in this study. These PDS used a different osmotic agent, at a concentration of 1.36, 2.27 and 3.68 w/v. Fluids were composed of electrolytes (sodium, chloride, calcium, and magnesium); lactate (a buffer); and glucose as osmotic agent, (the commercial name was Baxter -Dianeal PD Peritoneal Dialysis Solution).

Brand of the dialysis fluids

Dianeal PD4 Glucose 1.36% w/v, 13.6 mg/ml

Dianeal PD4 Glucose 2.27% w/v, 22.7 mg/ml

Dianeal PD4 Glucose 3.86% w/v / 38.6 mg/ml

The solute compositions of these PD4dialysis solution are in Table 2.3

	1.36% Glucose (76 mmol/l)	**2.27% Glucose (126 mmol/l)**	**3.86% Glucose (203 mmol/l)**
Na^+ (mmol/L)	132	132	132
Ca^{2+} (mmol/L)	1.75	1.75	1.75
Mg^{2+} (mmol/L)	0.75	0.75	0.75
Cl^- (mmol/L)	102	102	102
Lactate (mmol/L)	35	35	35
Osmolarity (mosmol/L)	350	410	490
pH	5.5	5.5	5.5

Table 2.3 Compositions of the Dialysis Solutions Used

2.1.1.2.2 Human Peritoneal Dialysis (HPD)

Each HPD sample was of around 100 ml volume, and when received was aliquoted to 20 parts, to avoid repeat freeze-thaw deterioration of the HPD proteins and microbial contamination. The aliquots were kept at -20 °C until required. The patient clinical data for these CAPD-HPDs are in Table 2.1 and the data for the follow up 18 month samples in Table 2.2.

2.1.2 Bacterial Strains

Species and Strain Name: *Staphylococcus epidermidis* (Tü3298)

Source: Dr Richard Haigh, Department of Genetics, University of Leicester.

2.1.3 Microbial growth media

S. epidermidis was taken from original glycerol stocks, plated onto a Luria broth **(**LB) plate; plates were maintained at 4ºC.

Luria broth (LB): Luria broth (Bertani, 1951) consists of the following ingredients: 1 % (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl, adjusted to pH 7.0 with 1M NaOH.

Luria agar (LA) consisted of LB solidified with 1.5% (w/v) agar (Difco).

2.1.4 Sterilisation of media

The media used for bacterial culture were sterilised by autoclaving at 121°C and 15 psi for 15 min, and stored at room temperature. Luria agar was melted in the microwave oven, allowed to cool to 50°C and then poured into sterile plastic Petri (approximately 20-25 ml per plate) .The plates were dried at 37°C and stored at 4°C.

2.1.5 Storage and maintenance of bacteria culture strains.

The *S. epidermidis* were overnight in LB at 37°C with shaking and then were mixed 1:1 with 50% ($^{v}/_{v}$) glycerol, mixture stored at - 80°C. The bacterial working cultures were re-cultured from original stocks every 3-4 weeks.

2.1.6 Catecholamines (CAs)

The catecholamines used were purchased from SIGMA, UK: (−) -Epinephrine (+)-bitartrate salt (EPI), E4375; (±)-Epinephrine hydrochloride (EPIHCL), E4642; L-(−) – Norepinephrine salt monohydrate (NE), A9512; dopamine hydrochloride (DOP), H8502.

2.1.7 Proteins

Human transferrin (Siderophilin, partially saturated) (T-3309) was purchased from SIGMA, UK. Peroxidase conjugated ChromPure human transferrin was purchased from Jackson Immuno Research laboratory, Inc, UK.

2.1.8 Antibodies

For transferrin detection, the primary antibody used was anti-human transferrin, developed in goat (T-6265), which was obtained from SIGMA, UK. The cross recognition secondary antibody for transferrin was the anti-goat Immunoglobulins G (IgG) peroxidise conjugated developed in rabbit (A-5420), which was bought from SIGMA, UK.

2.1.9 Protein molecular weight estimation

The following were used to monitor movement of proteins on SDS PAGE gels and to help estimate the molecular weight of proteins with PAGE profiles: Prestained SDS-PAGE Standard, broad range, BIO-RAD, (catalogue numbr161-0318) and the Low Molecular Weight Calibration Kit for SDS Electrophoresis, Amersham, (catalogue number17-0446-01).

2.1.10 Chemicals

All chemicals were purchased from SIGMA, UK, Fisher Scientific, UK, BIO-RAD, UK or GE Healthcare, UK, unless stated otherwise.

2.1.11 Buffers and solutions

- **Polyacrylamide gels**

SDS PAGE: the flowing recipes are for 50ml gel volumes for Resolving gel and 10 ml for Stacking gel.

Resolving gel recipe:

Component	**12 %**	**7%**
H_2O	16.5 ml	24.87 ml
30 % acrylamide mix	20 ml	11.6 ml
1.5 M Tris (pH8.8)	12.5 ml	12.5 ml
10 % Sodium dodecyl sulphate (SDS)	0.05 ml	0.05 ml
10 % ammonium persulphate (APS)	0.05ml	0.05
TEMED (N,N,N',N'- tetramethylethlenediamie)	0.002 ml	0.035 ml

Stacking gel recipe:

Component	10 ml
dH_2O	6.8 ml
30 % acrylamide mix	1.7 ml
1.5 M Tris (pH6.8)	1.25 ml
10 % Sodium dodecyl sulphate (SDS)	0.1 ml
10 % ammonium persulphate (APS)	0.1 ml
TEMED (N,N,N',N'- tetramethylethlenediamie)	0.01 ml

● **SDS PAGE buffers**

1.5M Tris- HCl pH 8.8: 181.71g Tris base was made up to 1 litre with dH_2O, and the pH adjusted to 8.8 with HCl followed by autoclaving for 15 mins at 121ºC.

1M Tris-HCl pH 6.8: 121.14g Tris base were made up to 1 litre dH_2O and pH adjusted to pH 6.8 using HCl followed by autoclaving for 15 mins at 121ºC.

1M Tris-HCl pH 7.5: 121.14g Tris base were made up to l litre dH2O, the pH adjust to 7.5 with HCl followed by autoclaving for 15 mins at 121ºC.

10 % SDS: 100 g dissolved in 1l of dH_2O.

5M NaCl: 292.2 g dissolved in 1l of dH_2O.

5x SDS-PAGE running buffer: 15 g Tris base, 72 g glycine, 5 g SDS was dissolved in 1l of dH_2O. For use in experiments, the concentrate was diluted 1:5 with dH_2O.

Sample loading buffer (SB): 4ml dH_2O, 1ml 0.5 M Tris-HCL pH 6.8, 0.8 ml glycerol, 1.6 ml 10% ($^{v}/_{v}$) SDS, 0.4ml 0.05% ($^{v}/_{v}$) dithiothreitol (DTT), and 0.2 ml 0.05 % ($^{w}/_{v}$) bromophenol blue.

● **SDS-PAGE Gel Staining**

SDS-PAGE Gel Coomassie Blue staining solution: 0.1 % ($^{w}/_{v}$) Coomassie brilliant blue R250 dissolved in 40 % ($^{v}/_{v}$) methanol, 10 % ($^{v}/_{v}$) glacial acetic acid.

SDS-PAGE Gel Coomassie Blue De-Stain: 25% ($^{v}/_{v}$) isopropanol, 10% ($^{v}/_{v}$) glacial acetic acid.

SDS-PAGE Gel Silver staining protocol

Fixation solution: 40% ($^{v}/_{v}$) methanol and 10% ($^{v}/_{v}$) glacial acetic acid.

Oxidative solution: this was prepared according to the manufactor's instructions (BIO-RAD Silver Stain kit Ccatalogue number 161-0443). The oxidative stock is a 10-fold concentrate containing potassium dichromate and nitric acid, which has to be diluted 10% ($^{v}/_{v}$) in deionized distilled H_2O.

Silver Complex Solution: this is also supplied as a 10-fold stock solution which contains silver nitrate, and also needs to be diluted to 10% ($^{v}/_{v}$) in deionized distilled H_2O

Development Accelerator Solution: This was made at the manufacturer; recommended stock of 32grams of developer per l of deionized distilled H_2O.

Stopping Solution: 5% acetic acid ($^{v}/_{v}$).

SDS-PAGE Gel staining of proteins intended for sequencing

SDS PAGE gels were fixed, washed in water and then stained using the ready to use Calbiochem RAPIDstain Reagent, according to the manufacturer's instructions (Calbiochem, UK, catalogue number 553215).

Western Blotting buffer

For transfer of proteins from gel to membranes, 5.9 g Tris, 2.9 g glycine, 100 ml methanol, 3.4 ml 10 % SDS was added dH_2O up to 1l. This buffer was cooled on ice prior to use.

Western blot blocking buffer: 5 % **($^{w}/_{v}$)** BSA in western blot wash buffer. This buffer was freshly made and cooled on ice prior to use.

Western blot wash buffer: 150 mM NaCl, 10 mM Tris-HCl pH7.5, with 0.05 % ($^{v}/_{v}$) Tween-20.

2.2 Methods

All experiments were performed at least in triplicate unless stated otherwise.

2.2.1 Proteomic Methods

2.2.1.1 *Staphylococcus* protein fractions

The method used for Staphylococcal protein fractionation (membrain-associated, membrane and cytoplasmic) was based on protocols used by other researchers (Cheung and Fischetti, 1988). Overnight *Staphylococcus* cultures were diluted 1:100 into either LB, PDS or HPD and then grown statically in a humidified CO_2 incubator set at 37 º C for a further 18-24 hr. Bacterial culturals were harvested by centrifugation at 3000 rpm for 10 minutes at 4ºC, washed twice in two buffer depending on the cell protein fraction needed. Phosphate buffer saline (PBS) was used to extract whole cell associated proteins or 10mM Tris-HCl, pH 7.5 buffer was used for the other protein fractions. The bacterial cell pellets were then re-suspended in 500µl of lysis buffer (30% raffinose in 50 mM Tris, pH 7.5, 150 mM NaCl) containing 80 µg/ml lysostaphin) then incubated for 1 h at 37ºC with gentle shaking followed by centrifugation at 13,000 rpm for 10 minutes. To prepare membrane proteins, protoplasts were mixed with PBS, cooled on ice for 10 minutes, lysed by sonication for a total of 1-2 minutes (in 15 second sonication cycles) set at 6-8 microns using a fine sonicator probe, and centrifuged at 13,000 rpm for 10 minutes. Cytoplasmic proteins are contained within the supernatant fraction, and total membrane proteins within the pellet.

2.2.1.2 Determination of the protein concentration of samples

The concentration of the protein in peritoneal fluids and the staphylococcal protein fractions prepared in section 2.2.1.1 were determined using the BIO-RAD protein Assay kit. The kit was supplied with Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) at a known concentration as standard protein. This assay is based on the Bradford method of protein concentration measurement (Bradford, 1976), when the Coomassie Brilliant Blue stain is added to the protein samples and the absorbance measured using spectrometer or microplate reader set to 595 nm (Reisner et al., 1975, Fazekes De St. Groth et al., 1963, Sedmack and Grossberg, 1977). Different concentrations of BSA were made up in 800µl distilled H_2O and 200µl of the dye was added to make up the total volume to 1000µl to create a standard curve. The dye was thoroughly mixed to the protein in the test solution and the mixture was left for 15 minutes before taking the absorbance reading at 595nm. The protein samples of unknown concentration were estimated by comparison to the standard BSA curve which is plotted as absorbance against the known concentration. The samples final concentrations were multiplied by the dilution factor to give the concentration/ml.

2.2.1.3 Protein concentration and preparation for SDS PAGE.

The staphyloccoal protein fractions samples and HPD samples were when necessary concentrated by lyophilisation (using a ModulyoD free dryer, Thermo) and then diluted to the test concentration required. For SDSPAGE analysis, or Western blotting, proteins in the samples were denatured by the addition of 2X SB at ratio 1:2 followed by boiling

at 95 ºC for 10 minutes. The protein samples were then centrifuged at 10,000 rpm for 10 minutes to remove any precipated cell or protein debris and the supernatant then directly loaded on to different percentages (7% and 12%) SDS-PAGE gel, as was required. The protein samples were standardised with the co-adding of 10 µl of the protein molecular weight markers (203,115,93,48,34,28,21,7 kDa range)(BIO-RAD) which had been heated at 95ºC for 5 minutes.

2.2.1.4 SDS-PAGE gel Preparation

SDS-PAGE acrylamide gels were used for protein separation and analysis. Mini gels (8.6 cm x 6.8 cm) were used for initial investigations and larger format maxi gels (20 cm x 18 cm) were used for profile comparisons and for protein sequence identification. Two different percentage gel formulations (7 % and 12 % acrylamide) were routinely used, and for analysis of very low molecular weight proteins, a 15 % gel was sometimes used. All the SDS-PAGE gel were prepared as described in Sambrook *et al.* (Sambrook et al., 1989) which is consist of two gels, the resolving and the stacking gels. Firstly, the gel cast equipment was assembled very securely to prevent the gel leakage (checked with nano pure H_2O). The resolving gel was then prepared in a 20 ml universal tube or 50ml falcon tube depending on the volume needed by adding the reagents in the order recommended by Sambrook *et al*. (Sambrook et al., 1989) .The resolving gel was carefully poured into the separating gel plates leaving at least 2 cm below the top of the small plate rim. Nano pure H_2O was then carefully added on the top of the gel before it polymerised to avoid drying of the gel surface, to allow the stacker to bind more evenly, and to help straighten the surface of the gel; this water was removed just before the addition of the stacking gel. The resolving gel was left for at least 20 minutes for

polymerization and in the meantime, the stacking gel was prepared, and then poured on the top of the resolving gel. Very quickly after this the sample well combs were pushed into place, taking care not to trap any air bubble under the teeth of the comb. The whole SDS-PAGE gel was left to settle down at least 1 hour before loading the samples to allow complete polymerisation.

2.2.1.5 SDS-PAGE electrophoresis

Protein samples were loaded onto appropriate SDS-PAGE percentage gels and separated using a BIO-RAD ready gel system for mini gels or Large Format Gel Electrophoresis unit (BIO-RAD) at a constant 25mA/gel for approximately 1-2 hours (depending on percentage acrylamide) or at 50 mA per large format gel for approximately 6 hours. SDS-PAGE gels were all run in 1x SDS-PAGE running buffer. After electrophoresis, the gels were then rinsed and washed in water, and prepared for staining or western blotting.

2.2.1.6 Western blotting

Western blotting followed by immuno-analysis was used to check for the presence of transferrin either in HPDs or bound to the bacteria. Protein samples were separated using SDS-PAGE gel electrophoresis and then transferred in western blotting buffer onto membranes, either polyvinylidene fluoride (PVDF, Immobilon-P, Millipore, UK catalogue number IPVH00010) or nitrocellulose (Trans-Blot transfer medium, Bio-

RAD, UK, catalogue number 162-0097) using a Trans-Blot electrophoresis western transfer cell (BIO-RAD, UK) at constant current of 250 mA for 1 hour. The blotted membranes were washed in water to remove the transfer buffer, and blocked in blocking buffer at 4ºC overnight. Next day the membrane was washed three times using wash buffer for 10 minutes with continuous shaking and incubated in 1µg/ml of anti-human transferrin developed in goat 1:5000 with blocking buffer for 2 hours at RT on a shaker. The blot was removed from the antibody-blocking buffer, and in a separate box washed three times in wash buffer to remove non-binding antibody. The blot was then incubated in the anti-goat Immunoglobulins G (IgG) peroxidise conjugated developed in rabbit at 1:20,000 dilution in blocking buffer for 1 hour at RT on the shaker. Finally, the blot was washed three times, twice in washing buffer and once in washing buffer minus the tween detergent, and then developed using the ECL+plus Western Blotting Detection System (Amersham GE Healthcare, UK, catalogue number RPN2132) . The treated blot was visualised using an x-ray film (Amersham Hyperfilm ECL, GE Healthcare 28906837) as recommended (Sambrook et al., 1989).

2.2.1.7 Gel Staining

SDS-PAGE gels were stained for visualisation of protein profiles and for sequencing of proteins. Visualisation staining for protein profiles was performed using the Coomassie blue or silver stain while the Rapid gel stain was used for gels used for proteins to be sequenced (RAPID stain Reagent, Calbiochem, catalogue number 553215). Coomassie blue staining was done by placing the gel in large 90 mm diameter Petri dish, then covering it with Coomassie Blue staining solution for 1 hour. The stained gel was then washed several times with nano-pure H_2O and placed in Coomassie Blue destain

solution for at least 1 hour, and the destain replaced until the gel background was totally cleared. Silver staining was used to visualize gels with very low amount of protein concentration as it is sensitive to around 1ng/band of protein while the Coomassie sensitivity to detect protein is about 30-100ng/ protein band. SDS-PAGE gels for silver staining were handled very carefully with gloves as finger prints cause artefactual staining. The gel was fixed with the fixation solution for 30 minutes for small format gels and 60 minutes for the large format; the gel was washed many times with deionized water before using Silver stain reagent for 15 minutes for small gel and 30 minutes for large format gel. The stained gel was then washed once in water, and then developed using the manufacturer's developing solution (this solution can be stored, but should be changed when its colour turns to yellow or a smokey precipitate appears). The development of protein band colour was allowed to reach the desired intensity in relation to background, and the reaction stopped using 5 % acetic acid.

2.2.1.8 Protein sequencing

The HPD samples were run on 7 and 12 % SDS-PAGE gels using a normalised concentration of 450µg of total protein in order to better compare the protein profile between different HPD dwells (O.N and D.T) and between different patients. After electrophoresis the gel was washed with nano-pure H_2O and then stained using the RAPIDstain reagent. The gel was covered with the stain for 1 h until the protein bands become visible, and then rinsed with nano-pure H_2O and stored until required. The protein bands were sequenced using an Applied Biosystems 477 sequencer, with the

kind help of Dr Andrew Bottrill of the University of Leicester; Protein Nucleic Acid Chemistry (PNACL).

2.2.1.9 Storage of Stained Gels

After staining was complete, the SDS PAGE gels were stored in sterile Petri dishes in dionized H_2O until required. Also, gels may be dried in a manual gel drier. In case of silver stained gels, to prevent continuation of the development and very high backgrounds, the acid stop solution needs to be changed at least two times to remove all of the developer solution before drying the gel.

2.2.1.10 Photographing Gels

SDS-PAGE gels and the western blot films were scanned and the bands were quantitatively analysed using an Imaging Densitometer connected to the GS-710 Quantity one software (BIO-RAD, UK).

2.2.2 Peritoneal dialysis characterization Methods

2.2.2.1 Measuring pH

The pH values of the CAPD peritoneal dialysis fluids were checked using a pH meter, which had been calibrated previously according to manufactures instructions using two buffers (pH 7 and 10). The pH probe was placed in an adequate amount of PDF and the measurement made at least three times; the mean was taken as the pH value of the HPD.

2.2.2.2 Measuring glucose levels of the HPD.

2.2.2.2.1 Glucose test strip assay

The glucose of the HPD fluids was determined using a DIBUR-TEST 5000 glucose dip stick test kit (Roche Diagnostic GmbH, catalogue number D-68298). This is a test based on a glucose oxidase-peroxidase reaction and is a semi quantitative test for glucose in urine up to 5000 mg/dl (278mmol/1, 5%). This kit is often used by patients, and so its utility was investigated. The kit was used according to test instructions. The test strip were immersed in the HPD sample for 1 second, the edge has been wiped against the rim of the specimen container to remove the excess sample fluid. The glucose reading was then taken after 2 minutes waiting by comparing the test strip colour to the calibration colour scheme chart on the label of the kit (see Figure 2.1) .The test accuracy is ≥ 75 % according to the manufacturer's literature.

Figure 2.1 Test strip assay of HPD glucose levels using the DIBUR-TEST 5000 Glucose measurement kit

2.2.2.2.2 Glucose (HK) Assay Kit

A more sensitive and quantitative assay of HPD glucose levels was carried out using the Glucose Hexokinase Assay Kit (SIGMA, UK). The PDF samples needed to be diluted with deionized water to 0.05-5 mg of glucose/ml to be within the concentration range of the kit. The Glucose Assay Reagent was add to the HPD test tube, then mixed together, and the reaction allowed to develop for 15 minutes at room temperature. The absorbance was measured at 340nm using a Varioskan spectrophotometer. Formulae to convert A340nm readings to concentration values were done using the formula provided by the Kit protocol.

2.2.2.3 ELISA measurement of transferrin levels in HPD

Transferrin was determined quantitatively using a commercial Assay Max Human Transferrin ELISA Kit (ASSAYPRO, catalogue number ET3105-1). This kit is a competitive sandwich enzyme immunoassay technique used to measure human serum transferrin in less than 2 hours at room temperature (20-30 ºC). The assay was performed as follows: HPD samples were diluted in the kit's Mix Diluent to be within the detection range of the assay; 25 µl of the provided positive control (Human Transferrin Standard) or HPD dilutions were add to the kit's 96 well polystyrene microtitre plate which was coated previously with polyclonal antibody against human transferrin. Biotinylated transferrin antibodies (1:100 dilution in Mix Diluents) were add to the samples directly and the plate gently mixed on a circulating rocker platform. The assay plate was incubated for one hour and washed 5 times with 200 µl of the washing buffer provided by the kit (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) by inverting the plate and sharply decanting the contents 4-5 times on to a paper towel to completely remove any excess solution. A Streptavidin–peroxidase conjugate (1:1000 dilution) (50 µl) was then added to each well and the plate was reincubated for 30 minutes. After removing the conjugate, and washing the plate, a Peroxidase chromogen Substrate (50 µl) was added according to the kit instructions, and the plate was incubated at room temperature for 10 minutes until colour development was optimal. The reaction (colour development) was stopped by the addition of H_2SO_4; the absorbance was read using Elisa reader (Dynatech) at 450nm.The curve of the standard absorbance reading of transferrin was plotted, the HPD sample's transferrin concentration were calculated by comparison to this standard curve.

2.2.2.4 Trace metal analysis

Duplicate 2 ml aliquots of PDS and HPD solutions were frozen at -80^{o}C and lyophilised using a freeze dryer until dry. The dry samples were suspended in 200µl of nano-pure H_2O. The samples were processed for Fe levels by adding 1 ml of concentrated HNO_3 acid to each sample; the samples were rinsed out into a new centrifuge tube using distilled H_2O and made up to 5 ml final volume. The treated samples were then left overnight, centrifuged for 15 minutes at 300 rpm, and the trace metal content analysed using Inductively-Coupled Plasma Optical Emission Spectrometry (on a JY Ultima 2 ICP-OES), at the Department of Geology, University of Leicester.

2.2.2.5 Cateholamine content of HPD samples

Aliquots of 10 ml of each patient HPD were divided to 2 tubes. After checking the pH, one of the tubes was acidified to pH to 2 using concentrated HCl or H_2SO_4. The HPD samples were sent to Nephrology unit at the Leicester General hospital to carry out catecholamine and catecholamine metabolite analysis using High Performance liquid chromatography (HPLC). Internal standards consisted of the usual renal excreted catecholamines.

2.2.3 Microbiology Methods

2.2.3.1 Staphylococcal growth

2.2.3.1.1. Responses to PDS growth with and without Fe and CAs.

Starter inoculums of *S. epidermidis* (Tü3298) were grown overnight in LB in a shaking incubator (180 rpm) set at 37^{o}C. Dilutions of this inoculum were add to different HPD or PDS without any additions (control); or with Fe (in the form of ferric nitrate) (100µM), and 100µM each of the catecholamines (NE, DO or EPI). The control and supplemented test cultures were grown statically in CO_2 incubator at 37 ^{o}C for 18 hours. After incubation, dilutions of the well mixed cultures were plated onto LA to be counted (CFU/ml) for viable density counts. Alternately, optical density (O.D600) readings of the cultures at 600 nm were taken using a spectrophotometer.

2.2.3.1.2 Analysis of the ability of PDS and HPD fluids to support staphylococcal growth

Inoculums of *S. epidermidis* were grown overnight in shaking incubator at 37°C. A 1:100 dilution of the inoculums growth was adds to different PDS or HPD samples with and without Fe (100µM) and 100µM of the Catecholamine (NE, DO or EPI). The O.D at 600 nm was taken after 18 hours incubation.

2.2.3.1.3 Time course of staphylococcal growth in PDS and HPD

An inoculum of *S. epidermidis* was grown overnight in shaking incubator at 37°C. A 1:100 dilution of the inoculum was added to the PDS and HPD in 150 µl triplicate volumes in a 96 well plate. Incubation of the cultures was done in a Varioskan spectrophotometer, set at 37°C and shaking at 180 rpm. The growth (increases in optical density at 600nm) was monitored for 24 hours, with readings taken every 15 minutes. The time points were plotted in Excel 2007 or Graph-Pad prism.

2.2.3.1.4 *S. epidermidis* binding to Tf present within HPD

S. epidermidis was grown for 18 hours at 37°C, in HPD of different CAPD dwells (O.N and D.T). The viable count of the culture was made, and the bacterial isolated by centrifugation at 3000 rpm for 10 minutes. Bacterial cell pellets were washed at least three times with PBS followed by re-centrifugation at 13000 rpm for 10 minutes. The washed cell pellets were resuspended in 500 µl PBS, and proteins binding to the bacteria released by the addition of 2X SDS sample buffer and the mixture boiled for 10 minutes. After centrifugation at 13000 rpm for 10 minutes, the supernatant was removed, and the samples run on a 12% SDS-PAGE gel and western blotted as described in section 2.2.1.6.

2.2.3.2 Staphylococcus virulence factors

2.2.3.2.1 Haemolyic activity

Haemolytic activity was examined by adapting slightly the method of Haigh et al., 1994. Briefly, the secreted protein and cell wall protein fractions (section 2.2.1.1) of *S. epidermidis* were prepared from bacteria grown in LB, and HPD. Serial two-fold dilutions of 50µl of the cell extract to be analysed were made in a round bottom 96 well microtitre plate. Erythrocytes obtained from sheep blood were prepared by centrifugation at 4000 rpm for 10 minutes, and the pellet re-suspended in PBS to give a 4 % suspension. Of this suspension, 50µl was added to each bacterial protein dilution well. The plate was incubated at 37 °C for 30 minutes; the result was taken as the reciprocal of the highest dilution causing 50% haemolysis of the erythrocytes.

2.2.3.2.2 Biofilm production in PDS and HPD

The ability of *S. epidermidis* to form biofilms *in vitro* was studied using the crystal violet attachment method described in Tendolkar *et al*. (Tendolkar et al., 2004). Briefly, *S. epidermidis* was grown overnight at 37 °C in LB. The cells were pelleted, re-suspended in fresh medium (PDS or HPD alone or with supplements), and normalized to an absorbance of 1.00 at 600 nm. The cultures were diluted 1:40 into PDS or HPD alone or with supplements, and 200 µL of cells dispensed into the wells of a flat-bottom polystyrene 96 well microtiter plate. After 24 hour of static incubation at 37 °C in a CO_2 5% carbon dioxide (CO_2) incubator, the culture supernatants containing planktonic bacteria were washed, and the wells washed 3 times with PBS and air-dried at room

temperature. Two hundred microliters of 0.2% crystal violet was added to each well, and after an incubation of 15 min, the wells were washed 3 times with PBS. Crystal violet bound to the bacterial biofilm attached to the well was extracted with 200 μL of an 80:20 mixture of ethanol and acetone. The absorbance of the extracted crystal violet was measured at 595 nm. All biofilm assays were run in 3 replicates and repeated twice.

The ability of *S. epidermidis* to form biofilms on plastic catheters relevant to CAPD patients was examined as well, using sterile 0.5 cm sections of a Tenckhoff catheter (kindly provided by Dr. Johanthan Barratt, Leicester General Hospital). The attachment of bacteria in O.N and D.T HPD was investigated, as was the addition of stress hormones. Catheter-bacteria cultures were grown statically for 48 hours in 24 well plates in a CO_2 incubator at 37 ^{o}C. Biofilm attachment was visualised using light microscopy.

2.2.4 Statistical analysis

Statistical analysis was performed for all measured parameters using a GraphPad Prism version 5 for windows (GraphPad Software, San Diego California USA). Results are presented as mean ± standard deviation (SD). One-sample t-test and Wilcoxon test has been used to compare one group to a hypothetical value (P value). Also, t test for unpaired group and Mann-Whitney test were used to compare two unpaired groups while one-way ANOVA analysis used to compare three or more groups. All P values below 0.05 were considered statistically significant.

Chapter 3.Characterization of the protein content of CAPD dialysates

3.1 Introduction

The dialysis fluids used for peritoneal dialysis are sterile and have been designed for intraperitoneal administration only. The most commonly used fluids are composed of electrolytes (sodium, chloride, calcium, and magnesium); lactate (a buffer to stablise dialysis fluid pH); and glucose, the osmotic agent, at a concentration of 1.36, 2.27, or 3.68 w/v (Dianeal PD Peritoneal Dialysis Solution manufacturer). While sterile, the dialysis fluids do not contain any specific bacteriostatic agents. In terms of routine practice, most patients use four 2 litre exchanges of 1.36 % glucose.

The transport of host molecules across the peritoneal membrane into the peritoneal dialysis fluid varies between different patients, and also within an individual patient over time (Oreopoulos & Rao, 2001). Along with water, host waste compounds and a variety of proteins enter the peritoneal dialysate and are lost following each dialysis exchange. Such proteins would not normally be lost in the urine in health (Emmanuel et al., 1981; Gail, 1989). Not surprisingly then, studies have established a relationship between peritoneal dialysis dwell, protein loss and malnutrition. The inflammatory and damaging effect of glucose on the integrity of the peritoneal membrane is thought to increase this protein loss **(**De Mutsert et al., 2009; Holmes & Lewis, 1991; Lewis& Holmes, 1991; Coronel et al., 2009). Few studies have investigated and characterized over time the profile of proteins released into the dialysis fluids of CAPD patients (Lin et al., 2008 ; Wang et al., 2010).

The aim of this chapter is therefore to characterize the protein profiles in the HPD of different CAPD patients and to see whether any differences exist between proteins present within an individual patient's samples, and between different groups of patients.

Peritoneal fluids from CAPD patients were characterized by proteomics methods. Tests included 1D SDS-PAGE of HPD samples, followed by protein sequencing, comparative protein densitometry and where appropriate immunoanalytical quantification of specific proteins.

3.2 Results

3.2.1 Patients and clinical data analysis

3.2.1.1 HPD dwell distribution

As already mentioned (Chapter 2, Materials and Methods), the HPD samples in this study have all been obtained from Leicester General Hospital patients with renal failure. This is a prospective study of patients commencing peritoneal dialysis at the John Walls Renal Unit, who were PD naïve, and agreed to follow-up samples of PD fluid being collected during the duration of the study. Of the patient cohort, a complete set of clinical data (age/drug usage etc) and follow-up PD fluid 1 year following commencement of CAPD was available for 14 patients (representing 25 different dwell samples). The common reasons for failure to obtain follow-up PD samples were transfer of clinical care, transfer to haemodialysis, kidney transplantation and patient death. In each case we collected aliquots of the overnight (O.N) and first morning (D.T) PD dwells. Universally, new patients to CAPD were commenced on 1.36 exchanges and therefore all first PD samples, both D.T and O.N were 1.36 exchanges. Figure 3.1

shows the distribution of the types of dwell samples used in this chapter. In a number of instances patients failed to deliver their O.N PD exchange and we were left with only the D.T dwell hence the excess of D.T dwells included in this analysis.

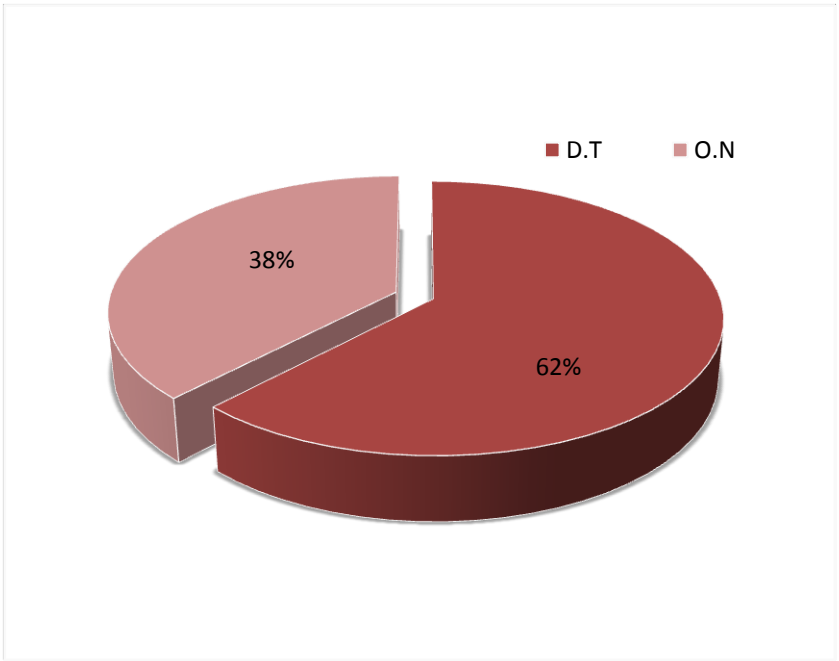

Figure 3.1: Distribution of the CAPD dwell samples studied in this chapter (37 samples). D.T and O.N represent daytime and over night HPD samples.

3.2.1.2 Clinical data on the CAPD patients involved in this study.

In this proteomics study the proteins within the HPD of 14 CAPD patients (8 female and 6 male) with a mean (± SD) age of 60.29 ± 9 years were investigated; their clinical information is presented in Table 3.1. In terms of pre-existing health conditions, 4/14 of the patients had diabetes while 6 patients were hypertensive, taking either beta blocker drugs or (the other 5 patients) alpha blockers. All of the patients in Table 3.1 were

dialyzed using the standard peritoneal fluid composition containing 1.36mmol glucose/L.

Clinical Parameters	Patient numbers
No of patients	14
Age	60.29 ± 9
Gender (F/M)	8/6
Diabetes/non diabetes	4/10
Taking oral iron/not	2/12
Taking beta blocker/not	6/8
Taking alpha blocker/not	5/9

Table 3.1 Relevant clinical data on the patients involved in this study.

3.2.2 Comparative analysis of peritoneal protein loss in CAPD patients

The protein concentration of the HPD samples (D.T and O.N dwells) from the 27 patients of the cohort (37 dwell) was determined using the Bradford protein concentration assay (Chapter 2, section 2.2.1.2). The total protein per HPD dwell was calculated, and the variation between patients and comparison between D.T and O.N loss is shown in Figures 3.2 and 3.3, panel A respectively. The two sets of data show that both daytime and overnight HPD fluid contained substantial amounts of protein, approximately 2.58 g/2l for O.N fluid samples (n=14 dwells) and 0.61 g/2l D.T (n=23

dwells). Figure 3.2 shows that there was a significant variation in protein loss between individual HPD samples. Figure 3.3, panel A shows that there was a significant increase in the total amount of protein released into the HPD during longer O.N dwell times compared with the shorter D.T dwell. Assuming an average of 4 D.T dwell changes and 1 O.N dwell, patients were losing on average (4x 0.61) + (1 x 2.58) = 5 g of protein per day. As stated in Table 3.1, 4/10 of the CAPD patients in this study were diabetic, and so a comparison was made of protein loss from diabetics versus non-diabetics (Figure 3.3, panel B).

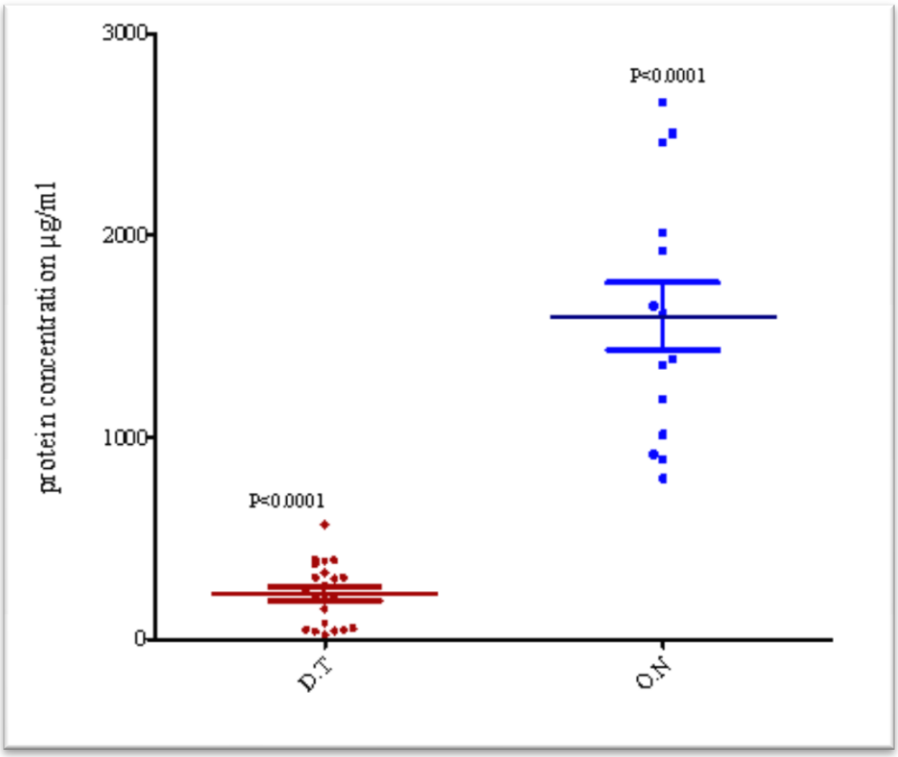

Figure 3.2 Protein concentration variations between HPD samples. The scatter plot shows the variation in protein concentrations of CAPD patient HPDs taken from daytime (D.T) and overnight (O.N) dwells.

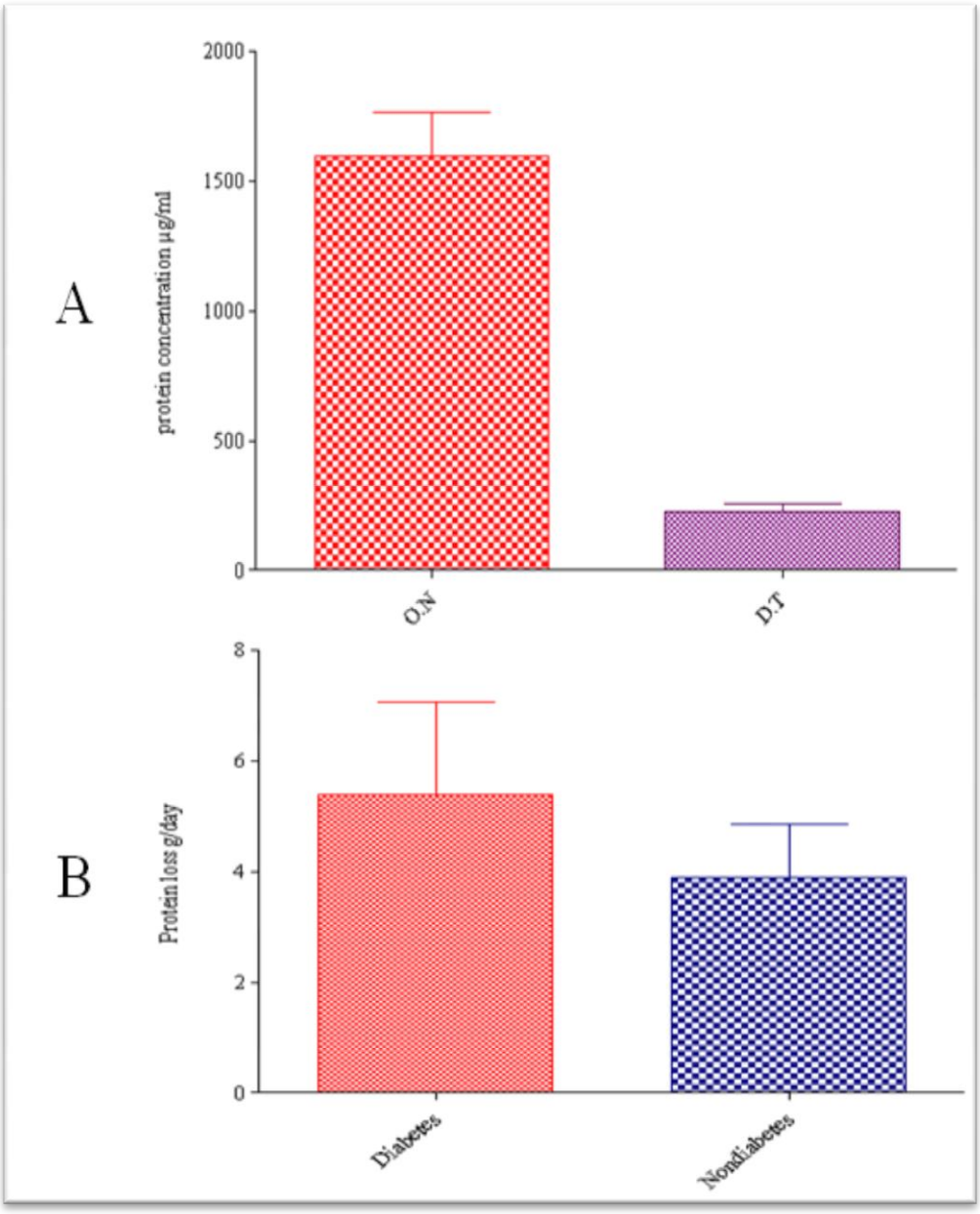

Figure 3.3 Histograms comparing total protein levels of overnight vs. daytime and HPDs, and diabetic vs. non-dabetics. Panel A, O.N, D.T: overnight and daytime HPDs: Panel B, comparison of total HPD protein loss in diabetics and non-diabetic CAPD patients.

3.2.3 Characterization of the proteins lost by CAPD patients

3.2.3.1 Analysis of the protein profile of CAPD patients

Protein profiles for all patients were initially determined using 7 and 12 % acrylamide 1-D SDS-PAGE gels. The different percentage gels were used to profile high and low molecular weight proteins, as most of the proteins in CAPD dialysates will be drawn from the blood. Also, initially, for all the samples analysed a range of volumes (5-20 µl) of dwell fluid were run out on gels in order to visualise low and high abundant proteins. It was found that 20 µl loaded onto 12% gels was a good compromise in terms of sample volume and protein profile. The next three Figures (Figure 3.4 a, b and c) show 12% SDS-PAGE gels for daytime and overnight HPDs for 14 patients using the same volume loaded per lane (20 µl). The protein bands on these gels showed the presence of proteins with molecular weights of between 100 kDa and around 20 kDa. In terms of HPD distribution, 11 patients had 2 dwell (overnight and daytime), and the remainder were one dwell only.

The protein profiles in Figure 3.4 show the daytime (A) and overnight dwell (B) protein profiles. The figure shows that the abundance of proteins and the number of protein bands depend on the dwell time and total amount of protein in the dwell sample. For each patient, the protein profile pattern was usually different between O.N and D.T HPDs. Differences were also seen between CAPD patients as well (Figure 3.4). All O.N dialysis samples of the patients had more protein(s) than the four hour (D.T) dwell.

Although all O.N samples had the highest protein concentration in all patients, the protein bands distribution was very variable. In Figure 3.4a, the O.N dwell of patient no 1 has the highest protein concentration at 1170µg/ml of all the samples analysed; this patient also had more proteins in his HPD than the dwells from the other patients. Figure 3.4b, shows the lowest D.T HPD concentration at 14µg/ml (patient 8). A comparison of the gels shows some similarity in the patterns of protein profiles between patients when using a similar volume of HPD per lane. However, some protein bands appear to be more prevalent than others (for instance, the protein of 68 kDa). Although the protein profiles as determined by relative migration distance of the protein molecular marker were different between all D.T and O.N samples, they all showed the presence of the 68 kDa protein. A comparison of all the main proteins within the HPDs is shown in Figure 3.4.

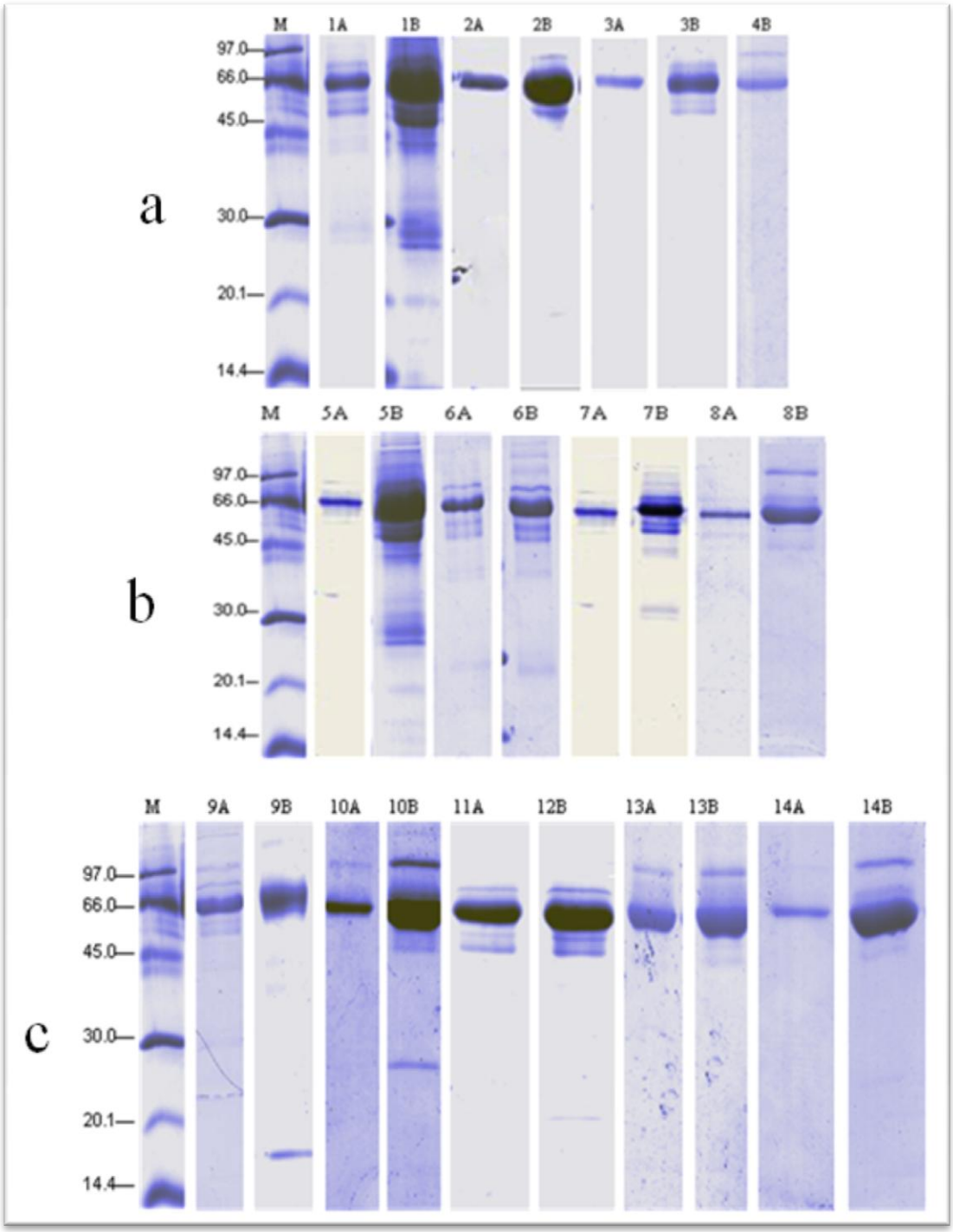

Figure 3.4 A, B and C. Protein profiles of CAPD patients. A, B and C show 12 % SDS-PAGE gels for the proteins from HPD from 14 patients with two CAPD dwells (13 D.T and 12 O.N). A constant volume of 20 µl was loaded per lane. Key: M, molecular weight markers; A, proteins in patient D.T dwell; B, proteins in patient O.N dwell.

3.2.3.2 Comparison of HPD proteins from different patients

As a consequence of the protein profile differences between CAPD patients, a comparison SDS PAGE gel was run using a fixed concentration of 450 µg total protein per lane. Because the CAPD patient's samples varied considerably in protein concentration, due to differences in the dwell time and individuals peritoneal membrane permeability, it was necessary to lyophilise the HPD; protein concentrations of the concentrated HPDs were determined using the Bradford concentration assay. HPD samples requiring lyophilisation were diluted to the original concentration and run side by side on SDS PAGE gels with the original HPD to confirm that there had not been any loss of protein during the concentration process (gels not shown).

Figure 3.5 shows the protein profiles for the 14 CAPD patients in Figure 3.4 run side by side on a single gel, following loading of 450µg of total protein per lane. It is clear that despite differences in protein loss between different patients, when a similar amount of protein is separated, there are greater degrees of similarity between the protein profiles. This similarity applies to both day and night time HPDs of individual patients, and also profiles between patients. To compare the protein profiles in Figure 3.5 each lane of the gel was subjected to a densitometry scan using a BioRad G701 image analyzer to determine if there were any differences in the numbers of proteins between the D.T and O.N HPDs, and between individual patients. The data obtained showed that the average number of proteins in the HPD profiles was fairly constant at 19.60±2.12 proteins per profile.

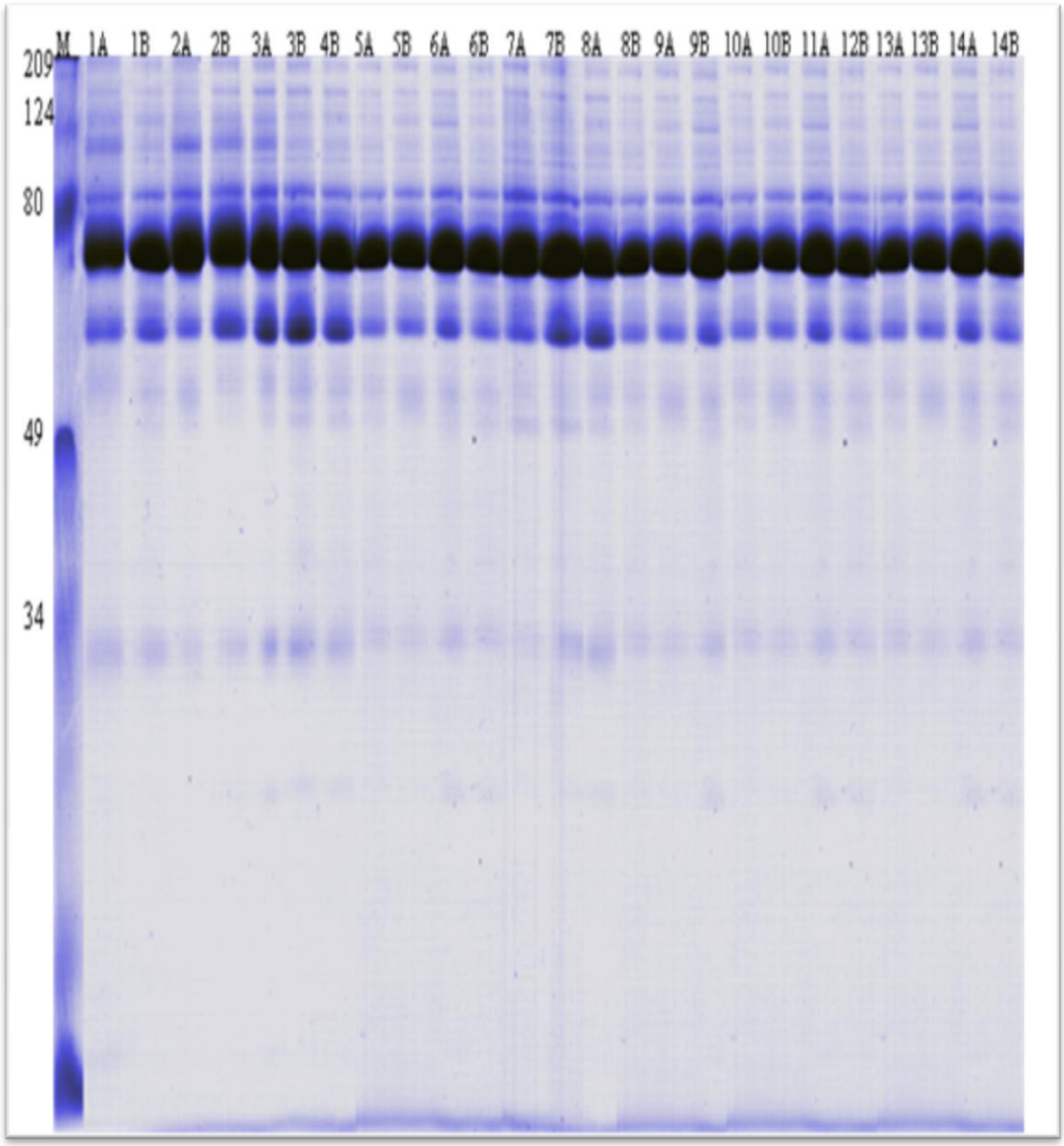

Figure 3.5 Comparative protein profiles of CAPD patients. The Figure shows a 12 % SDS-PAGE gel of the HPD proteins from 14 patients with two CAPD dwells. A constant amount of 450 µg of total protein was loaded per lane. Key: M, molecular weight markers; A, proteins in patient D.T HPD; B, proteins in patient O.N HPD.

3.2.4 Identification of HPD proteins

The proteins shown in Figure 3.5 were identified with the assistance of the University of Leicester PNACL facility. The proteins were numbered as shown on the gel in Figure 3.6 (sequenced proteins are represented by red dots), excised robotically, digested with trypsin, and the resultant peptides sequenced using mass spectroscopy (thanks to Dr Andrew Bottrill, PNACL).

The sequenced peptides were assigned identities by comparison with sequences on the Matrix science database. The identities and functions of the numbered proteins in Figure 3.6 are shown in Table 3.4. The data in Table 3.4 shows the proteins common in all patients protein profile and shows the presence in CAPD dialysate of proteins of the type that are normally found in serum. These included the carrier protein albumin (alone or complexed with myristate or azaproprazone), immune defence proteins (IgG-immunoglobulin, alpha 2-macroglobulin), and iron and copper binding proteins (transferrin and ceruloplasmin, respectively). Technically, IgG was detected as the Kappa light chain (dot ≠ 10). Alpha 2-macroglobulin was observed both as an Alpha-2-M (dot≠3) and inter-alpha (globulin) inhibitor H1. Transferrin had a molecular weight of 80kDa (dot ≠ 5), while ceruloplasmin was detected as 151kDa (dot≠ 4). The complement component 3 precursor was visualized as well (dot ≠7). Fibrin beta was observed as a protein of about 50 kDa (dot ≠ 8). The data also shows that 3 of the proteins sequenced (dots ≠ 11, 12 and 13) did not have any matches in the database and were therefore labelled as unknown function.

3.2.4.1 Comparison of the levels of major HPD proteins

The levels of three of the most abundant proteins found in the gel in Figure 3.7 (indicated by the arrows) were compared using densitometry analysis (Table 3.3). It can be seen that even though similar amounts of protein were loaded onto the gel, the three proteins (transferrin, and albumin alone or complexed) are present in different amounts between patient's protein profile. Figure 3.8 shows the degree of differences in levels of these proteins

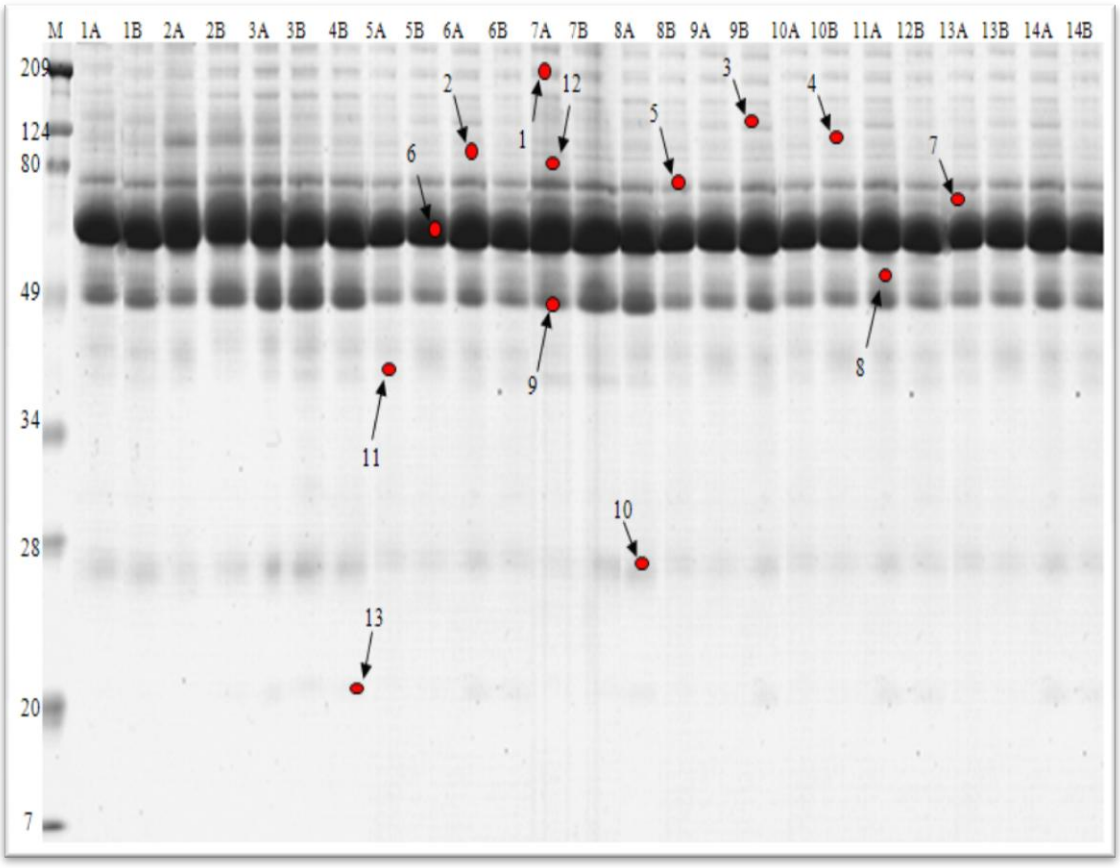

Figure 3.6 Identification of proteins in CAPD patient dialysate . The Figure shows a 12 % SDS-PAGE gel of the proteins from HPD of 14 patients with two CAPD dwells. A constant amount of 450 µg of total protein was loaded per lane. Key: M, molecular weight markers; A , proteins in patient D.T dwell; B, proteins in patient O.N dwell. The red dots represent the proteins that were sequenced.

Protein dot ≠	HPD Protein identity	Protein function
1	Inter-alpha(globuin) inhibitor H1	Play a role in inflammation, wound healing & cancer metastasis
2	Human serum albumin in a complex with myristic acid and tri-iodobenzoic acid	Serum protein which binds a wide variety of metabolites and drugs
3	Alpha-2-macroglobulinprecursor (Alpha-2-M)	Inhibitor all four classes of proteinases
4	Ceruloplasmin	Copper-carrying blood protein
5	Transferrin	Iron binding protein
6	Albumin	Responsible for much of the plasma colloidal osmotic pressure and serving as a transport protein for large organic anions.
7	Complement component 3 precursor	Plays a central role in the complement system and contributes to innate immunity (De Bruijn & Fey,1985)
8	Fibrin beta	Has an important role in haemostasis, fibrin (ogen) and has central regulator role of the inflammatory response (Flick et al., 2004)
9	Human serum albumin in a complex with myristate and azapropazone.	Serum protein which binds a wide variety of metabolites and drugs
10	IgG kappa chain	Binds antigen as part of immune protection of the host (Kappa is the light chain).
11	Unnamed protein product	**? Function not known**
12	Unnamed protein product	**? Function not known**
13	Unnamed protein product	**? Function not known**

Table 3.2.Identity and function of the patient HPD proteins sequenced in Figure 3.6.

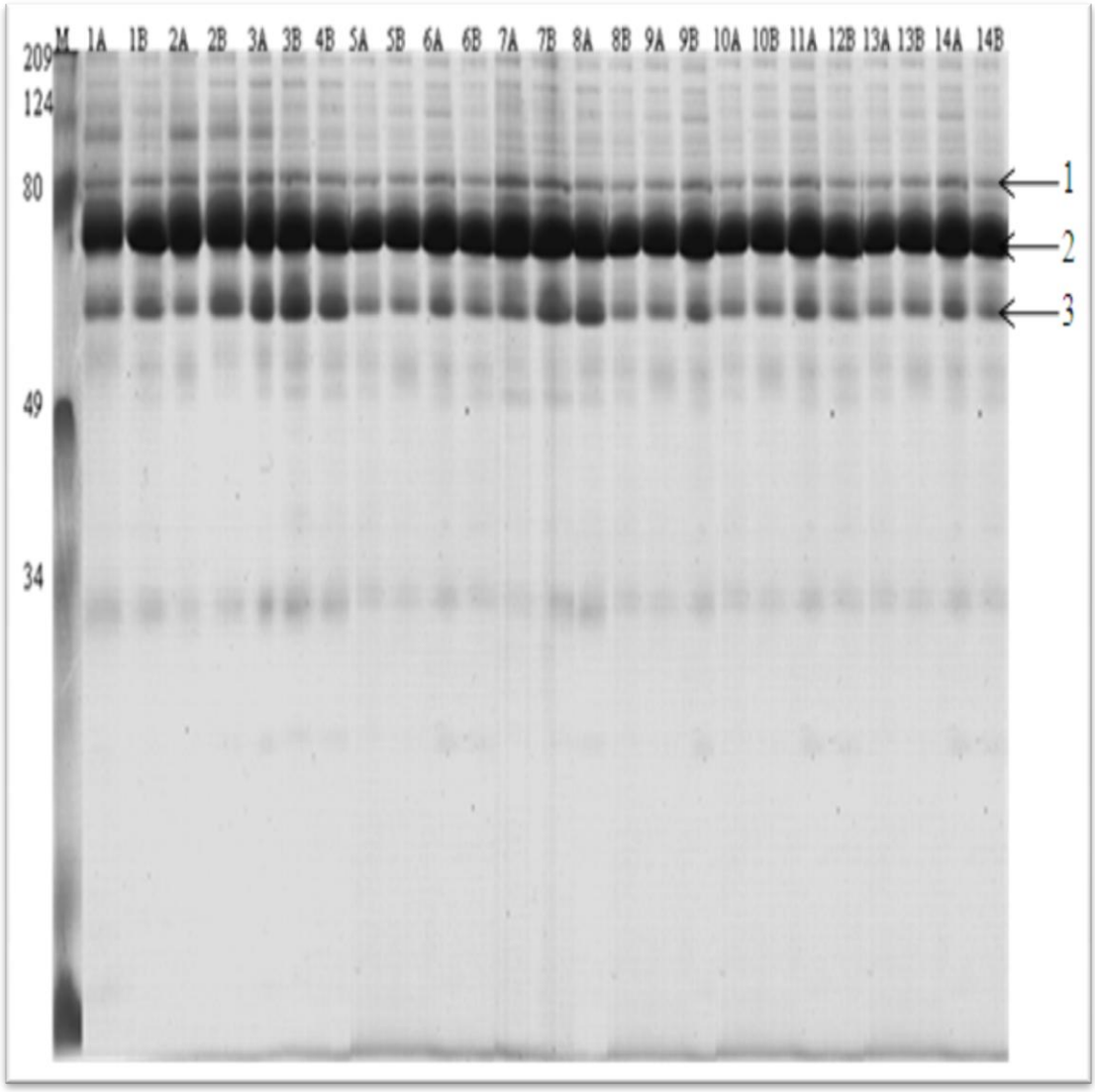

Figure 3.7 Comparison of the levels of major HPD proteins.The Figure shows a 12 % SDS-PAGE gel of the proteins from PDF of 14 patients with two CAPD dwells. A constant amount of 450 µg of total protein was loaded per lane. Key: M, molecular weight markers; A, proteins in patient D.T dwell; B, proteins in patient O.N dwell. The arrows identify the 3 most abundant proteins in the PDF.

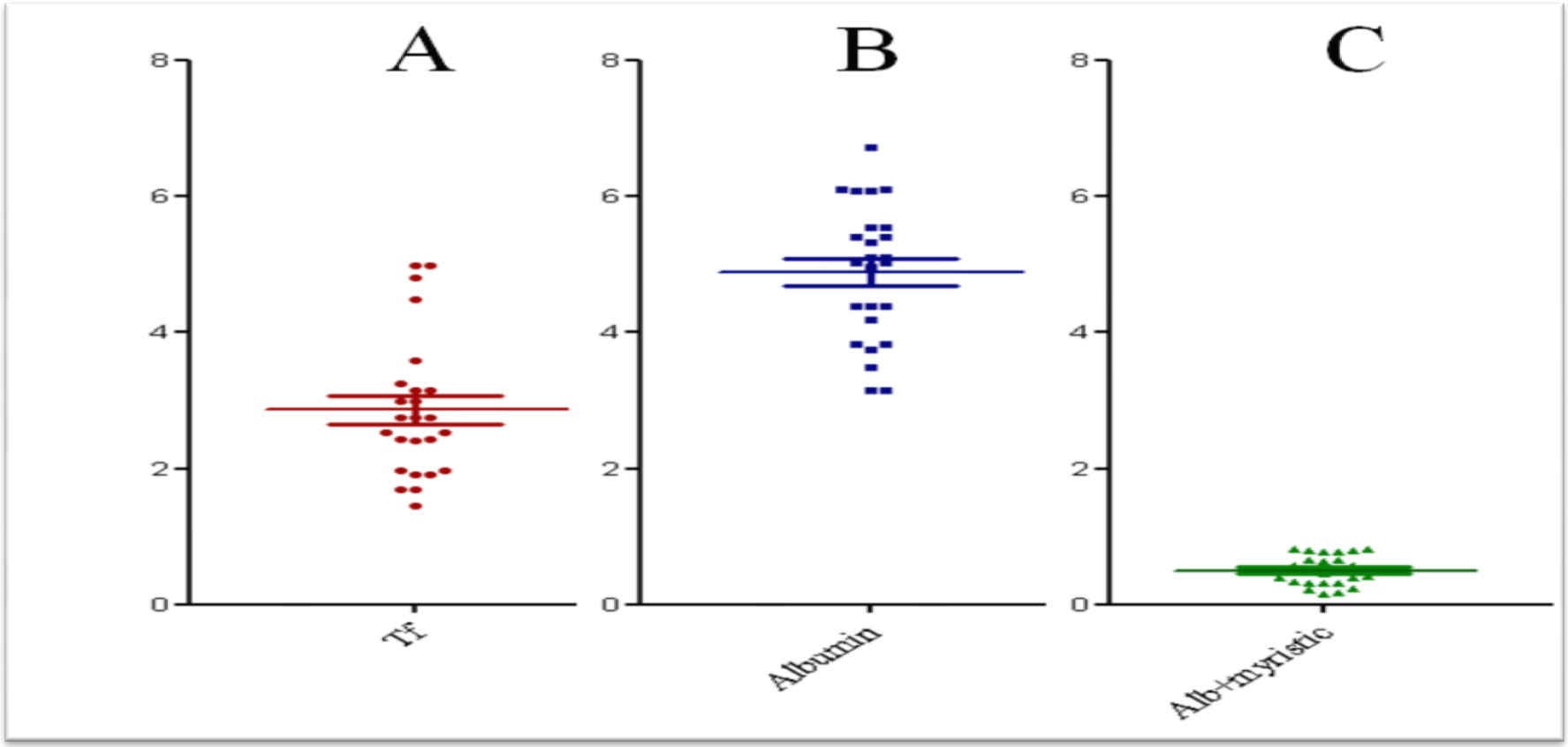

Figure 3.8 Differences in the levels of three proteins common between patients The densitometry analysis showed in the scatter plots used a background subtraction programme (Bio-rad G701 densitometer). The y axis is a relative value scale. Key: A, transferrin; B, albumin; C, albumin complexed to myristate and azapropazone. Numerical data for this figure is shown in Table 3.3.

Parameters	Tf	Albumin	Albumin complex with myristate and Azapropazon
Minimum	1.452	3.146	0.1608
Maximum	6.483	7.091	0.8028
Mean	2.943	4.964	0.5012
Std. Deviation	1.217	1.126	0.2163
Std. Error	0.2434	0.2251	0.04326
P value	< 0.0001	< 0.0001	< 0.0001

Table 3.3: Numerical data for the plot shown in Figure 3.8. The values shown under the Tf and Albumin columns represent scan areas of the bands analysed from graph 3.7, also it shows the maximum and minimum of the 3 proteins (Tf, Albumin and Albumin complex with myristate and Azapropazone) were analyesd, The table show the 25 sample dwell range and the significant differences within the each protein compared with zero.

3.2.5 The release of transferrin

Transferrin is a protein of particular interest due to its highly important iron sequestering and bacteriostatic role in serum and blood. The densitometry analysis in Figure 3.8 suggested that there were differences in the levels of this protein between patients. The presence of transferrin was confirmed using western blotting (Chapter 4), and immunoassay and the actual levels between D.T and O.N were measured using a commercial transferrin ELISA kit. Figure 3.9 compares D.T HPD transferrin levels with those in O.N HPD and shows that there was a significant difference in the levels of transferrin in the HPD. The D.T mean± SEM was 214.3±27.16 ng/ml (N=12) and O.N mean± SEM was 864.5 ± 127.4ng/ml (N=13).

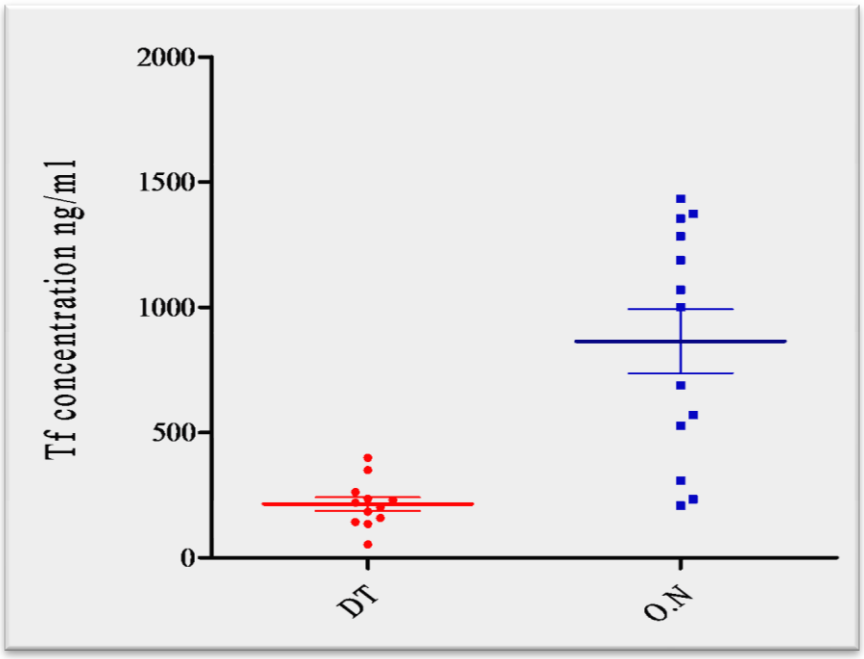

Figure 3.9.The transferrin concentration in the HPD of 14 patients.

3.2.6 Discussion

One of the major side effects of peritoneal dialysis treatment is protein loss into the dialysis fluid. This study confirmed that substantial amounts of protein were lost by all the patients included in this study, particularly from overnight dwells. The total protein loss calculated for the patients analysed was 30.55± 7.046 mg/100ml (0.61g/2l) for D.T dwells (N=23) and 129.1± 18.89 mg/100ml (2.58g/2l) for O.N dwells (N=14). These values differ somewhat from that reported by Eason *et al*. (Eason et al., 1996) who reported 90±50 mg/100 ml. However, Eason *et al*. (Eason et al., 1996) did not state which type of dwell sample was being analysed, which type of dialysis solution was used and did not mention how many dwell per day. In the current study, total protein loss per 2L of dialysate was around 0.6g for D.T HPD and 2.58g for O.N HPD. These

values are similar to those reported by Steinhauer *et al*. who reported 2.62g of protein loss but did not state the duration of dwell or type of PDS used (Steinhauer et al., 1992). Westra *et al.* found that peritoneal dialysis patients lost protein averaging D.T=3.9±0.4g, and O.N 6.2±0.6g per dwell, with an average 24-hour loss of 10.0±0.6g (Westra et al., 2007). In this study, assuming an average of 4 D.T dwell changes and 1 O.N dwell, patients were losing on average (4x 0.61) + (2.58 x 1) = 5.0 g of protein/day, which is within the region of both of these studies.

It was found that there was a significant increase in the total amount of protein lost during longer dwell times (overnight) than the shorter periods of dialysis (daytime). This finding is similar to the results reported by Kagan *et al*. who concluded that a longer dwell time resulted in greater loss of protein (Kagan et al., 1990). In general terms the range of the total protein loss per day is varied from 1.5 to 9 g/day, which is within the range of the 5 to 15 g/day losses reported by Blumenkrantz *et al*. (Blumenkrantz et al., 1981). However, these studies of protein loss in CAPD patients did not specify the type of dialysis fluid solution used and how long the PDS had dwelled in the patients, so making closer comparisons of HPDs difficult. The osmotic agent used can significantly influence protein loss, as Imholz *et al*. (Imholz et al., 1993) and Coester *et al*. (Coester et al., 2009) both demonstrated that high glucose (high osmolarity) PD fluids resulted in significantly higher transcapillary ultrafiltration and net ultrafiltration which will have a major influence on protein loss.

Proteins released into the CAPD dialysate were characterized using proteomic methods. SDS-PAGE showed that the protein profile of D.T HPD was different from O.N dialysate in terms of protein abundance. This agrees with Kabanda *et al* who

demonstrated that peritoneal loss of proteins of large molecular weight was dwell time dependent (Kabanda et al., 1995). In this study it was found that there were differences in the apparent numbers of proteins, and relative protein concentrations, depending on the dwell time length (Figures 3.4 A, B and C). There were also marked differences between the HPD profiles of individual patients. Different patients showed considerable differences in terms of protein loss, whether this was the daytime or overnight dwell. This variation has not been reported before, possibly because of small patient cohort sizes. Also of interest was the finding in Figure 3.3, panel A, that diabetic patients lost more protein over all, than non-diabetics. This may be due to enhanced peritoneal permeability related to the generalised vasculopathy and hyalinisation associated with longstanding diabetes.

A comparison of the HPD protein pattern shows that the most abundant protein in all the HPD samples was the serum osmotic stabliliser and general carrier protein albumin. This finding is consistent with Jones *et al*. (Jones et al., 1997) who showed that nearly 60% of the total protein concentration in CAPD dialysate was albumin Figure 3.6 showed that by normalising the amount of protein analysed the patient's protein profiles showed a generally similar pattern. This finding differs from the result of Eason *et al*. (Eason et al., 1996) who demonstrated that using either a similar amount of protein per lane or similar volume all produced the same relative patterns. This may be due to small patient sample size as the Eason study analysed HPDs from only 5 CAPD patients while this study analysed HPD from over 14 patients. In this study the average number of protein bands per HPD 19.36±1.997, which is different from the Eason *et al*. study which found that the mean number proteins released was 37.6 ±5.0 (Eason et al., 1996). However, the Eason *et al.* study used gradient gels and it is not clear what type of gel

staining was used (this study used colloidal coomassie blue stain which can visualise protein bands of 10ng and less), or if the protein profiles shown might also have included proteolytic fragments.

Sequencing the proteins released into the HPD showed that those being lost consisted of serum proteins, some of which are involved in the first line of defence against microbial pathogens such as transferrin, IgG and complement C3. These proteins were lost from the systemic circulation in to the peritoneal fluid through the peritoneal membrane, and as well as the effects on metabolism of such constant general protein loss, reduction in levels of immune defence proteins may affect susceptibility to infection. It is reasonable to speculate that the greater the levels of protein lost, overall, potentially the greater the reduction in immune defence against infection, and perhaps, the greater the provision of peritoneal nutrients (including iron) to bacteria introduced into the peritoneal cavity during a PD exchange.

Several of the HPD proteins identified in this study have also been found by others investigating protein loss in CAPD patients. For instance, Yanagisawa *et al*. used radiolabeled antibody staining to show that albumin, transferrin, IgG and fibrinogen were deposited on Tenckhoff catheters (Yanagisawa et al., 2004). Furthermore, alpha-2-macroglobulin has also been identified previously by Kabanda *et al.* (Kabanda et al., 1995). The presence of complement C3 in HPD was reported by Blumenkrantz *et al*. (Blumenkrantz et al., 1981). In the current study, identifying ceruloplasmin as a HPD protein, and showing the presence of serum albumin in a complexed form (with myristate and azapropazone, and with myristic and tri-Iodobenzoic acid) are both unique discoveries.

Surprisingly, low molecular weight proteins ~ 40 kDa were not very abundant in the HPD samples analysed in this study. Cueto-Manzano, *et al*. reported the presence of five low molecular weight proteins using a sensitive immunoassay (Cueto-Manzano et al., 2001). It may have been that these proteins were at too low a concentration to detect on SDS-PAGE gels (limit of detection 10 ng using silver staine). Interestingly, Kagent *et al*. reported that higher molecular weight proteins were lost into the CAPD dialysate faster than lower molecular weight proteins (Kagent et al., 1990).

Although it has been found that the presence of transferrin can affect the bacteriostatic activity of CAPD dialysates (McGregor et al., 1987), no one has directly measured transferrin levels. Use of a transferrin ELISA showed differences in transferrin levels between D.T and O.N dwells. There were also significant differences between patients. Yanagisawa *et al*. found that in a study of 5 CAPD patients the dialysate transferrin content was very high at 290±35 µg/ml (Yanagisawa et al., 2004). In contrast, the current result for 14 CAPD patients was 1.07 ± 0.23 µg/ml for O.N dwells and 0.231 ± 0.04 µg/ml for D.T dwells. The differences in these transferrin values may be due to the detection method used also Yanagisawa *et al.* used different PD samples and the age group was lower than the one used in this study. The Yanagisawa study used densitometer scanning to analyse the band area of the transferrin protein, while the current study used a more specific quantitative ELISA. Since transferrin is a potentially important source of iron for many bacteria, including those most likely to cause peritonitis, its importance will be examined in more detail in later chapters.

Chapter 4.Factors affecting Staphylococcus growth in peritoneal dialysis solution and Human Peritoneal Dialysate

4.1 Introduction

As mentioned in Chapter 3 protein loss is a major concern in peritoneal dialysis treatment, not just because of the general effect on physiology and nutrition, but also because the loss of first line defence proteins such complement and transferrin. Loss of these first line defence proteins may help bacteria to grow in infused HPD, increasing the risk of peritonitis. Peritonitis is one of the major complications of peritoneal dialysis and one of the most common causes of treatment failure (Woodrow et al., 1997). The coagulase- negative staphylococci (C-NS) are the most frequent bacterial species causing peritoneal dialysis related infections.

For a long time C-NS were thought of as benign skin commensal flora, and not considered to be pathogenic, however, because of their ability to form biofilm on the plastics of indwelling medical devices such as intravenous catheters, they are now considered to be significant nosocomial pathogens (Spencer, 1998; Williams et al., 1995). *S. epidermidis* (followed by *S. aureus*) are by far the most commonly found cause of infections of the peritoneum of patients on peritoneal dialysis, with nearly 50-80% of catheter-associated infections caused by *S. epidermidis* (Archer and Climo 1994; Spencer; 1998; Levey and Harrington, 1982; and von Graevenitz and Amsterdam 1992).

Few studies have investigated host factors that might influence the composition of infused PD fluid and thereby the risk of bacterial growth. McDonald *et al*. (McDonald et al., 1986) studied *Staphylococcus* growth in peritoneal dialysis solutions with increasing glucose concentrations (0.5, 1.5, 2.5 and 4.25 % glucose), while Levy *et al*.

(Levy et al., 1990) examined the importance of physical factors on risk of PD peritonitis. He found that surgical procedures including pyelostomy or colostomy were not a contraindication or predisposing factor for subsequent development of peritonitis, and that age, sex, diaper usage, and low serum albumin similarly did not seem to predispose to the development of infection in CAPD patients. This chapter therefore examines the changes that occur in PD fluid during a PD exchange, and investigates factors in commercial PDS fluid and HPD fluid following a standard PD exchange that might influence C-NS growth.

4.2. Results

4.2.1 The importance of inoculum size on the growth of *S. epidermidis* in PDS

The objective of the first part of this study was to look at the ability of PDS to support the growth of *S. epidermidis*. The PDS used were the Dianeal PD4 Peritoneal Dialysis solutions with 1.36, 2.27 and 3.86 % glucose. These were first compared to see if there was any effect of differences in glucose concentration on the ability of *S. epidermidis* to grow in PDS. Initially, a very low inoculum of less than a 100 CFU/ml was used to inoculate the cultures, as this has been shown to be more representative of the numbers of bacteria likely to be present at the start of an infection (Freestone et al., 2008). However, this inoculum size did not grow in PDS, at any of the glucose concentrations (data not shown). Figure 4.1 shows an *S.epidermidis* time course of growth over 24 h in

1.36 % glucose PDS using an inoculum of 10^8 CFU/ml diluted up to a hundred fold (10^6 CFU/ml). As can be seen, only a very slight growth of *S.epidermidis* took place in the PDS inoculated with 10^6 CFU/ml (1:100 dilution) of bacteria. Bacterial proliferation increased with inoculum size (the 1:10 dilution, 10^7 CFU/ml and even more with the 1:4 dilutions, equal to $2.5x10^7$ CFU/ml). A similar set of results was obtained for the 2.27 and 3.86 % glucose PDS, and also for HPD fluids following a standard CAPD exchange (both D.T and O.N, data not shown). It is therefore clear that for experimental reasons – that is, to develop a reliable test for the growth of *S. epidermidis* in PDS or HPD, an inoculum of 10^7 CFU/ml needed to be used for all growth assays.

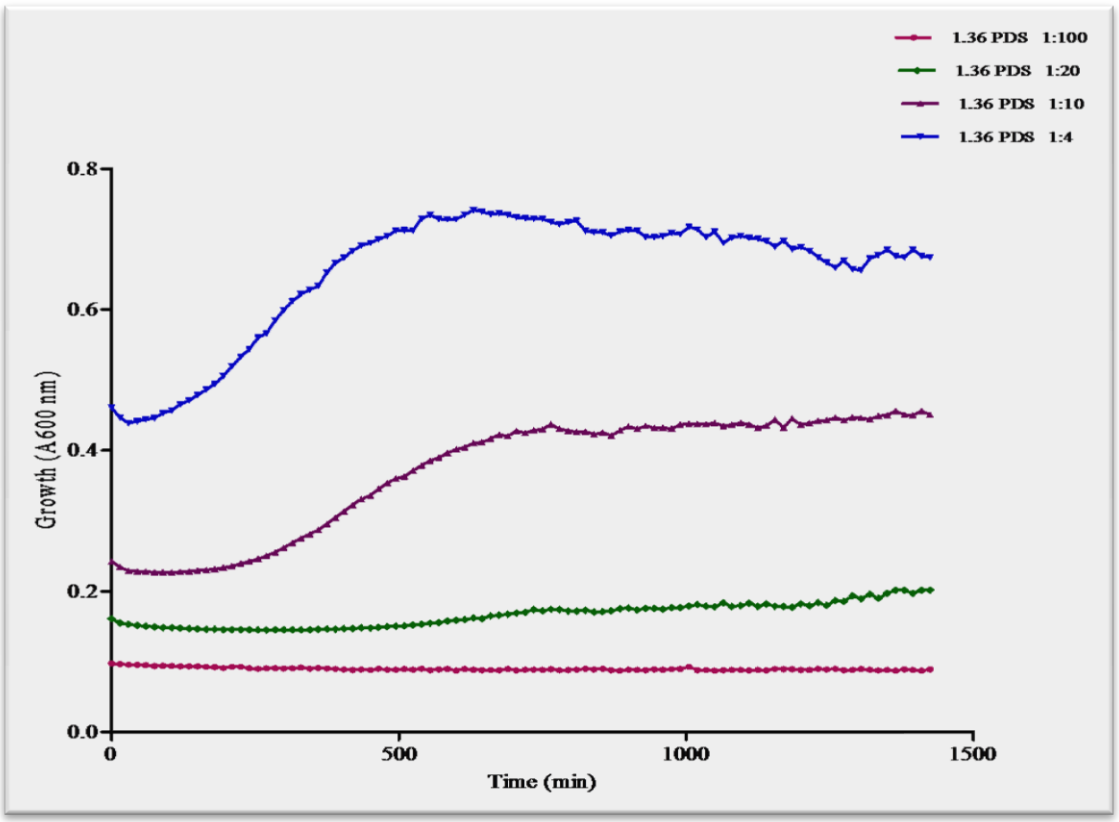

Figure 4.1 the time course growth of *S. epidermidis* in 1.36% PDS at different inoculum sizes. An overnight culture of *S. epidermidis* was used to inoculate (10^7 CFU/ml) PDS 1.36% glucose (1.36 % PDS) at the culture dilutions shown (1:100 to 1:4). Growth was carried out at 37°C in a Varioscan spectrophotometer; increase in cell numbers was monitored by taking optical density readings at 600 nm every 15 minutes.

4.2.2 The importance of glucose and iron availability on the growth of S. epidermidis in PDS and HPD

Most bacteria require both a carbon source (such as glucose) and iron for growth. Figure 4.1 shows that increasing the glucose concentration of the PDS increased the growth levels of the *S. epidermidis* ($p<0.01$). In order to study the iron-availability in PDS and HPD, trace metal analysis in the form of Inductively Coupled Plasma Optical Emission Spectrometry (ICPOES) was carried out in the University of Leicester Geology Department in order to measure the Fe levels of 1.36, 2.27 and 3.86 % glucose PDS (thanks to Lin Marvin of the Geology Department, University of Leicester for assistance with the analyses).

This date is shown in Table 4.1. It can be seen that the 1.36% glucose PDS has the highest Fe level at 0.27μg/ml, which is equivalent to an iron concentration of around 5 μM. Increasing the glucose concentration apparently decreases the iron detected by a factor of about 4, to around 1.25 μM. Bacteria need around 1 μM to grow, which is present in all the PDS formulations. However, Figure 4.2 shows addition of iron (100 μM) still stimulated growth of *S. epidermidis* in all the PDS glucose formulations. This indicates that PDS is iron limited, which is important to the infectious disease process, as iron availability, particularly limitation, is known to be an important regulator of bacterial virulence (Ratledge and Dover, 2000).

PDS	Fe concentration µg/ml
PDS glucose 1.36 %	0.271566
PDS glucose 2.27 %	0.06841245
PDS glucose 3.86 %	0.061500

Table 4.1 PDS Fe concentrations. The iron concentrations of 5 samples from each of the PDS glucose formulations was determined by trace metal analysis using a JY Ultima 2 ICPOES (see Materials and Methods, chapter 2, section 2.2.2.4 for details)

It was agreed to use two different methods (endpoint and time course growth) to study the bacterial growth with and without the addition of Fe $(NO_3)_3$. Both the CFU/ml endpoint and the time course of *S. epidermidis* growth in PDS (Figure 4.2) again showed that for all of the PDS glucose formulations the growth rate and final growth levels of the bacterial cultures were increased in the presence of added iron. One –way ANOVA was used and showed P.value< 0.0001.

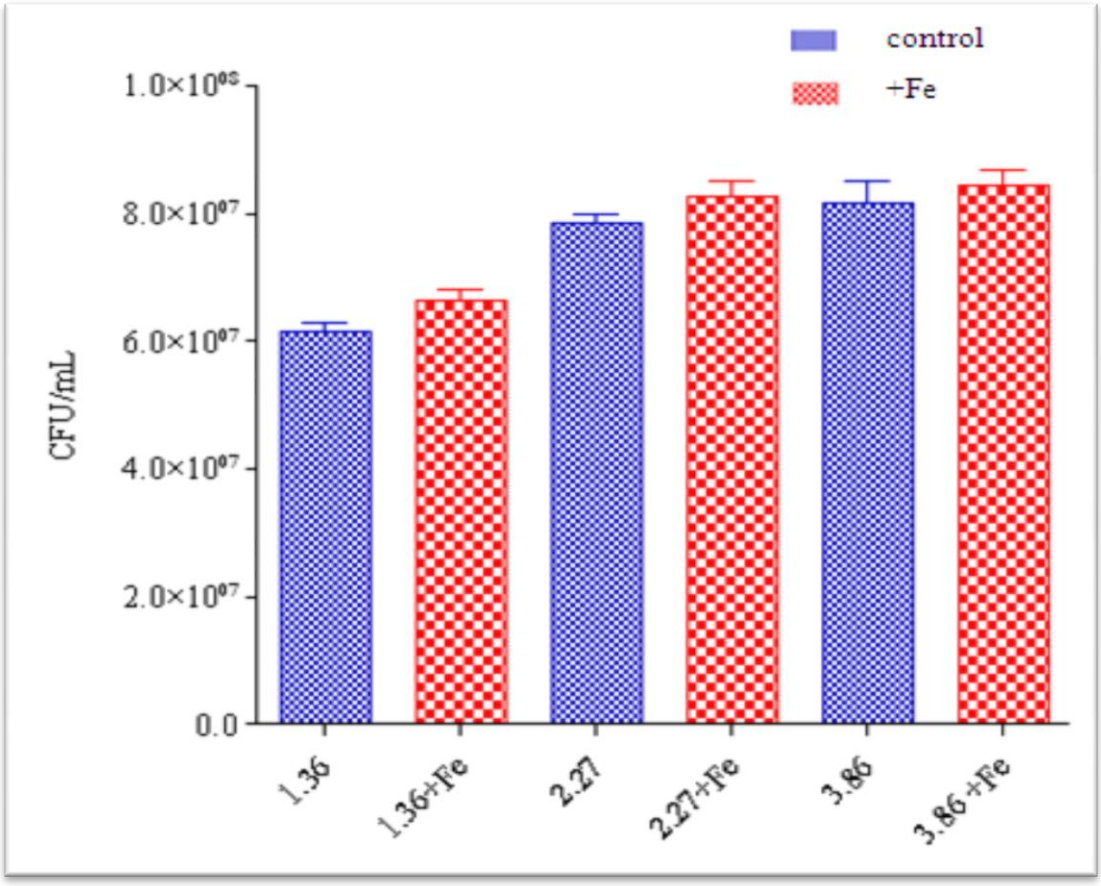

Figure 4.2 The growth of *S. epidermidis* in PDS +/- $Fe(NO_3)_3$ .The histograms show tthe endpoint growth levels of an inoculum of 10^7 CFU/ml *S.epidermidis* added to the PDS shown (1.36, 2.27 and 3.68 % glucose) without and with Fe, (100 µM $Fe(NO_3)_3$), and incubated statically 37°C in a humidified CO_2 incubator.

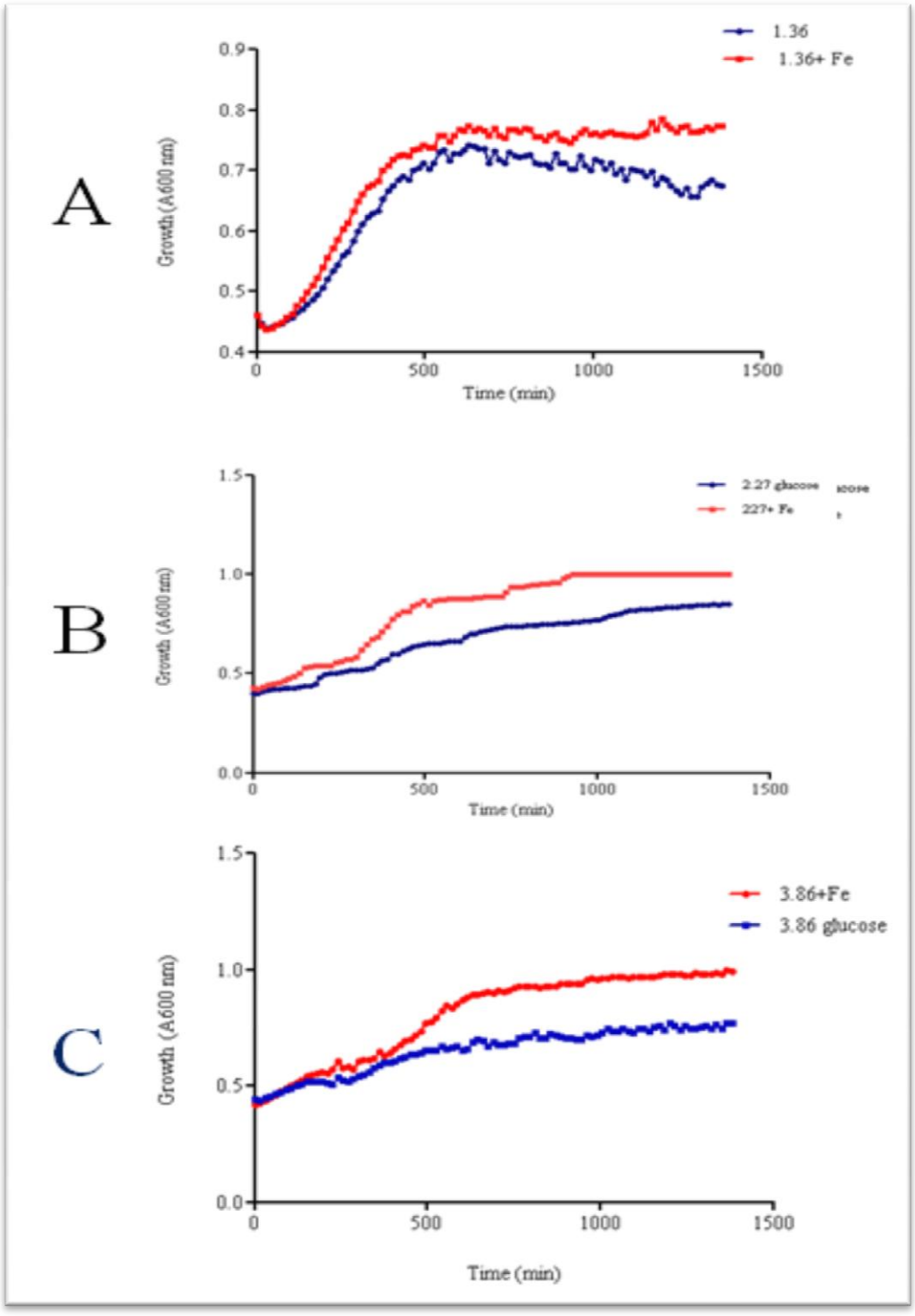

Figure 4.3 The time course growth of *S.epidermidis* in PDS with and without $Fe(NO_3)_3$. An overnight *S. epidermidis* culture was used to inoculate ($2.5x10^7$ CFU/ml) the PDS shown. Growth was carried out at 37°C in a Varioscan spectrophotometer; increase in cell numbers was monitored by taking optical density readings at 600 nm every 15 minutes.

4.2.3 The effect of stress hormones on growth of *S. epidermidis* in PDS.

The kidneys are responsible for elimination of catecholamine stress hormones (norepinephrine, epinephrine and dopamine) and their metabolites. However, in the case of PD patients, these metabolites are eliminated into the HPD. Stress hormone release has been shown to directly affect both the efficiency of the immune system as well as bacteria, increasing both their growth and virulence (Freestone et al., 2008) both of which could influence the development of an infection. Therefore, it was investigated if stress hormones influenced the growth of *S. epidermidis* in PDS, by analysing how the bacteria grew in the 3 different PDS glucose formulations with and without the addition of catecholamines. Figures 4.3 and 4.4 show the end point growth levels and time course of growth of *S. epidermidis* and it can be seen that the catecholamines have a generally stimulatory effect, particularly in the higher glucose PDS.

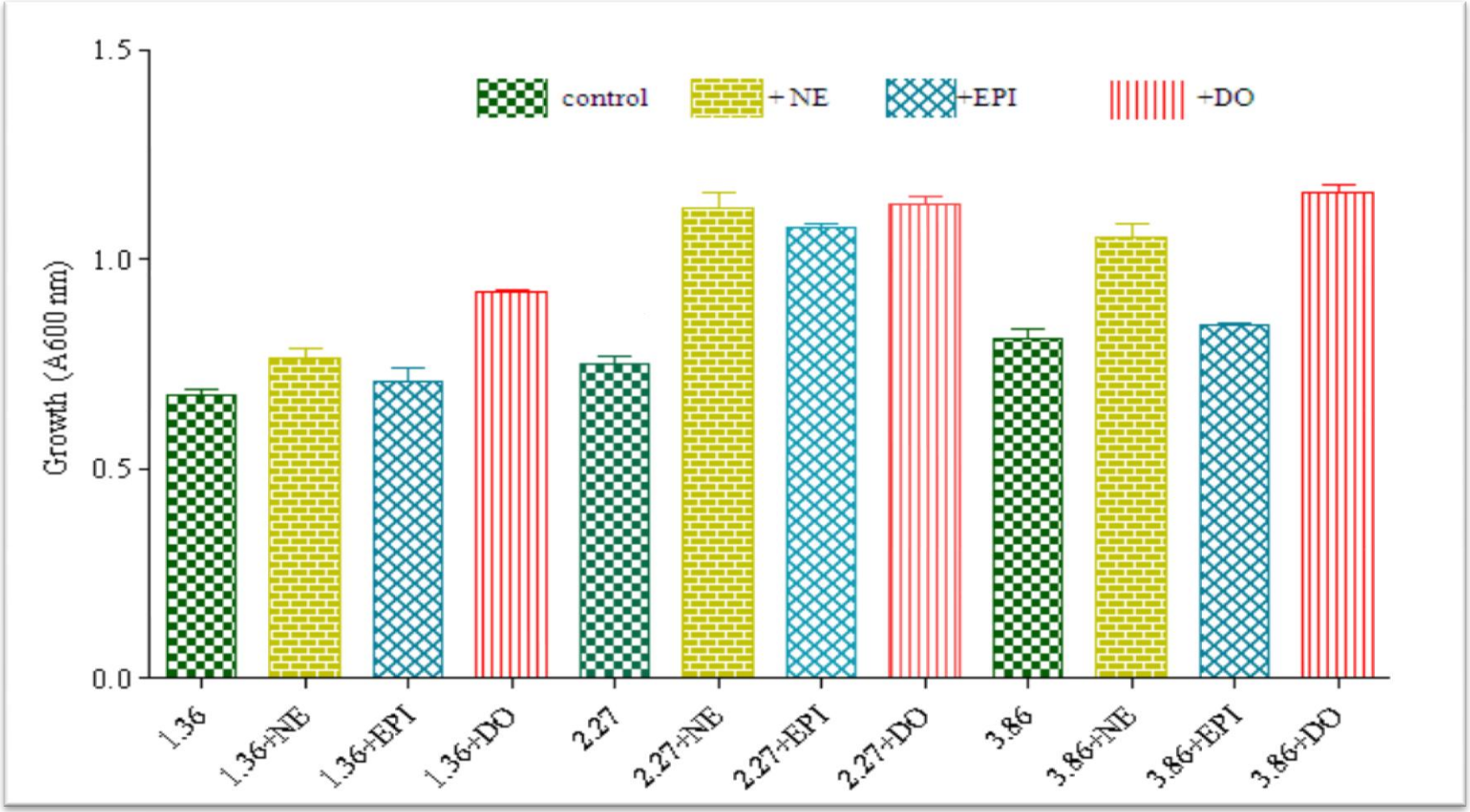

Figure4.4. The effect of catecholamines on the growth of *S. epidermdis* in PDS.The histograms show the endpoint growth levels of an inoculum of 10^7 CFU/ml *S.epidermidis* added to the PDSs shown without (1.36, 2.27 and 3.68 % glucose only) or with Norepinephrine (NE), Epinephrine (EPI) or dopamine (DO); all catecholamines were used at 100 µM. The cultures were incubated for 24 h with vigorous shaking (220 rpm) at 37°C and growth levels measured by monitoring optical density (A600nm). The values shown are the means of triplicate assays.

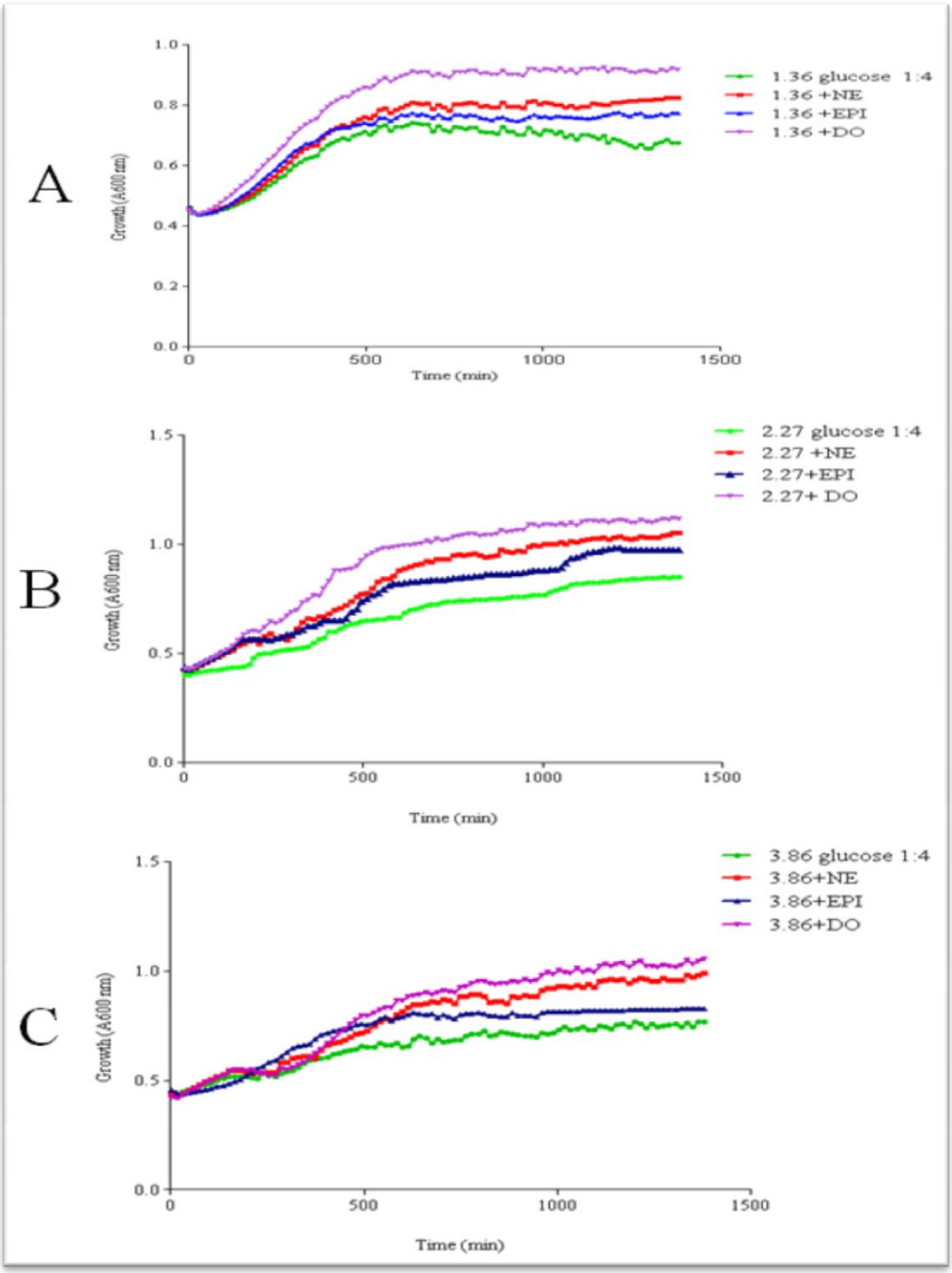

Figure 4.5 The time course growth of *S. epidermidis* in PDS in the presence of catecholamines

The timecourses show the growth responses of an inoculum of ($2.5x10^7$ CFU/ml) *S.epidermidis* added to the PDSs shown without (1.36, 2.27 and 3.68 % glucose only) or with norepinephrine (NE), epinephrine (EPI) or dopamine (DO); all catecholamines were used at 100 µM. The cultures were incubated for 24 h at 37°C in a Varioscan spectrophotometer and growth levels measured by monitoring optical density (A600nm) every 15 mins.

4.2.4 Analysis of the growth stimulatory characteristics of human peritoneal dialysates

In order to investigate if there were any differences in *S. epidermidis* response between different HPD dwells and within patients, growth analyses were carried out on the HPD from daytime and overnight dwells for the 14 CAPD patients whose protein loss was analysed in Chapter 3. Figure 4.5 shows that *S. epidermidis* was able to grow in all the HPDs. Unlike PDS which has shown inhibit the growth of S.epidermidis in low inoculums (Figure 4.1 and 4.3).

It was shown in Chapter 3 that there were for almost all the CAPD patients investigated differences in the protein composition of overnight and daytime HPD dwells. Similarly, in terms of the ability of bacteria to grow in HPD, there was a significant difference in the growth levels between the D.T and the O.N dwells ($p<0.01$). Bacteria grow consistently better in the overnight HPD for all 14 patients. Figure 4.5 shows that there was also considerable variation in the levels of growth of *S. epidermidis* in the O.N HPD dwells.

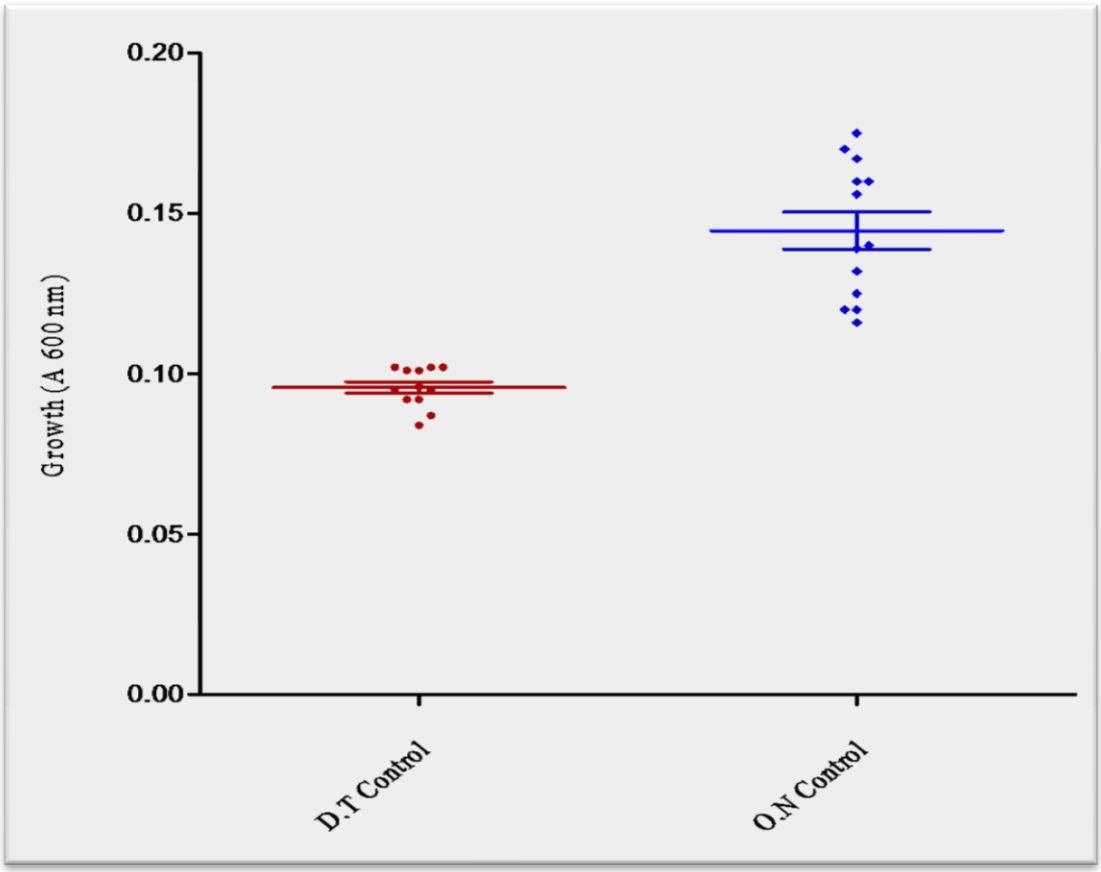

Figure 4.6 the growth of *S. epidermidis* in HPD

The data points show the endpoint growth levels of an inoculum of 10^7 CFU/ml *S.epidermidis* added to the HPDs shown. The cultures were incubated for 24 h with vigorous shaking (220 rpm) at 37°C and growth levels measured by monitoring optical density (A600nm). The values shown are the means of triplicate assays.

Differences in the degree to which bacteria can grow in HPD might explain why some CAPD patients are more susceptible to infection than others. Therefore it was important to investigate the biochemical differences in the composition of daytime and overnight HPDs.

4.2.4.1 Investigation of pH changes in PDS during CAPD

The glucose based PDS solutions used in this study are buffered to have a pH of around 5.5, which is not optimal for the growth of many pathogenic bacteria, who prefer a more neutral culture environment. To identify if there were changes in the acidity of the PDS after being in the human peritoneal cavity, the HPD pH for each CAPD patient was measured 3 times (Figure 4.6). Both D.T and O.N dwells show significant increases in pH over the starting 1.36% PDS, going from pH 5.5 to the pH range 7.3- 8.5 (P=0.0001). Overnight dwells had higher mean pH values than daytime samples, as well as showing greater variation in pH (7.5-8.5) than daytime dwells (7.3-7.7). Neutralising the originally acidic PDS would be expected to improve its bacterial culture ability, but the pH ranges of both D.T and O.N are similar, which suggests pH differences are not the reason for increased *S. epidermidis* growth in O.N HPD.

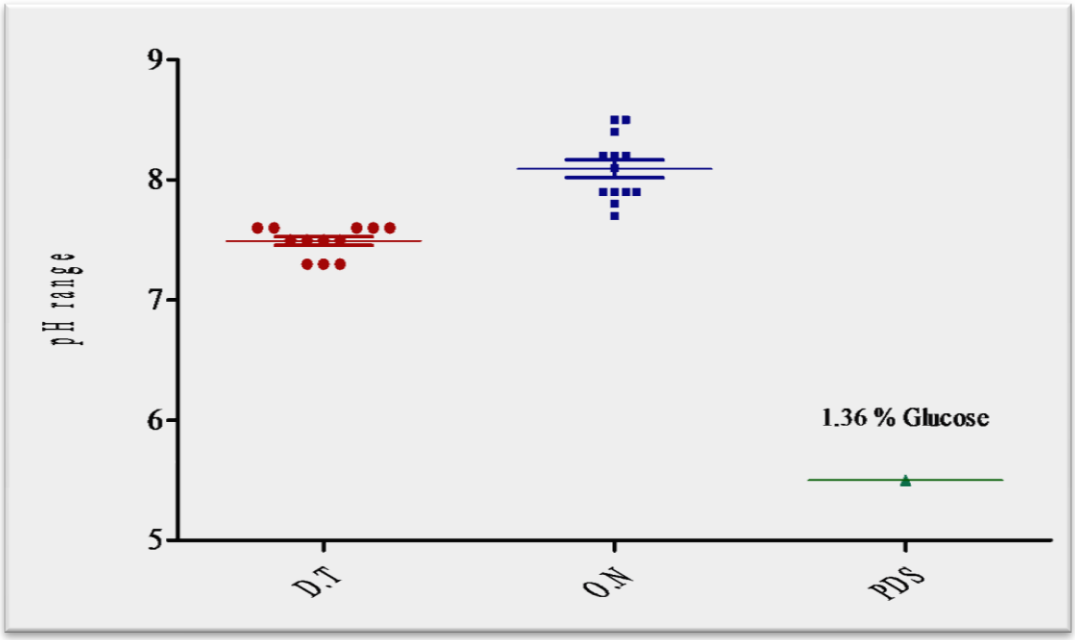

Figure 4.7 pH values of daytime and overnight HPD: pH values of the daytime (D.T) and overnight dwells (O.N) along with the control PDS (1.36% glucose) were measured as described in Materials and Methods; values shown are means of triplicate readings.

4.2.4.2 The importance of glucose on the growth of *S. epidermidis* in HPD

As mentioned before, the CAPD patients in this study used a 1.36% glucose PDS. Another point of interest is that 4 out of 10 patients in the study were also diabetics. Increases in glucose levels were shown in Figure 4.1 to increase the growth of *S. epidermidis* in PDS. Therefore, measuring glucose levels in the HPD may provide some insight into the underlying reason for differences between how *Staphylococcus* bacteria grow better in overnight than in daytime dwells, and also why there are growth variations between individual CAPD patients. To determine if PDS glucose differences might increase the susceptibility to infection, glucose concentrations were measured in the HPD using both a dipstick test, which could be used by CAPD patients, and a more precise hexokinase enzymatic test. The results for both tests are shown in Figures 4.7 and 4.8.

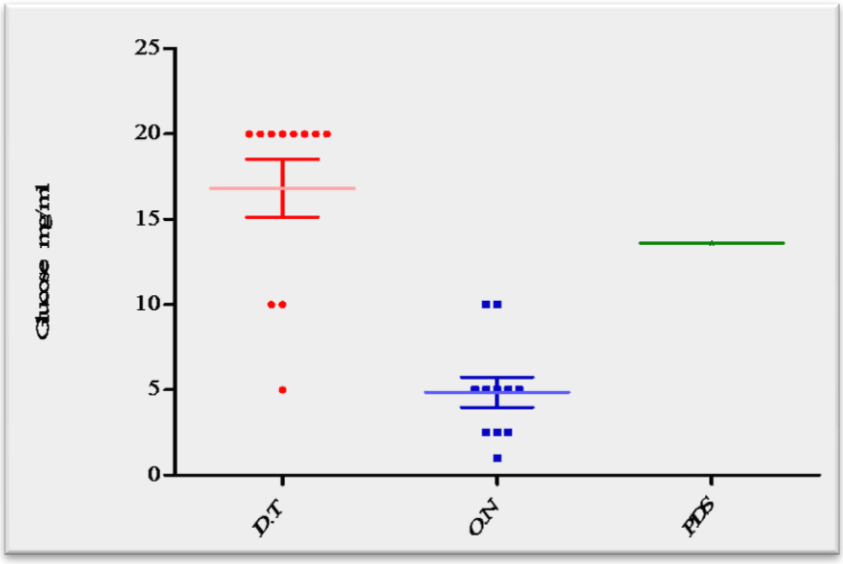

Figure 4.8 Glucose dipstick analysis test of D.T and O.N HPD compared to control 1.36% PDS. The scatter plot values shown are means of triplicate readings.

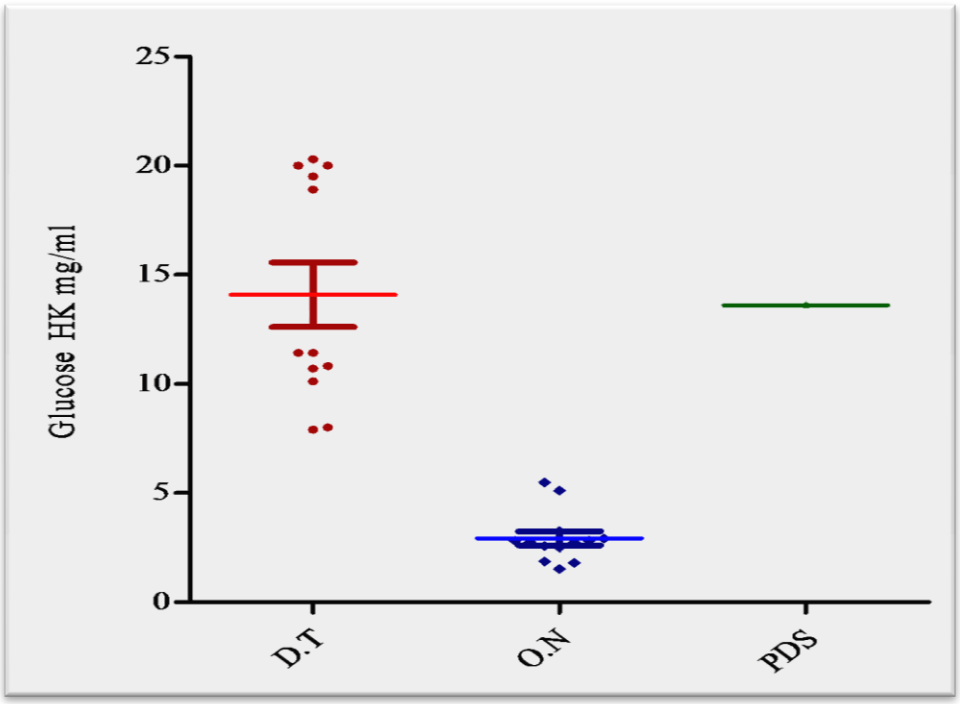

Figure 4.9 Glucose hexokinase analysis test of D.T and O.N HPD compared to control 1.36% PDS. The scatter plot values shown are means of triplicate readings.

Figures 4.7 and 4.8 both show that the CAPD patient's HPD glucose levels are different to that of the starting 1.36 % PDS. The dipstick test gives higher glucose values for the samples than the hexokinase assay, but the two are similar in terms of the HPD glucose concentration data spread. This means the dipstick could be used by patients with some accuracy confidence. In terms of the CAPD dialysates, generally, the glucose levels of the HPD from the D.T dwell patients were higher than the HPD of the O.N dwell (P=0.05), and closer to the 13.6 mg/ml of the starting 1.36% PDS. As expected, longer dwell times appeared to increase peritoneal uptake of glucose from the PDS, resulting in lower HPD levels, there was no significant difference between diabetic and non diabetic glucose absorbance. This is interesting, as it suggests that glucose is not the factor which makes O.N HPD more supportive of *S. epidermidis* growth than the dialysate from D.T dwells (Figure 4.5).

4.2.4.3 Fe levels in HPD

Peritoneal dialysate (HPD) is composed of various salts, host released proteins and other molecules including glucose and various trace elements such as iron and zinc (Thomson et al., 1983 and Padovese et al., 1992). Iron was shown to increase *S. epidermidis* growth in PDS and HPD, so in order to study the iron-availability of HPD and PDS, ICPOES analysis was also carried out on the dialysates from different PDSs and the CAPD patients in this study. Figure 4.9 shows that the 1.36 % PDS has a higher concentration of iron than either set of patient HPD, and that a PD fluid dwell is associated with a fall in iron concentration and therefore there is a much more iron limited environment towards the end of a PD dwell than at the beginning. This may in part be due to a dilution effect of ultrafiltration during an exchange. Low iron levels are sensed by infectious bacteria as indicative that they are within their host, causing bacteria to up regulate virulence and expression of iron scavenging molecules such as siderophores (Ratledge and Dover, 2000) Figure 4.9 also shows that there were significant differences between patients within the two HPD groups ($P < 0.0023$), and that Fe was present at a higher concentration in the O.N-HPD compared to the D.T-HPD ($P < 0.0001$).

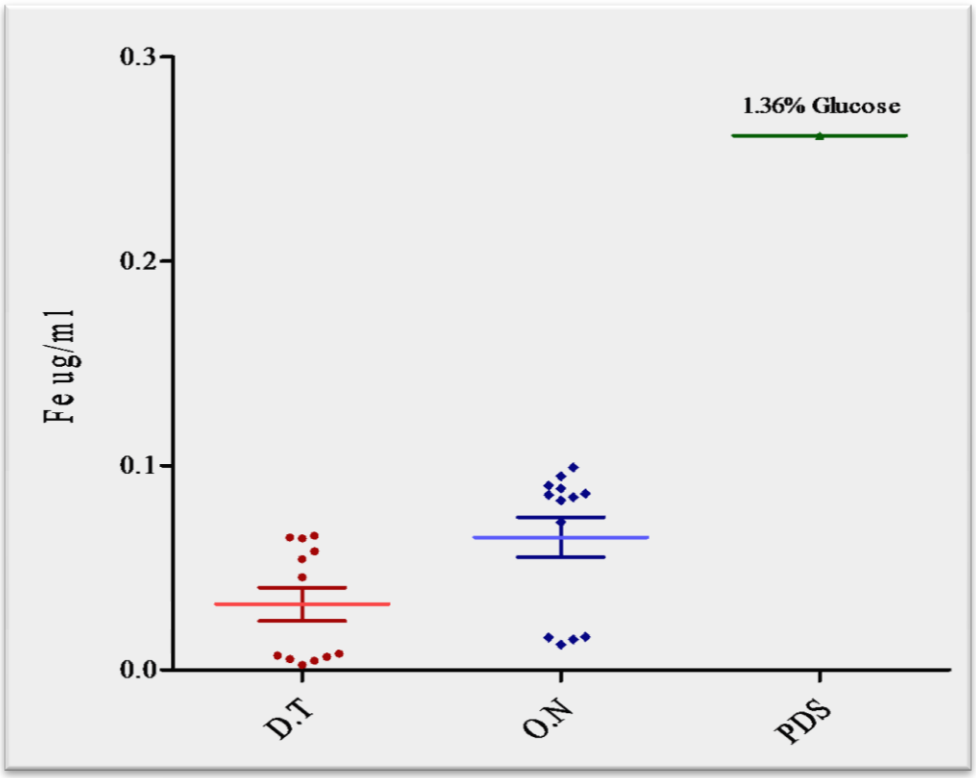

Fig. 4.10 Fe levels in HPD compared to 1.36% PDS. The scatter plot values shown are means of at least duplicate readings.

In order to investigate the importance of iron in the ability of staphylococci to grow in HPD, all the CAPD dialysates were incubated overnight with 10^7 CFU/ml *S. epidermidis*. The results show that there was again a significant difference in bacterial growth levels between D.T and O.N HPD ($P<0.0025$), and that there were also significant differences within the patient samples groups $P<0.0001$. In addition, it can be seen that all of the CAPD patients HPD are iron limited, since the growth levels of the bacteria are in all cases greater if iron was present.

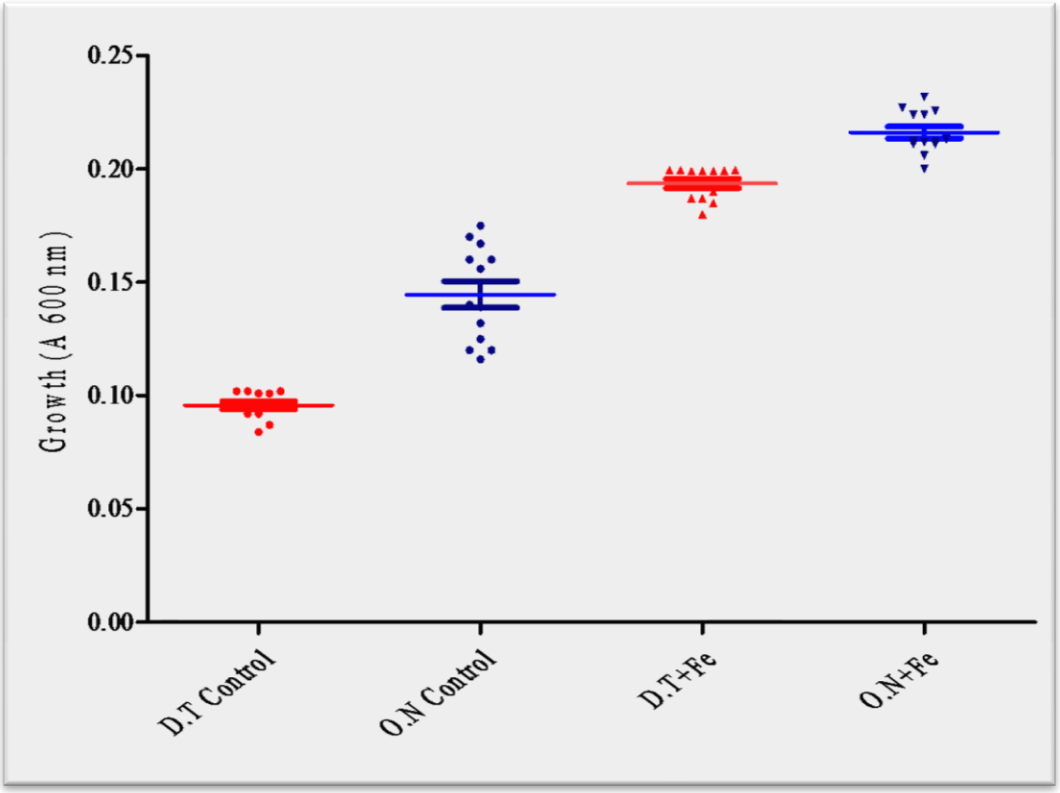

Figure 4.11 Growth of *S. epidermidis* in HPD with and without Fe .The data points in the scatter plots show the endpoint growth levels of an inoculum of 10^7 CFU/ml *S.epidermidis* added to the daytime dwell HPD (D.T), overnight HPD (O.N), without (Control) or with 100 µM Fe $(NO_3)_3$ (Fe). The cultures were incubated for 24 h with vigorous shaking (220 rpm) at 37°C and growth levels measured by monitoring optical density (A600nm). The values shown are the means of triplicate assays

The timecourse of growth in HPD in the absence and presence of iron is shown for a representative HPD in Figure 4.11. Once more, final growth levels for both D.T and O.N HPD are increased in the presence of iron.

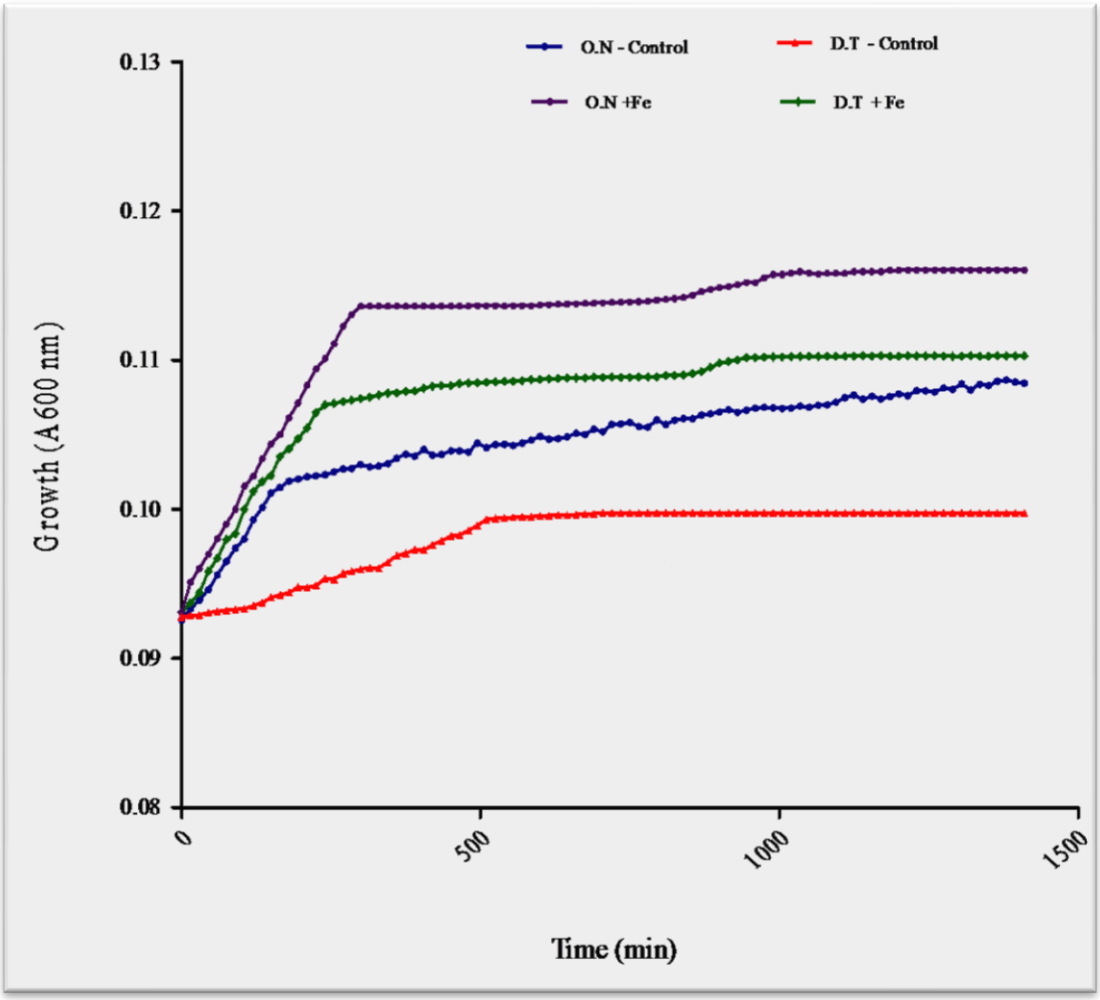

Figure 4.12 Time course of growth of *S. epidermidis* in a representative HPD with and without Fe.The timecourses show the growth responses of an inoculum of 10^7 CFU/ml *S.epidermidis* added to the daytime dwell HPD (D.T), overnight HPD (O.N), without (Control) or with 100 µM Fe $(NO_3)_3$ (Fe). The cultures were incubated for 24 h at 37°C in a Varioscan spectrophotometer and growth levels measured by monitoring optical density (A600nm) every 15 mins

4.2.4.4 Effect of catecholamine stress hormones on growth of *S. epidermidis* in HPD

Efforts were made to directly measure catecholamine levels in HPD using HPLC and mass spectroscopy. However, despite repeat efforts the levels of catecholamines in both D.T and O.N HPD samples were below detection. This is probably because catecholamines and their metabolites are unstable during storage and tend to decompose, especially at increased pHs such as occur in HPD (Raggi et al., 2003; Kushnir et al., 2003). This was unfortunate, as catecholamine and their metabolites are both able to stimulate growth and bioflim formation of *S. epidermidis* and other C-NS (Freestone et al., 1999; Neal et al., 2001; Freestone et al., 2002; Lyte et al., 2003). Since catecholamines and their metabolites would most likely have been released into HPD, especially during patient stress, experiments were carried out to determine if peritoneal dialysate becomes more supportive of bacteria growth if stress associated hormones are present. HPD samples from D.T and O.N dwells of the 14 CAPD patients were inoculated with *S. epidermidis* and the stress hormones norepinephrine, epinephrine and dopamine (Figure 4.12).

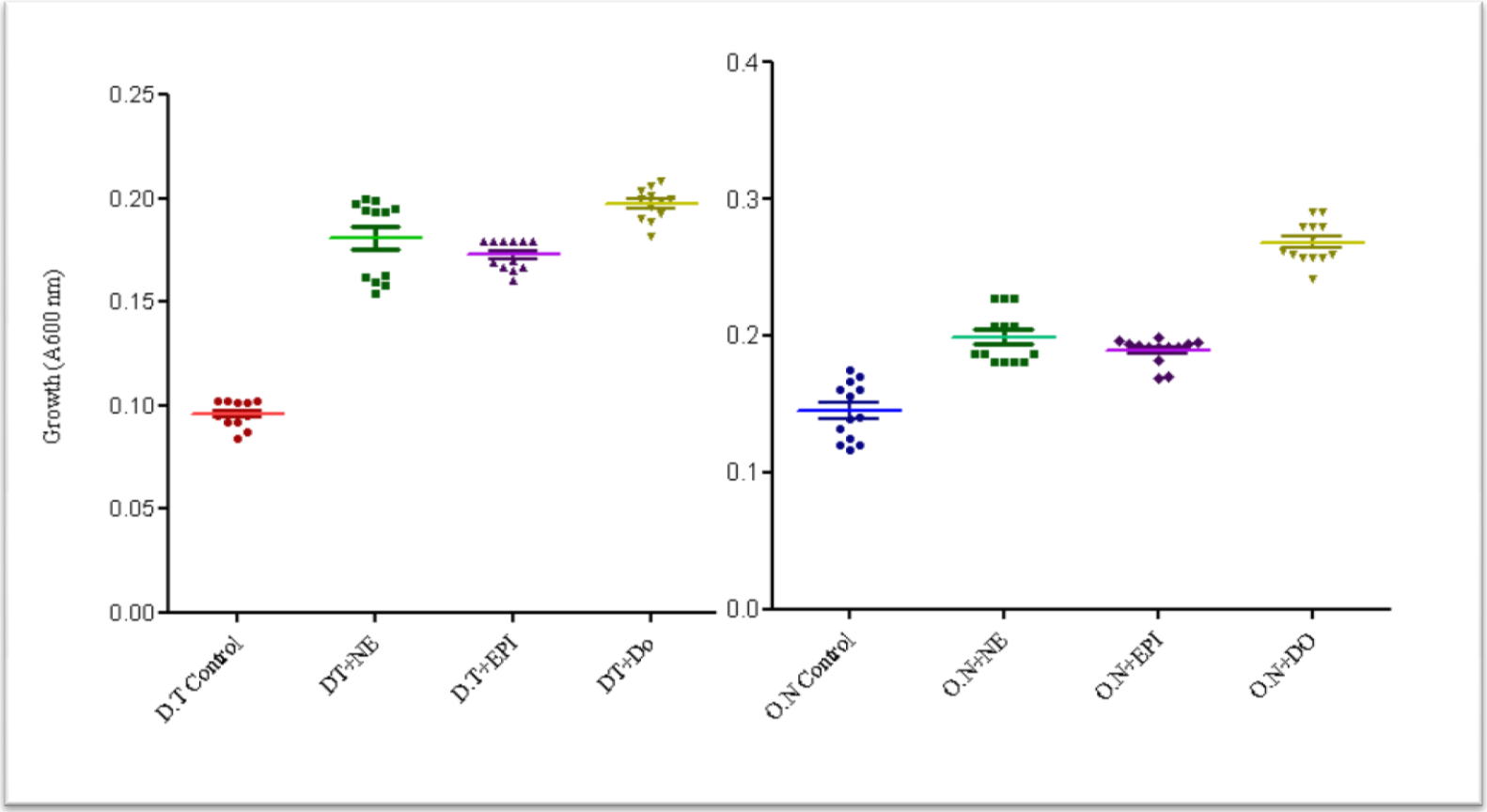

Figure 4.13 Growth of *S. epidermidis* in HPD in the presence of catecholamines .The scatter plots show the growth responses of an inoculum of 10^7 CFU/ml *S.epidermidis* added to the HPDs shown without (Control) or with Norepinephrine (NE), Epinephrine (EPI) or dopamine (DO); all catecholamines were used at 100 µM. The cultures were incubated for 24 h with vigorous shaking (220 rpm) at 37°C and growth levels measured by monitoring optical density (A600nm). The values shown are the means of triplicate assays.

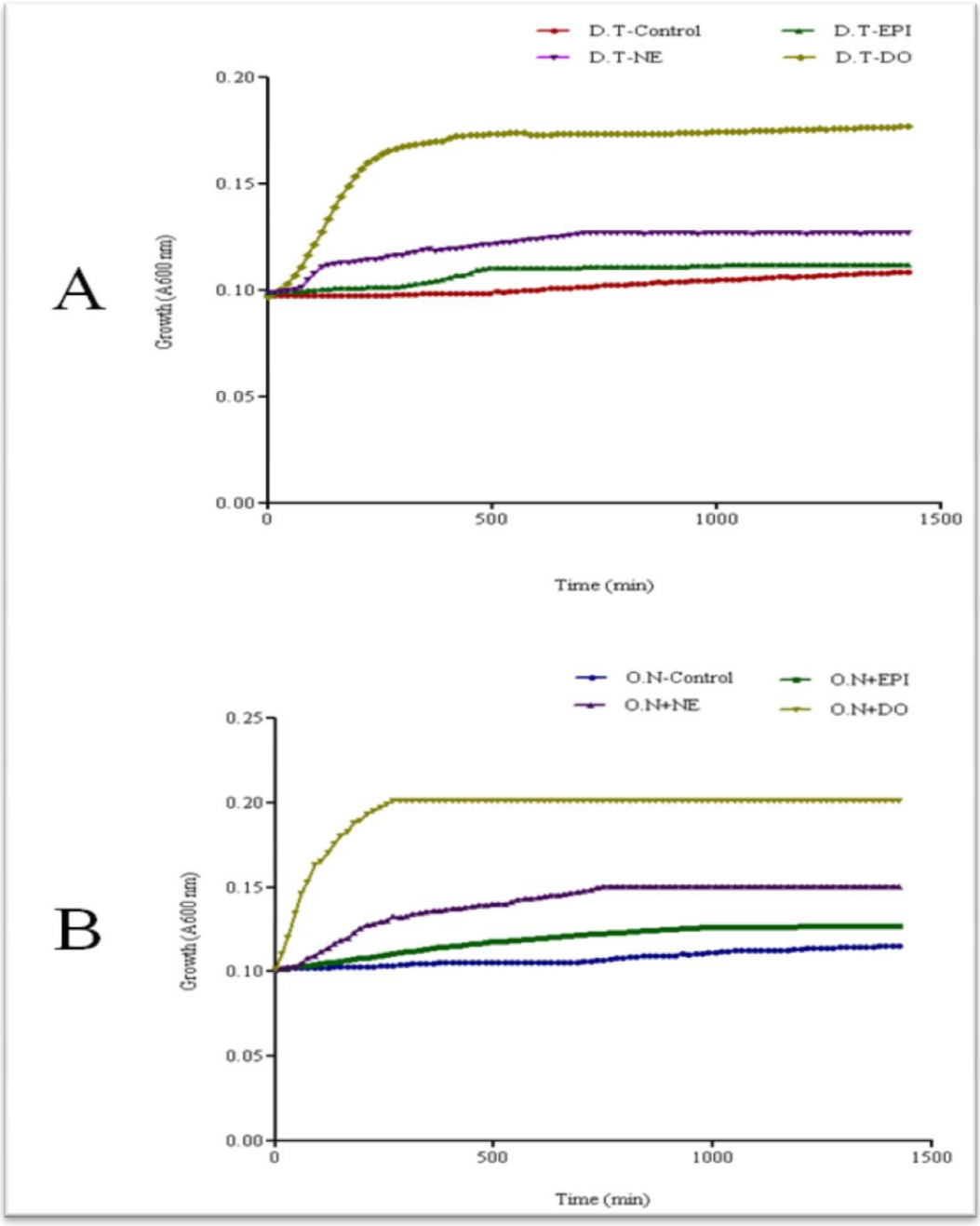

Figure 4.14 Time course of growth of *S. epidermidis* in HPD in the presence of catecholamines The timecourses show the growth responses of an inoculum of 10^7 CFU/ml *S.epidermidis* added to daytime (A) and overnight (B) HPDs .The additions to the HPDs shown were nothing (Control) or norepinephrine (NE), epinephrine (EPI) or dopamine (DO); all at 100 µM. The cultures were incubated for 24 h at 37°C in a Varioscan spectrophotometer and growth levels measured by monitoring optical density (A600nm) every 15 mins.

4.2.4.4.1 Investigation of the mechanism by which catecholamine stress hormones might improve growth of S. epidermidis in HPD

How might the stress hormones be increasing bacterial growth in the HPDs? It was previously shown that iron appears to be an important factor in the growth of *S. epidermidis*, as both daytime and overnight dwells were both very iron limited. Stress hormones have been shown to stimulate growth in serum and plasma by providing iron from transferrin, which is of interest as the proteomics investigations in Chapter 3 showed that transferrin is present in both D.T and O.N HPDs. *S. epidermidis* has also been shown to bind transferrin (Modun et al., 1998; Lyte et al., 2003; Freestone et al., 2008; Freeston et al., 2010; this study), as part of the mechanism by which it can acquire iron. To investigate if the bacteria were using transferrin bound iron to grow in HPD; the *S. epidermidis* were cultured in D.T and O.N HPDs**,** the bacteria harvested and bacterial cell wall proteins separated by SDS-PAGE, blotted and immunoprobed with transferrin antibodies, as described in Materials and Methods. The developed blots are shown in Figure 4.14 A and B, and show that considerably more transferrin is bound by bacteria grown in the O.N HPD. It has been shown that *S. epidermidis* needs this direct contact with transferrin to obtain the iron bound by the protein (Freestone et al., 2008 ;Fresston et al., 2010), combined with the observation that stress hormones allow *S. epidermidis* to obtain more iron from transferrin. Therefore, increased transferrin availability could explain why bacteria grow better in overnight dialysates.

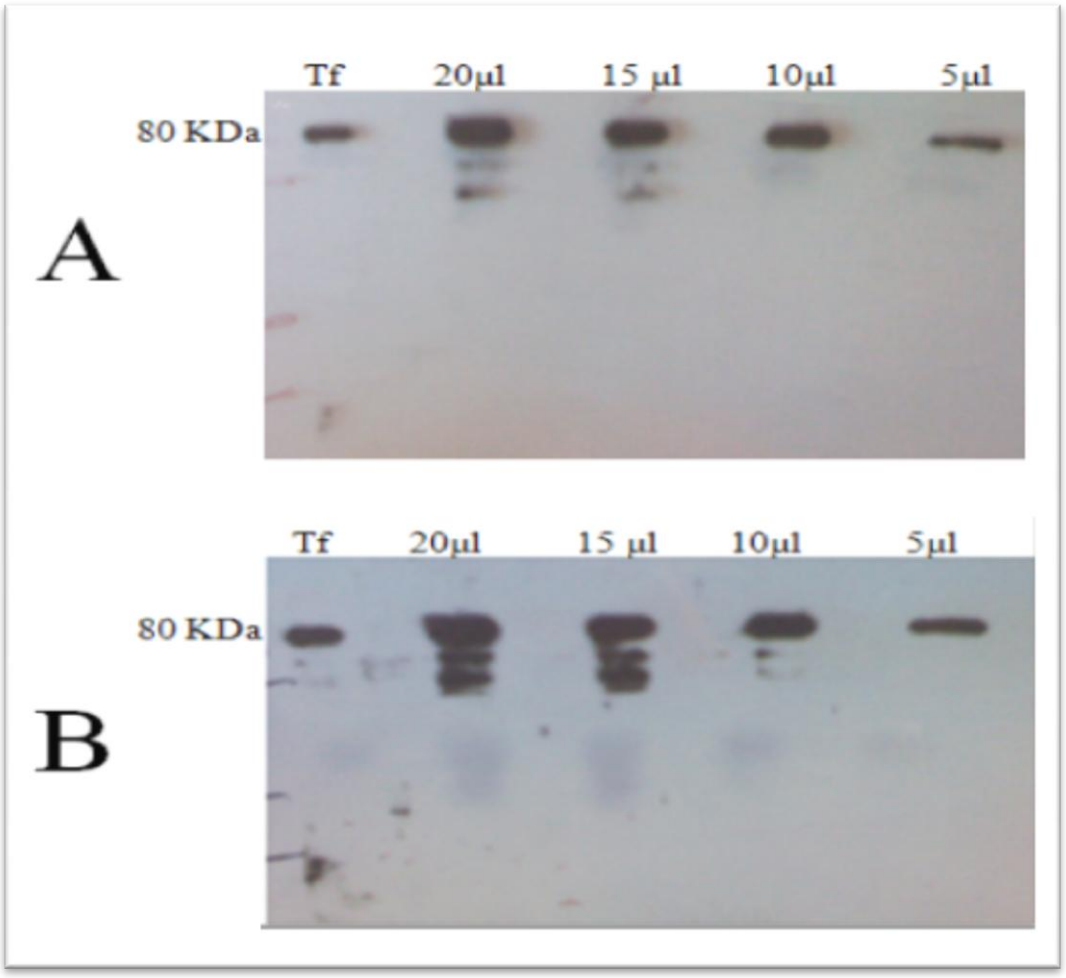

Figure 4.15 *S. epidermidis* binds transferrin in HPD.

Staphylococcus epidermidis was cultured for 24 h in D.T and O.N HPDs**,** bacteria were then harvested and surface associated proteins extracted as described in Materials and Methods. A range of volumes of the cell wall protein extract was loaded onto 12 % SDS-PAGE gel and eletrophoresed. The gels were western blotted, immunoprobed and developed using anti-human transferrin primary antibody developed in goat, anti-goat IgG peroxidise-conjugated developed in rabbit, and ECL. Protein extract from bacteria not grown in HPD did not produce a Tf- associated signal (Data not shown) .A, shows transferrin binding by *S.epidermidis* incubated in daytime dwell HPD, and B, overnight dwell HPD. Key Tf: transferrin standard protein, 20-5 µl: volume of normalised protein extract loaded onto the gel. The multiple bands beneath the main tranferrin band represent proteolytic breakdown products

4.3 Discussion

Few studies have evaluated host factors that might support bacterial growth in HPD. Those that have been undertaken have investigated factors in peritoneal dialysate that support the growth of Staphylococcus after the development of peritonitis (Alexander et al., 1987; Park et al., 2005; Kotsanas et al., 2007). Few studies have examined the peritoneal dialysate in healthy patients at the start of their PD career, and before any episode of catheter-related infection, to identify risk factors for future peritonitis (George et al., 1997; Caravaca et al., 1998; chung et al., 2003). In this chapter factors in HPD that might affect bacterial growth have been systematically investigated *in vitro*, factors studied were dialysate pH, the use glucose as an osmotic agent, and the presence of iron as trace metal in PDS, as well as the effect of catecholamine hormones. Comparisons were also made of staphylococcal growth in different patient HPDs.

The peritoneal dialysis solutions used in these studies are acidic (pH=5.5), however, during the dwell the fluid becomes modified in various ways and the pH rises to neutral or alkaline due to the movement of urea, creatinine, amino acids and protein into the PDF. This rise in pH occurs early, within 30 minutes of a PD dwell, (Duwe et al., 1981). Duwe *et al*. (Duwe et al., 1981) examined the pH *in vivo* during a 4.25% Dianeal dwell and found that pH rose rapidly from 5.2 to approximately 6.8 within 30 minutes and equilibrated at around 7.2 after 1 hour.

In general terms most bacteria grow best in the pH range from 6-8 which means bacteria survive in alkaline pH better than acidic. Coagulase-negative staphylococci grow readily on commonly used media under a broad range of growth conditions (Huebner & Goldmann, 1999). Alexander & Rimland, (Alexander & Rimland, 1987) reported that there were no significant differences in *S. epidermidis* growth in commercial dialysis fluid with altered pH (pH=4.95, 5.75, 6.35, 7.75) however, they did not study bacterial growth in peritoneal dialysate. A more recent study (Chaieb et al., 2007) examined *S. epidermidis* strains which were isolated from peritoneal dialysate and examined their ability to produce extracellular polysaccharide in media at various pH levels (3, 5, 7, 9 and 12). Chaieb *et al.* found that *S. epidermidis* slime production was pH dependent suggesting dialysate pH can affect *S. epidermidis* pathogenicity.

Another factor that might influence the risk of infection is glucose availability, as all bacteria need a carbon source. So, the total glucose of HPD was determined, and the effects of its availability on *S. epidermidis* growth investigated

Glucose of variable concentrations is used as an osmotic agent in PD solutions; however, a substantial amount of this glucose is absorbed from the peritoneal cavity leading to unwanted metabolic effects. (Holmes & Shockley, 2000; Sayarlioglu et al., 2004). Consistent with this, the glucose concentration tests in this chapter showed higher levels of glucose in the D.T dwell over the O.N HPD, which means the longer the PDS stayed within the patient the more glucose was absorbed, with the final glucose concentration more closely resembling the serum glucose concentration than that seen in the PDS.

One of the factors we thought might influence bacterial growth was trace metal availability, particularly the availability of iron. Iron availability in peritoneal dialysate has been studied before but with conflicting results (Scancar et al., 2003; Rottembourg et al., 1984 and Blake et al., 1996). Milacic and Benedik (Milacic and Benedik., 1999) studied Fe levels in a series of spent HPD (48 samples) using electrothermal atomic absorption spectrometry, which is a different method to that used in this study (Plasma Optical Emission Spectrometry). They showed the iron range for 45 samples was between 0.005-0.035 µg/ml while 3 samples were higher (0.075-0.135 µg/ml). Scancar *et al*. (Scancar et al., 2003) reported iron concentrations for 12 patients of 0.0034-0.0049 µg/ml, both studies have not stated which PDS been used making comparison hard. By contrast, Wallaeys *et al*. (Wallaeys et al., 1986) found the range was < 0.157µg/ml and increased after 2 months to < 0.21µg/ml. It is likely that, the Fe level range was vary in all studies depending on the method used. However, this study range 0.0025-0.09 µg/ml was in the same range of Milacic and Benedik study (Milacic & Benedik, 1999).

In order to study the growth of bacteria in both PDS and HPD, I established a standard set of conditions to study bacterial growth (number of bacteria and pH). Two different assays were used: endpoint growth and a continuous time course. Although, commercial PDS does not stimulate staphylococcal growth, probably as a result of both low pH (Sheth et al., 1986) and high sodium lactate concentration, the presence of Fe and catecholamines could support the growth of the bacteria to some extent.

Appleby and John, (Appleby and John, 1982) suggested that a 1-log drop in CFU is evidence of bacteriostatic activity. Studies performed to examine the growth of *S. epidermidis* and other organisms in sterile PD fluid prior to instillation into the peritoneal cavity have suggested that PDS is bactericidal (Diskin et al., 1983, Flournoy et al., 1983 ; Verbrugh et al., 1984), however, none of these earlier studies confirmed complete absence of bacterial cells during the time of incubation. McDdonald *et al.* (McDdonald et al., 1986) demonstrated a 1- to 1.5 log decrease in CFU during a 24 h incubation period in PDS suggesting that while PDS is at least bacteriostatic, it does not have the ability to completely eliminate bacteria. The MaDonald *et al*. study also reported that changing the glucose concentration and pH of PDS did not affect bacterial activity. By contrast, my findings are consistent with those of Sheth *et al.* (Sheth et al., 1986) and show that PDS can allow the survival of both coagulase-negtive staphylococci and *S.aureus* In this study, though the number of cells showed an increase during the first 24 hours of incubation, the overall numbers of viable bacteria decreased with time, with small numbers of residual organisms still present at 72 and 96. Unfortunately, Sheth *et al*. (Sheth et al 1986) did not mention which type of PDS was studied and therefore direct comparison with my data is not possible.

Both PDS and HPD are iron limited (Modun et al., 1998; McGregor et al., 1989; Williams et al., 1988), as evidenced by the fact that adding iron to the PD fluid resulted in bacterial growth stimulation. Pathogenic bacteria require a minimum iron concentration of 0.4 ~ 4.0 μM for growth, which is less than the free iron concentration in the human body (10^{-18} M), as most iron is either bound to iron-binding glycoproteins, such as transferrin and lactoferrin, or is intracellularly sequestered (Park et al., 2005). Therefore, in order for pathogenic bacteria to grow and cause human infections, they

must be able to free bound iron using iron-uptake system (IUS) (Ratledge and Dover 2000). The iron-uptake system in Staphylococci is divided into two classes: siderophore- and hemoprotein receptor-mediated iron uptake systems (IUS) (Cabera et al., 2001; Sebulsky et al., 2000; Mazmanian et al., 2003; Dale et al., 2004 ; Sellman et al., 2005). There is little data on the expression of these two IUS by Staphylococci *in vivo*, and none relating specifically to Staphylococci exposed to PD fluid (Chung et al., 2003). Shin *et al*. (Shin et al., 2001), have demonstrated that the transferrin receptor of *S.aureus is* expressed in body fluids. As HPD is not as complex as other human bodily fluids, particularly blood, and it is a relatively enriched medium for bacterial growth compared to artificial PDS, it should be possible in the future to use the *ex vivo* system I have established to study Staphylococcal behaviour and IUS during a peritoneal dialysis dwell (McDonald et al., 1986; Chung et al., 2003; Park et al., 2005).

Yamaji *et al*. (Yamaji et al., 2004) suggested that iron release from the transferrin that enters PD fluid during a dwell is pH dependent. Despite iron release being optimal at pH 5.2 the cases of peritonitis reported by Yamaji *et al* were all associated with PD fluid with an alkaline pH. Other factors that may influence availability of free iron in PF fluid are the levels of catecholamine hormones. It has been noted for some time that bacterial growth may correlate with the level of catecholamines (Renaud et al., 1930; Cooper et al., 1946). A number of studies (Lyte et al., 2003; Neal et al., 2001; Freestone et al., 2000; Lyte et al., 1993) have shown that *Staphylococcus* growth is stimulated by catecholamines present in human fluids such as plasma, and that the underlying mechanism involves iron removal from transferrin. It is clear from the results in this chapter that catecholamines stimulate bacterial growth in both PDS and HPD. The kidneys are responsible for elimination of catecholamines, and Ziegler *et al*. (Ziegler et

al 1990) has shown that norepinephrine and dopamine plasma levels are elevated in patients with renal failure. Furthermore, Martin *et al*. (Martin, et al., 2000) reported the presence of norepinephrine and dopamine in the urine of patients with hypotensive septic shock. While no precise measurement of peritoneal clearance of catecholamines in peritoneal dialysis has been documented it is likely that there will be appreciable movement of catecholamines into the PD dialysate during a dwell. This is likely to be increased during an episode of peritonitis as part of a systemic inflammatory response syndrome (SIRS).

Chapter 5.Analysis of the effects of exposure to HPD on production of *S.epidermidis* virulence factors

5.1 Introduction

The coagulase- negative staphylococci (C-NS) are the most frequent causes of CAPD infections (Piette and Verschraegen, 2009). The C-NS are normal skin commensal flora and for a long time were not considered to be pathogenic. However, because of their ability to form biofilms on the plastics that form indwelling medical devices, such as intravenous catheters, heart valves and replacement hips, they are now considered to be serious, if accidental, nosocomial pathogens (Spencer, 1998; Williams et al., 1995). *S. epidermidis* is the most commonly found C-NS involved in causing infections of the peritoneum of CAPD patients (Bint et al., 1987; Spencer, 1998; Verbrugh et al., 1986). Smith *et al,* (Smith et al., 1991) suggested that the ability of Staphylococcus to adapt and survive within HPD was because of their capability to: (i) grow in PDS (Verbrugh et al 1984; Wilcox et al 1990; Williams et al 1988) (ii) colonize intraperitoneal or plastic catheter surfaces (Hogt et al 1983; Spencer, 1988; Verbrugh et al., 1986) (iii) avoid phagocytosis or phagocytic killing by immune cells such as peritoneal macrophages and infiltrating polymorphonuclear leukocytes (McGregor et al., 1989; Verbrugh et al., 1983; Verbrugh et al, 1986). More recent studies emphasise the importance of *S. epidermidis* to adhere to artificial surfaces and to assemble large biofilm consortia as factors in the involvement of CAPD infections (Rohde et al., 2010; Gotz , 2002; Mack et al., 2006; Rohde et al., 2006).

Chapter 4 demonstrated that release of host factors in HPD converts PDS into a more supportive growth medium and examined the growth supportiveness of different patient HPD. It was shown that HPD represents an iron-restricted environment for infecting pathogens, and that addition of iron or catecholamines can increase bacterial growth.

Earlier studies have shown that growth of staphylococci in HPD induces production of two, iron-regulated cytoplasmic membrane proteins (Williams et al., 1988; Modun et al., 1992; Smith et al., 1991; Wilcox et al., 1991). Both iron limitation and exposure to catecholamines can increase bacterial virulence (Ratledge and Dover, 2000; Freestone et al., 2008). Since both factors clearly affected growth of staphylococci in HPD, it was important to investigate if virulence of the bacteria was also influenced. This chapter therefore analyses the effect of culture in HPD on production of the principal virulence factors of the main infecting agent in CAPD, *S. epidermidis*.

5.2 Results

5.2.1 HPD effects on production of staphylococcal virulence factors.

5.2.1.1 Haemolytic activity

The main haemolytic toxin made by *S.epidermidis* is the N-formylated alpha-helical peptide δ-toxin (McKevitt et al., 1990) which causes the lysis of erythrocytes by forming pores in the cytoplasmic membrane (Gemmell & Thelestam, 1981). Although both α and γ haemolysins have been detected in *S.epidermidis* isolates (Gemmell et al., 1997). Michelim *et al*, (Michelim et al., 2005) stated that little is known about the importance of haemolytic activity as a pathogenicity factor in *S. epidermidis.*

In this chapter, haemolytic activity in *S. epidermidis* was examined using cell wall protein fractions of the bacteria, where haemolytic activity is usually associated. These fractions were prepared by culturing the bacteria in both D.T and O.N HPD, washing the cell cultures and extracting the cell wall associated proteins using lysostaphin digestion, as described in Materials and Methods (Section 2.2.1.1). *S. epidermidis* cultured in Luria broth was the HPD culture control. The filter sterilised culture supernatants of the *S. epidermidis*-HPD/Luria broth cultures were also tested for secreted haemolytic activity, but none was found (data not shown). The cell wall fractions were normalised for protein concentration and then serially diluted in PBS in two fold dilutions. All of the 25 CAPD patient HPDs were analysed for their influence on haemolytic activity of *S. epidermidis*. Representative examples of haemolysis activity are shown in Figure 5.1 and the results of the complete study is summarised in Table 5.1.

Figure 5.1, Plate A shows the *S. epidermidis* haemolytic activity of cell wall extracts taken from bacteria grown in Luria broth, or patient HPD for D.T and O.N. Luria broth-grown bacteria (panel A) did not produce any toxin activity during the course of the assay. A trace of activity was produced in D.T grown bacteria (panel B), but the most striking results of all were seen with the O.N HPD grown bacteria (panel C). Very substantial amounts of haemolysin were produced that completely lysed the red blood cells. Plate B, panel A, and Plate C, panel A each show the assay results for the D.T and O.N HPD alone and by absence of any cell lysis confirms that the haemolysis seen is not due to any activity naturally present in the dialysates. Comparative analysis of all the patient HPDs showed the same difference between D.T and O.N dwells, (the results are not shown because of their similarity, but are summarised in Table 5.1).

A small trace of haemolytic activity was present in some of the D.T samples, and so investigations were made of what host factors might be increasing production of toxin by the bacteria grown in the O.N patient HPDs. Figure 5.1, Plate B shows that addition of either iron or the catecholamine stress hormones to *S. epidermidis* grown in D.T HPD significantly increased production of the haemolysin. Figure 5.1, Plate C shows that iron and the stress hormones also increased production of haemolysin from O.N-dwell grown bacteria, but less so than for the D.T HPD-grown bacteria.

Table 5.1 shows the summary of the haemolytic assays for *S. epidermidis* grown in the CAPD patient HPDs. Among the 14 CAPD patients tested (12 D.T and 13 O.N HPD samples in total) all the O.N samples were positive for induction of haemolysin, either with or without the addition of any stimulus such as Fe or stress hormones. In the case of the D.T samples, some were negative for haemolysin induction of *S. epidermidis* unless the bacteria were co-cultured with Fe or the catecholamines (patients 2, 4, 6 and 9). Interestingly in the case of patients 1, 7, 8 and 11-14, even addition of Fe or stress hormones did not induce production of the haemolysin. Since the catecholamines are clearly inducing *S. epidermidis* to make more haemolysin, it was hoped that a correlation could be made with HPD catecholamine levels. However, as explained in Chapter 4, since catecholamines and their metabolites are very unstable in alkaline media, or to storage un-frozen, by the time the samples arrived for analysis, it appeared the catecholamines had decayed to below detectable levels.

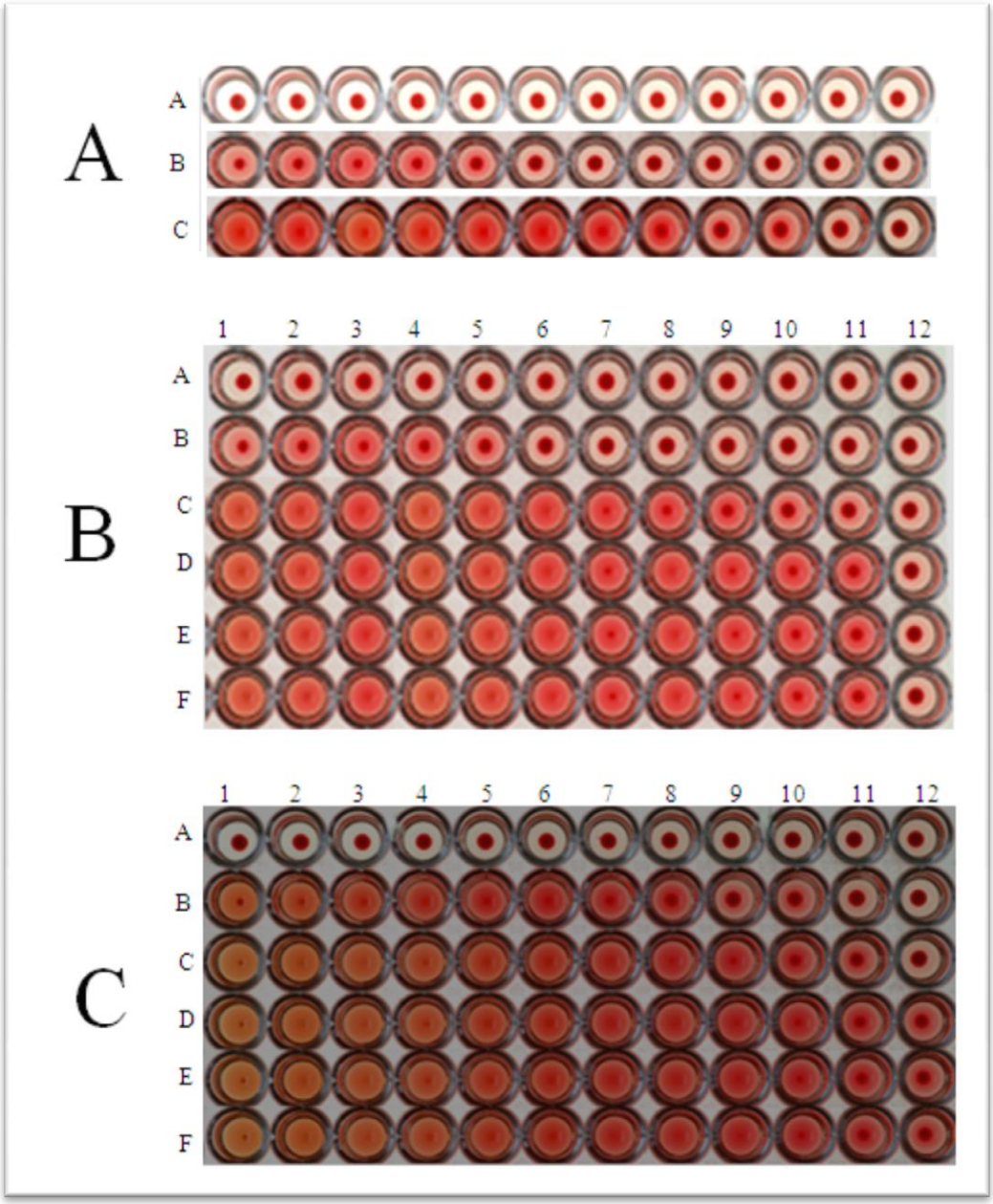

Figure 5.1 Haemolytic activity assays. Plate A: row A= Two fold serial dilution of *S. epidermidis* (Cell wall-associate protein) from bacteria grown in LB as negative culture control, row B = *S. epidermidis* was grown in D.T HPD, row C= *S. epidermidis* was grown in O.N HPD. Plate B: row A= HPD-D.T only; row B= *S. epidermidis* grown at D.T HPD. C= *S. epidermidis* grown in HPD-D.T+Fe (100µM); D-F= *S. epidermidis* was grown in HPD-D.T with 100µM catecholamines (Norepinephrine , D , Epinephrine ,E and Dopamine ,F). Plate C: row A= HPD-O.N only; row B= *S. epidermidis* grown in O.N HPD. C= *S. epidermidis* grown at O.N HPD +Fe (100µM) D-F= *S. epidermidis* was grown in HPD-O.N with 100µM catecholamines (Norepinephrine D, Epinephrine, E and Dopamine, F). The plates were incubated at 37 °C for 30 minutes;

Samples	D.T					O.N				
	Control	Fe	NE	EPI	DO	Control	Fe	NE	EPI	DO
1	-	-	-	-	-	+	+	+	+	+
2	-	+	+	+	+	+	+	+	+	+
3	-	+	+	+	+	+	+	+	+	+
4	------					+	+	+	+	+
5	-	-	-	-	-	+	+	+	+	+
6	-	+	+	+	+	+	+	+	+	+
7	-	-	-	-	-	+	+	+	+	+
8	-	-	-	-	-	+	+	+	+	+
9	-	+	+	+	+	+	+	+	+	+
10	-	-	-	-	-	+	+	+	+	+
11	-	-	-	-	-	------				
12	------					+	+	+	+	+
13	-	-	-	-	-	+	+	+	+	+
14	-	-	-	-	-	+	+	+	+	+

Table 5.1 Comparison of haemolysin induction by *S. epidermidis* grown in D.T and O.N HPD.C= control, no additions to HPD; or HPD with 100µM additions of Fe (Fe) Norepinephrine (NE), Epinephrine (EPI), E or Dopamine (DO). The – symbol indicates slight or no haemolytic activity (equivalent to Plate B, panel B); + indicates strong induction of haemolysin production (equivalent to Plate B, panel C).

5.2.1.2 Biofilm production.

As mentioned, an important factor in the ability of *S. epidermidis* to cause infections of CAPD patients is thought to be its capacity to form biofilms on indwelling plastics. The second virulence factor that was studied was therefore the influence of growth in HPD on biofilm production. As was done for the haemolysin study, HPD from all of the CAPD patients was investigated for its influence on *S. epidermidis* biofilm formation. A photometric crystal violet assay was used to investigate attachment to the well of a polystyrene culture tray. Analysis of the effects of inclusion of Fe and the catecholamine stress hormones were also included, as these have been shown by Lyte *et al*. (Lyte et al., 2003) to increase *S. epidermidis* biofilm formation in blood-like media.

The results in Chapter 4 showed Fe and the catecholamines affected *S. epidermidis* growth in PDS, and so as a first step biofilm formation of *S. epidermidis* in PDS with and without these factors was investigated. The *S.epidermidis* were cultured statically at 37°C in a humidified CO_2 incubator in different PDS glucose formulations (1.36, 2.27 and 3.68 % glucose) for 24 h and after processing of cultures as described in Materials and Methods, the degree of attachment (biofilm formation) was measured using the crystal violet assay and measuring absorbance of attached bacteria at 600 nm. The results in Figure 5.2 show that the *S. epidermidis* produced biofilm in the PDS, although in contrast to growth, the amount of bacterial attachment was not influenced by the glucose concentration. Also, the catecholamines all stimulated increases in *S. epidermidis* attachment to the plastic.

Figure 5.3 shows *S. epidermidis* biofilm production in D.T and O.N HPD. For all of the CAPD dialysates more attachment was observed in HPD than in PDS (Figure 5.2). Also, the bacteria cultured in the O.N HPDs showed greater levels of attachment than the D.T dwell HPD. Addition of Fe to the HPD increased attachment for both dwell types. The results also show variation in degree of attachment within the individual types of HPD dwell. Since *S. epidermidis* grows to a higher level in O.N HPD, the apparent increase in attachment in the O.N HPD-cultured bacteria, and the cultures to which iron was added could be due to increased culture density.

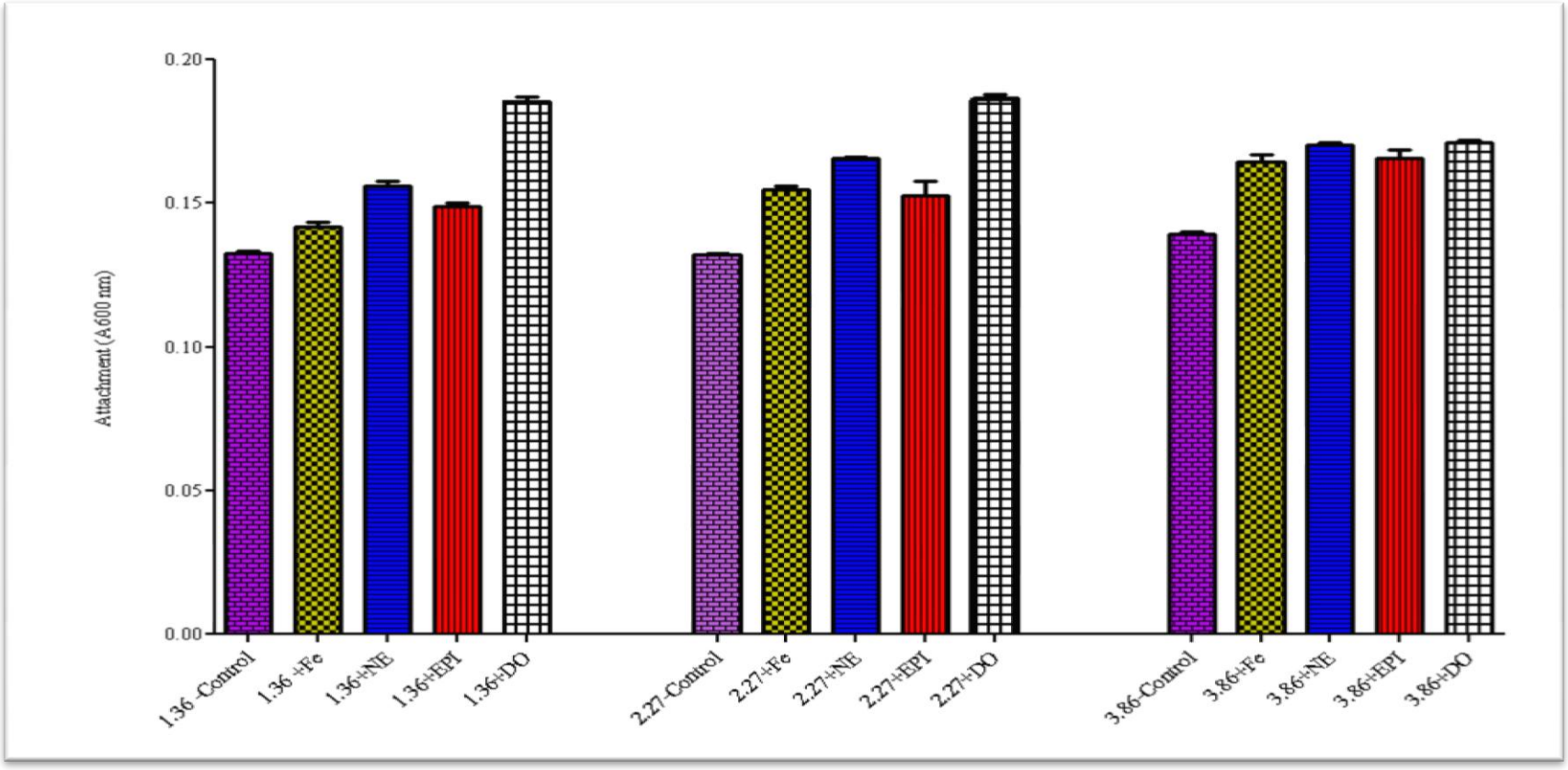

Figure 5.2 Biofilm formation by *S. epidermidis* cultured in PDS with and without Fe and catecholamines. 1.36, 2.27 and 3.68 represent PDS glucose formulations. C, unsupplemented control (PDS only), Fe, NE, EPI and DO, represent 100 µM additions to the PDS shown of $FeNO_3)_3$ (Fe), Norepinephrine (NE), Epinephrine (EPI), or Dopamine (DO).

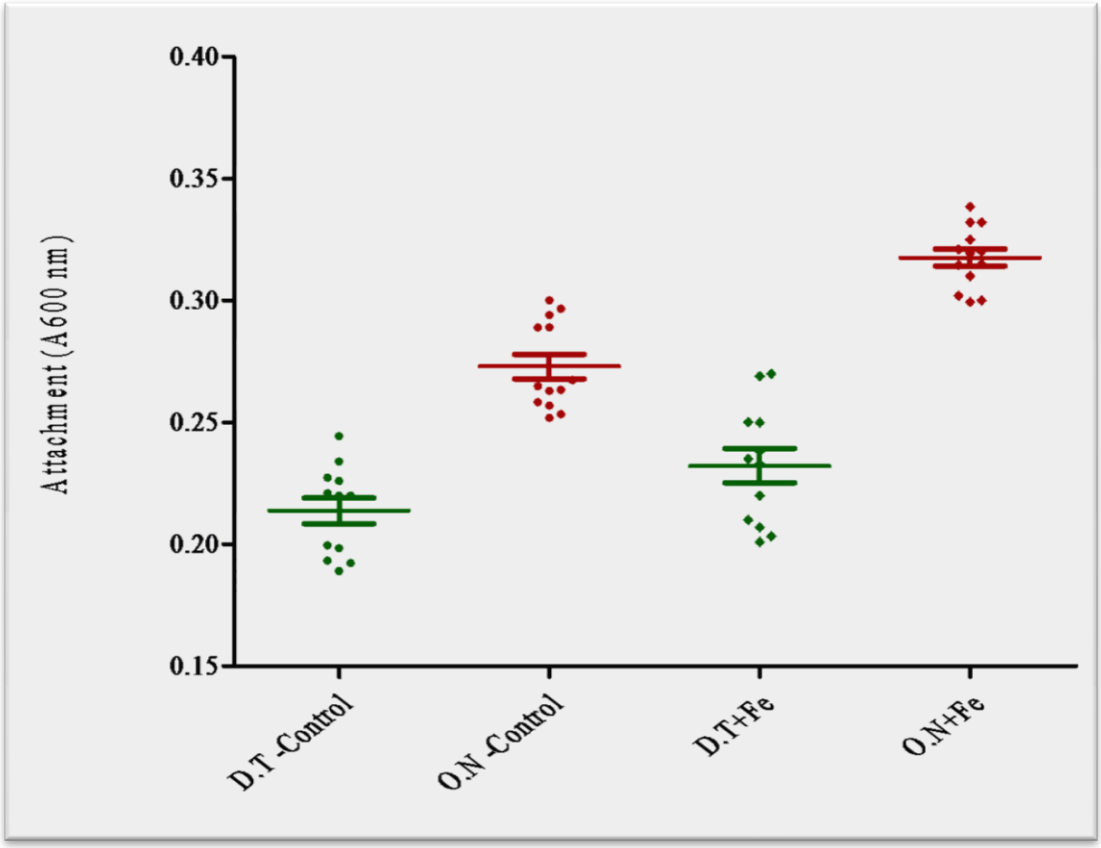

Figure 5.3 Biofilm formation by *S. epidermidis* cultured in HPD with and without Fe

S. epidermidis was cultured in the HPD shown with no additions (D.T-Control, O.N-Control) or HPD supplemented with 100 µM $FeNO_3)_3$) (D.T+Fe, O.N+Fe).

S.epidermidis biofilm production was also examined in the different HPD (O.N and D.T) supplemented with different catecholamines. The results in Figure 5.4 show that the stress hormones not only stimulate the bacteria to grow better, their presence in HPD also stimulates the bacteria to make more biofilm formation.

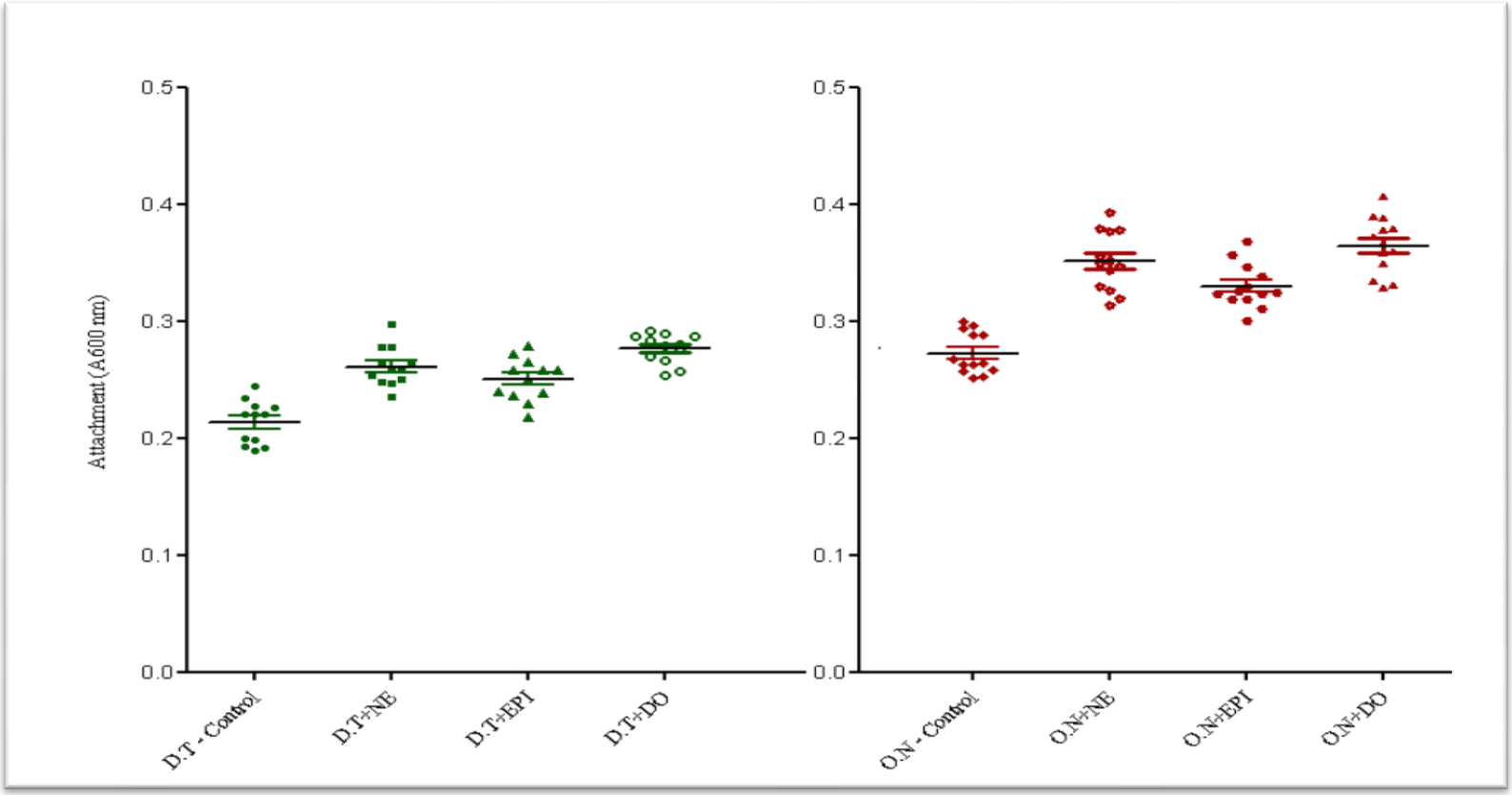

Figure 5.4 Biofilm formation by *S. epidermidis* cultured in HPD with and without catecholamine stress hormones. *S.epidermidis* was cultured in the HPDs shown with no additions (D.T-C, O.N-C) or HPD supplemented with 100 µM Norepinephrine, Epinephrine and Dopamine (D.T-Control, +NE, +EPI, +DO; O.N-Control, +NE, +EPI, +DO).

The influence of HPD on *S. epidermidis* biofilm formation on sections of Tenckhoff catheters plastic was also investigated. Figure 5.5 shows the ability of *S.epidermidis* to form biofilm in catheters grown in the presence of HPD (O.N and D.T dwell). The light microscopy pictures show that biofilm occurred in both dwell types, but that coverage of the plastic by *S. epidermidis* was more extensive in bacteria grown in the O.N HPD.

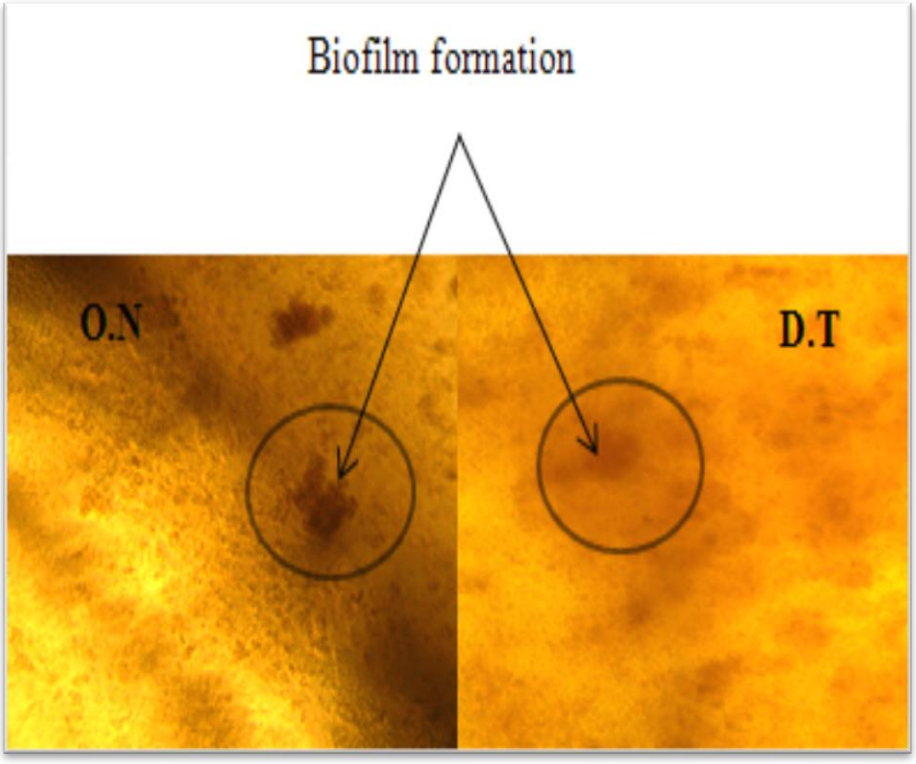

Figure 5.5 Influence of HPD on *S. epidermidis* biofilm formation on Tenckhoff catheter plastic. *S. epidermidis* was grown for 24 h in D.T or O.N HPD from patient with the highest total protein concentration , washed in PBS, and photographed using a digital camera attached to a inverted microscope Olympus ckx41(100 magnification). The areas of *S. epidermidis* biofilm are circled.

5.2.2. Investigation of the effects of culture in PDS and HPD on *S. epidermidis* cell wall protein profiles

PDS becomes chemically modified during dialysis due to the release of host proteins and other molecules. Chapter 4 showed that *S. epidermidis* becomes coated with the human iron binding protein transferrin during incubation in HPD, while section 5.2 showed that exposure to HPD also increases *S. epidermidis* biofilm formation. Since bacterial attachment to surfaces is at least partially mediated by cell wall proteins, these observations suggest that changes in cell wall associated proteins in *S. epidermidis* are likely to have occurred following exposure to HPD. An investigation therefore was made of the effects of incubation in HPD on the cell wall-associated proteins of *S. epidermidis.* Bacteria were cultured in the HPD, and cell wall associate proteins obtained by lysostaphin digestion as described in Chapter 2, Materials and Methods. Figure 5.6 shows the protein profiles of *S. epidermidis* grown in Luria broth, PDS (1.36%) and D.T and O.N dwell HPD. What is most striking are the differences between the profiles. Different culture media each produced different *S. epidermidis* protein profiles. For the HPD grown bacteria, some of the proteins shown are likely be of human origin. The binding of HPD-derived transferrin has already been shown (Chapter 4), and coating with other HPD proteins is also possible.

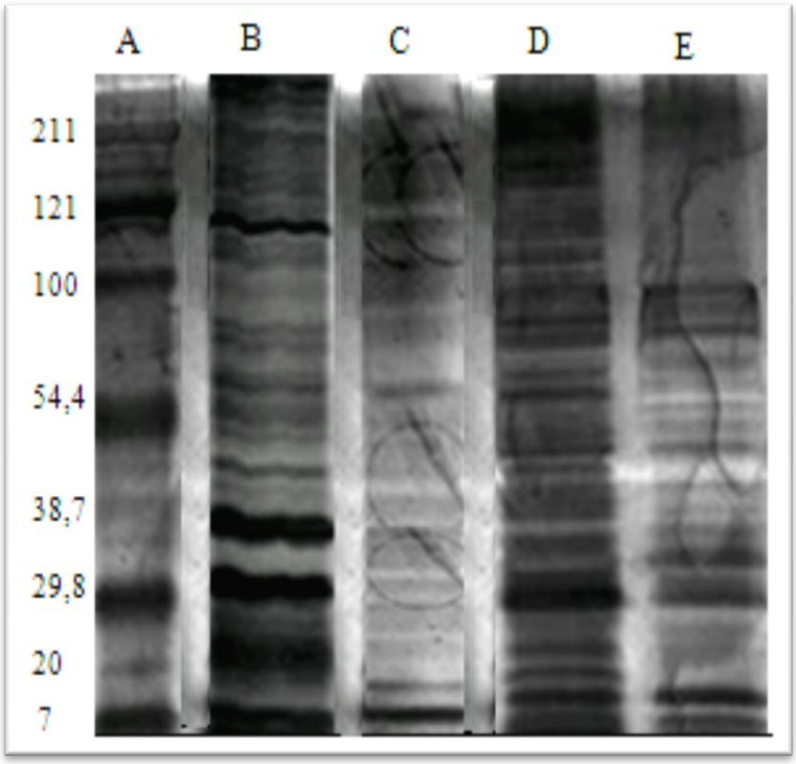

Figure 5.6 Cell wall protein profiles of *S. epidermidis* grown in PDS and HPD. Cell wall associated proteins were extracted from *S. epidermidis* grown in Luria broth (B), 1.36% PDS (C), O.N HPD (D) or D.T HPD (E). The molecular weight markers are shown in A and their sizes shown down the side in kDa.

Since Fe and the catecholamine stress hormones were found to increase *S.epidermidis* surface attachment and biofilm formation, the cell wall protein profiles of bacteria grown in HPD in the presence of noradrenaline, adrenaline and dopamine were analysed (Figure 5.7).

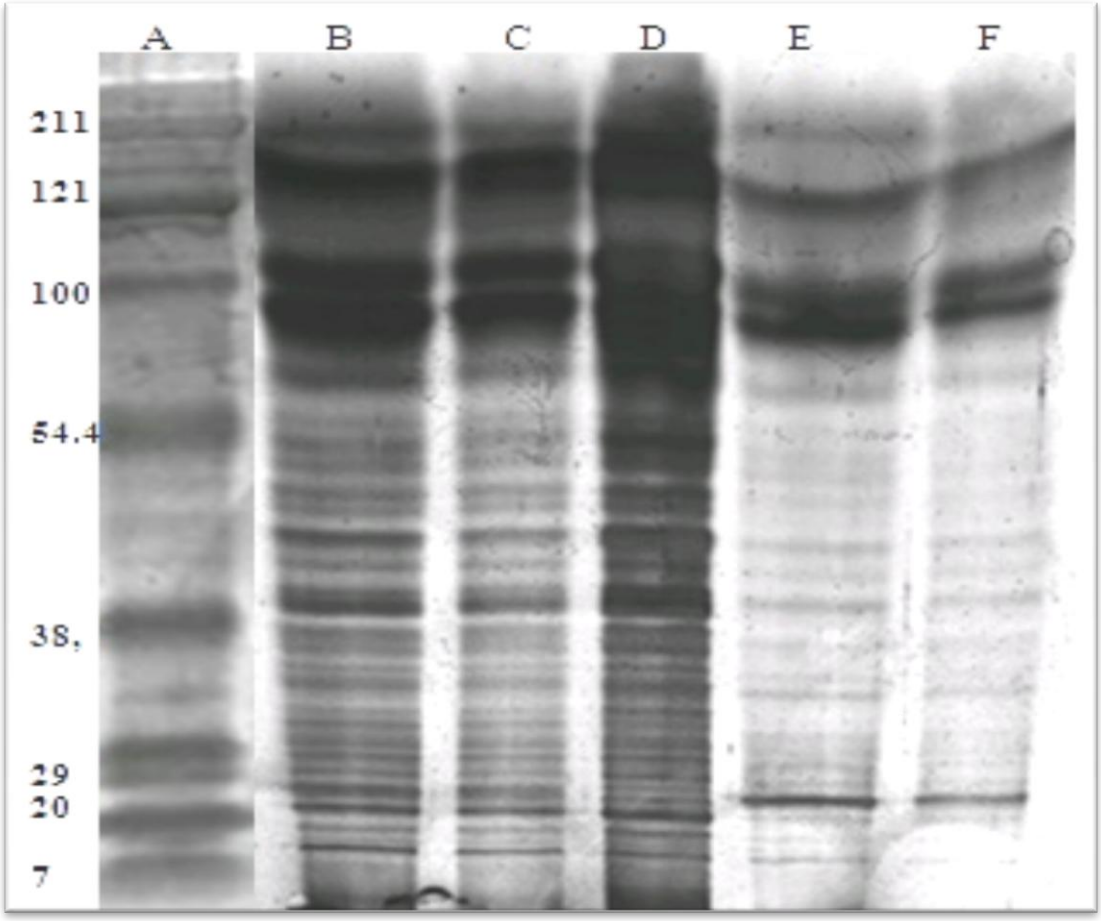

Figure 5.7 Cell wall protein profiles of *S. epidermidis* grown in HPD (O.N) with Fe or catecholamine stress hormones. Cell wall associated proteins were extracted from *S. epidermidis* grown in HPD only (B), or HPD supplemented with 100 µM each of $Fe(NO_3)_3$ (C), Norepinephrine (D), Epinephrine (E) or Dopamin (F) .The molecular weight markers are shown in A, and their sizes shown down the side in kDa.

5.3 Discussion

S. epidermidis is one of the most frequently isolated bacteria pathogens in hospitals in general, and is the most common pathogen involved in nosocomial infection of indwelling catheters (Vuong et al., 2002; Domingo & Fontanet, 2001; Tacconelli et al., 1997). Pathogenicity of *S.epidermidis* has been linked to three factors according to Michelim *et al.* (Michelim et al., 2005) (i) their resistance to antimicrobial agents (Boyce, 1999) (ii) production of invasins (Gemmell, 1996; Hebert & Hancock, 1985; Vuong & Otto, 2002) and (iii) biofilm formation (Pei & Flock, 2001; Vuong & Otto, 2002; Kristinsson et al., 1987).

Haemolytic activity has been detected in some *S. epidermidis* strains, with α-haemolysin having been reported to be associated with neurotoxin activity and γ-haemolysin with severe inflammatory responses (Dinges et al., 2000; Vuong & Otto, 2002). Also, some studies report the presence of a β–haemolytic activity in *S. epidermidis* (Bowden et al., 2005) Although the role of haemolytic activity in the pathogenicity of *S.epidermidis* is not clear, Michelim *et al.* (Michelim et al., 2005)found that clinical isolates have a significantly higher incidence of haemolytic activity than community isolates.

Molnar *et al.* (Molnar et al., 1994) found that different strains of *S. epidermidis* yielded considerably different haemolysin activity when different culture fluids were used. However, his study used strains isolated from different patients. The haemolytic activity analyses of chapter 5.1 showed that the toxin levels of *S. epidermidis* grown in D.T dwell were less than from bacteria grown in O.N HPD. From the analyses in Chapters 3 and 4, the main differences between the HPD types appeared to be related to glucose

levels and protein concentration. However, other factors could be involved in vivo, such as catecholamines as these were strong inducers of haemolysin production in bacteria grown in low haemolytic activity inducing D.T HPD. Interestingly, there were no correlations between D.T or O.N samples and protein concentration, as well as patient's status.

The second virulence factor investigated was biofilm formation, which is considered to be the main virulence factor in *S. epidermidis* infections related to indwelling medical devices such as catheters (Oasgupta eta al., 1987; Raad et al., 1998; Huebner & Goldmann., 1999; Frebourg et al., 2000). Biofilm formation on peritoneal catheters is commonly recognised as a potential reservoir of infection (Da Cunha Mde et al., 2004; Dasgupta & Costerton, 1989). Skin bacteria are the primary origin for bacteria which colonize peritoneal dialysis catheters, the material of catheters allows bacteria to adhere and the PD environment provides the condition for growth and proliferation of biofilm formation (Dasgupta, 2002).

The analyses in this chapter showed that O.N HPD induced the greatest level of *S. epidermidis* attachment and biofilm formation. Catecholamines also stimulated biofilm formation, possibly by delivering the iron from the host protein transferrin to *S. epidermidis* as had been shown by Lyte *et al*. and Freestone *et al*. (Lyte et al., 2003 and Freestone et al., 2008) To grow in iron limited conditions and establish an infection, pathogenic bacteria must be able to evolve mechanisms for obtaining iron from the host (Paul et al., 1988; Martinez et al., 1990). Most of these mechanisms involve producing low molecular weight compounds with high affinity for iron called siderophores, or development of proteins to steal iron from iron binding proteins such as transferrin or lactoferrin (Ratledge and Dover, 2000).

As mentioned before (Chapter 4), HPD is an iron-restricted medium. Iron limitation is an inducer of bacterial virulence including haemolysins (Ratledge and Dover, 2000), so the iron limited nature of HPD could be contributing to the induction of activity shown in Section 5.2. However, addition of Fe also stimulated production of haemolysin, which is inconsistent with this hypothesis. Catecholamines induce growth of bacteria by delivery of iron from host iron binding proteins (Freestone et al., 2000, 2002, 2003, 2008, Sandrini et al., 2010), but also have been shown directly to affect gene expression, including virulence factors such as toxins or surface attachment factors (Freestone et al., 2008).

Unfortunately, it was not possible for technical reasons to measure catecholamine levels in the HPD samples obtained. However, future studies should focus on determining if there is a correlation between catecholamine concentrations and induction of staphylococcal virulence factors. It is also possible that the inducers of haemolytic activity are neither catecholamines nor proteins, and are some other molecule yet to be identified. What has also become apparent in the investigations in Chapters 3, 4 and 5 is that there are significant differences within individual CAPD patient HPDs which can affect bacterial growth and virulence. Chapter 6 looks in more detail at those CAPD dialysates which were more or less able to induce growth or virulence. Most of the HPDs analysed were from patients in their early stages of CAPD, so Chapter 6 looks at several HPDs taken 18 months after the initial sample to investigate if changes in HPD characteristics occur over time.

Chapter 6.Follow up HPD studies

6.1 Introduction

Although peritoneal dialysis has been described as a simple, cheap and available method for most renal failure patients (Cozad, 1986; Sennfalt et al., 2002), the percentage of successful applications of the therapy over the long term is low due to persistent infections, and increased protein loss causing general muscle wasting and health. Some Nephrology units attempt to decrease the risk of peritonitis associated with the multiple dialysis bag connections and disconnections of daytime exchanges (Diaz-Buxo, 2004). Therefore some patients are changed from CAPD to continuous cycling peritoneal dialysis (CCPD) which is considered better than CAPD in terms of biochemical control (Alliapoulos et al., 1984). Infection prevention is considered essential for the successful maintenance of PD (Prowant, et al., 1993). Medical protocols for patients have set up a few strategies to decrease the infection risk including advice on catheter care, training patients to use aseptic techniques, monitoring patient's general stature and regular testing. Additional other methods to prevent infection include reducing the biofilm formation on catheters by including anti-microbials and treating any nasal *Staphylococcus aureus* with anti-microbial to prevent them acting as an infection reservoir (Keane et al., 2000).

Previous studies analysing the fate of CAPD patients have used a centre and registry data to study the factors affecting patient survival (Rumpsfeld et al., 2006; Churchill et al., 1998). There is a lack of studies using models that examine the combination of infection and other risk factors affecting the PD technique which can predict early possible PD technique failure (Tangri et al., 2008). One of the objectives set in the

beginning of this study was a follow up study of the characteristics of the HPD of CAPD patients after one year of peritoneal dialysis. Repeat HPD samples from 5 CAPD patients were kindly obtained from follow up visits at the Leicester General Hospital Nephrology unit. The follow up study in this chapter covers both patient related factors (protein characterization) and microbial-infection related factors (changes in the capacity of the HPD to support bacterial growth). During the 12 months after the start of dialysis 3/5 of the follow up patients had developed at least one episode of peritonitis. As mentioned earlier (Chapter 1, section 1.7), so an objective of the comparative analysis in this chapter is to determine if there are any distinguishing features of the follow-up patients HPD which might be useful as a prognostic marker(s) for susceptibility to development of infection.

6.2 Results

6.2.1 Characterization of peritoneal fluids from the follow up CAPD patients

6.2.1.1 Follow-up patient's clinical data

The 5 follow up patients in this chapter consisted of 2 female and 3 males with a mean (±SD) age of 59.6 ± 7.6 years. Table 6.1 shows the clinical data of the follow up patients, and show 3 out of 5 were diabetics. Most of the patients were also hypertensive, taking either beta or alpha blocker drugs. Several of the patient's PD

status had also changed after one year of dialysis and for clinical reasons 3 out of 5 were now on CCPD. During the 12 months following the start of dialysis 3/5 of the patients had developed at least one episode of peritonitis (patients 1, 3 and 4). Of these patients, two were diabetic (1 and 4); the other patient, 3, was non-diabetic.

Patient ID	**Type of HPD samples provided**	**Age**	**Sex**	**Diabetes?**	**Taking oral Fe**	**Taking a beta blocker**	**Taking an alpha blocker**	**Peritonitis in first 12 months?**
1	CCPD	67	M	Y	N	N	Y	**Y**
2	CCPD	58	F	Y	N	Y	N	N
3	CAPD (D.T,O.N)	54	F	N	N	Y	N	**Y**
4	CCPD	68	M	Y	N	N	N	**Y**
5	CAPD (D.T,O.N)	51	M	N	N	Y	N	N

Table 6.1 The clinical data of the 5 follow up patients. CAPD: D.T, daytime HPD dwell, O.N overnight HPD dwell, CCPD, continuous cycling peritoneal dialysis.

The follow up patients all used 1.36 % glucose PDS formulations in both types of PD treatment (CAPD and CCPD). Figure 6.1 shows the HPD sample type distribution used in this chapter. The highest percentages were the O.N samples followed by D.T; 3 out of 5 of the patients were moved to use CCPD.

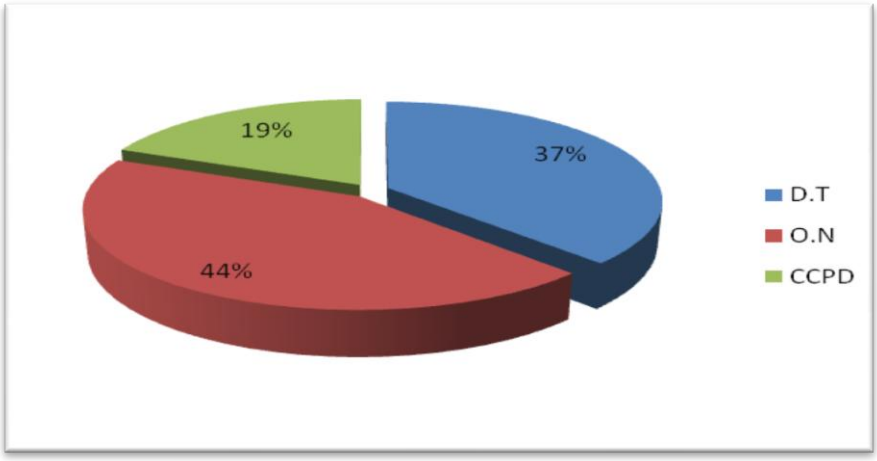

Figure 6.1 Distribution of the types of follow up HPD dwell samples CAPD: D.T, daytime HPD dwell, O.N overnight HPD dwell, CCPD, continuous cycling peritoneal dialysis.

6.2.1.2 Peritoneal protein characterisation of CAPD and CCPD patients

The protein concentrations of the HPDs from the CAPD and CCPD patients of the follow up cohort were measured using a Bradford protein concentration assay. Figure 6.2 shows the protein concentration in the 5 patients (16 dwell samples in total). It can be seen that in the two follow up CAPD patients the protein concentration, and therefore protein loss, has increased with the increased period of dialysis. Using the data in Figure 6.2, the protein loss (g/2l) of the HPD dwells was determined. Table 6.2 shows that in the D.T dialysis exchange patient no 3 has lost nearly 7 times more protein in his/her D.T dwell, and over 5 times in the O.N HPD in the 12 months following the start of the CAPD. After 12 months of CAPD, patient no 5 now loses 1.5 g/per day which is twice the value of the first HPD sampled. There were also significant differences in protein concentration between 1st and 12th month HPDs of two of the CCPD patients. Figure 6.2 compares the protein concentration of the CAPD HPD and the total concentration of the CCPD.

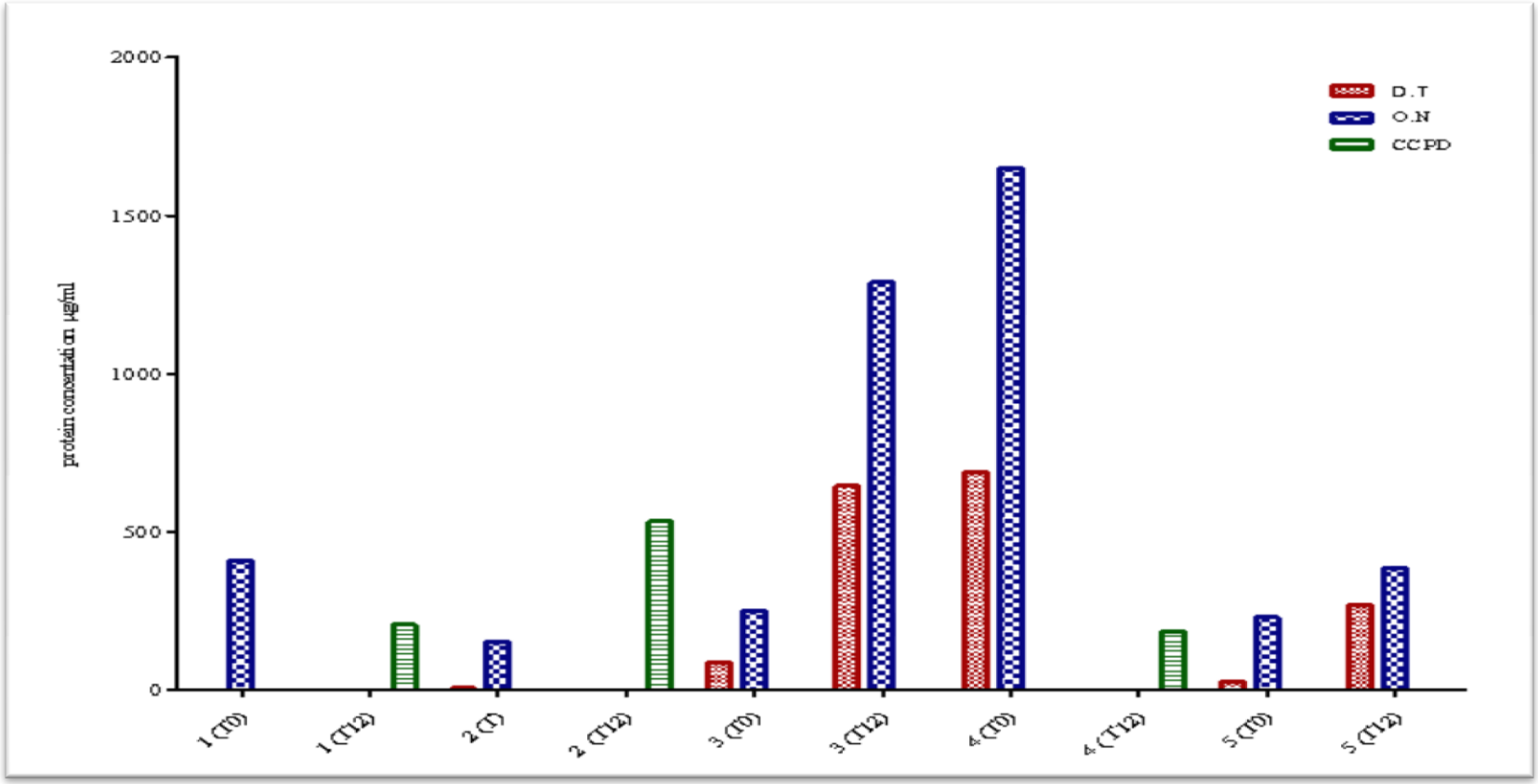

Figure 6.2 Protein concentration of peritoneal dialysates from CAPD follow up patients. The histogram shows the mean of two protein concentration measurements on HPD from the follow up CAPD patients (1-5) at the start of CAPD (T0) and after 12 months (T12);. For easier comparison purposes the data in the Figure is also shown in a table format in Table 6.2.

Patient ID	DT protein loss /2l)	O.N protein loss /2l)	CCPD protein loss /2l)
1 (T0)	--	0.818	--
1 (T12)	--	--	0.414
2 (T)	0.011	0.31	--
2 (T12)	--	--	1.066
3 (T0)	0.18	0.51	--
3 (T12)	1.29	2.58	--
4 (T0)	1.38	3.30	--
4 (T12)	--	--	0.37
5 (T0)	0.054	0.46	--
5 (T12)	0.54	0.77	--

Table 6.2 Protein losses per dwell of CAPD follow up patients. The data shows the HPD protein loss per 2l dwell from the follow up CAPD patients (1-5) at the start of CAPD (T0) and after 12 months (T12).

6.2.2 Characterization of the protein profiles of CAPD and CCPD patients.

Protein profiles for the 5 follow up patients were obtained using 12% SDS-PAGE gels. To check for differences between protein profile of the first (0) HPD samples and the 12 month follow up HPD (12), different volume of the HPD samples were analysed (5, 10, 15 and 20 µl). The 20 µl HPD tracks of the SDS PAGE gels for each of the patients are shown in Figure 6.3.

Figure 6.3 panels A and B, show the protein profile for the 5 follow up patients. Consistent with the increases in protein concentration shown in Figure 6.2 and Table

6.2, Figures 6.3 A and B show that for CAPD patients 3 and 5 the amount of protein present in the HPD has generally increased between the 1st and 12 months of dialysis, though the protein profile itself (numbers of proteins present) has not obviously changed. The protein profile of the CCPD HPDs from patient 1, 2 and 4 in Figure 6.3, C shows a similarity in trend to the CAPD results, though the CCPD 12th month HPD for patient 1 has less protein than the 1st O.N HPD.

In Figure 6.4, the protein concentration of the follow up HPDs was normalised, and similar amounts of protein (450µg/ml) run out on a large format 12% SDS PAGE gel, as described for Figure 3.7, to examine if there were differences in the types and numbers of protein released over time. Figure 6.4 shows the protein profile separation of 16 different dwell samples; the numbering at the top of the gel represents the patient and type of HPD, run next to each other for easier comparison. It can be seen that the profiles have undergone some changes, such as abundance – see the albumin-myristate complex protein levels. But, overall, the numbers of proteins are similar, and the protein profiles of the T0 and T12 have not undergone very major changes in terms of new proteins released. However, it is possible that there are novel proteins released into the HPD that are at very low levels that are not detected by the colloidal coomassie staining technique used to visualise proteins on the gels;

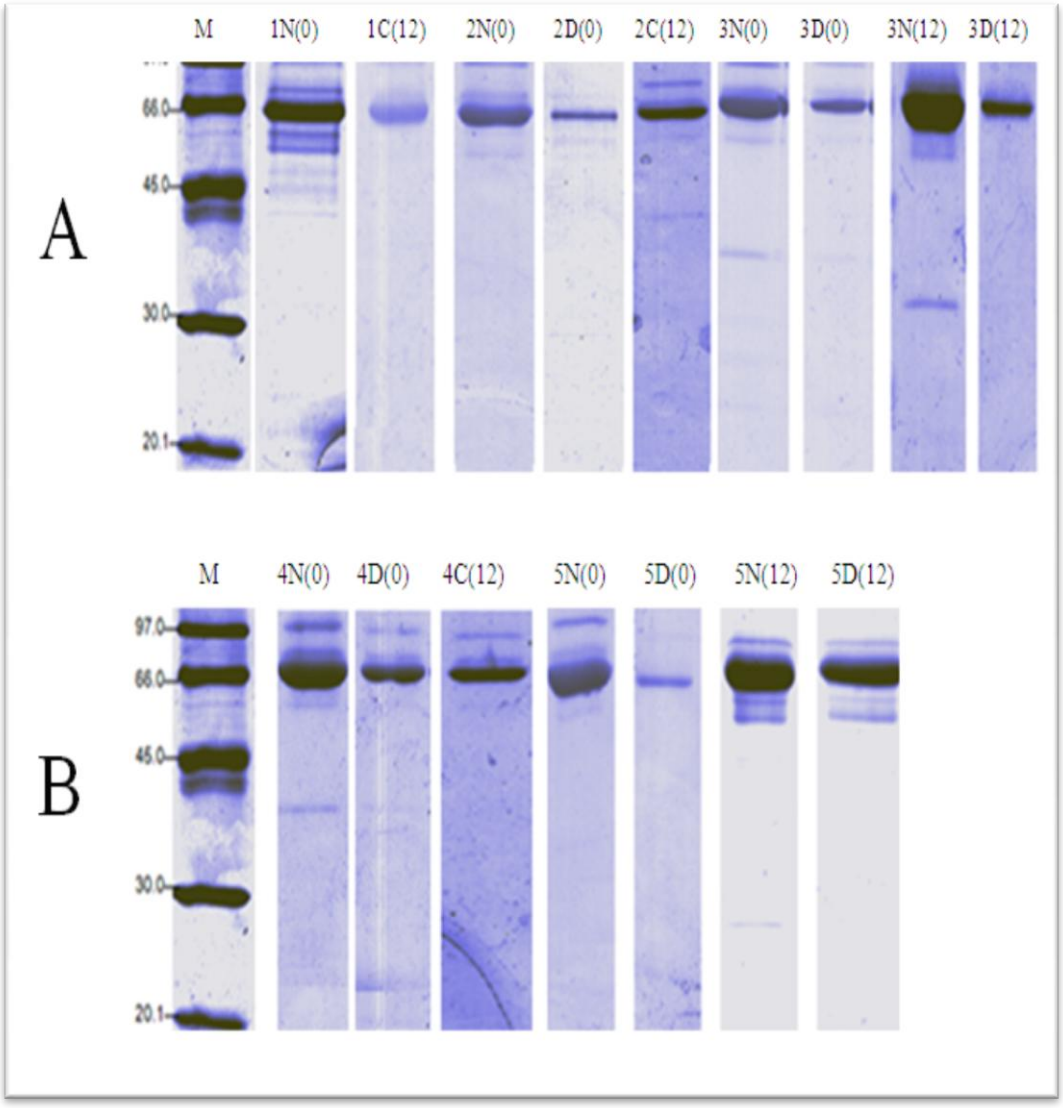

Figure 6.3 HPD protein profiles at the start of CAPD and after 12 months dialysis. Panel A: 12% SDS PAGE gel profile of dialysate from CAPD patients 1, 2 and 3; N = O.N, D = D.T first time HPD and C=CCPD. 0 and 12 represent HPD taken at the start of CAPD and the follow up HPD at 12 months. Panel B: 12% SDS for patients 4, 5.

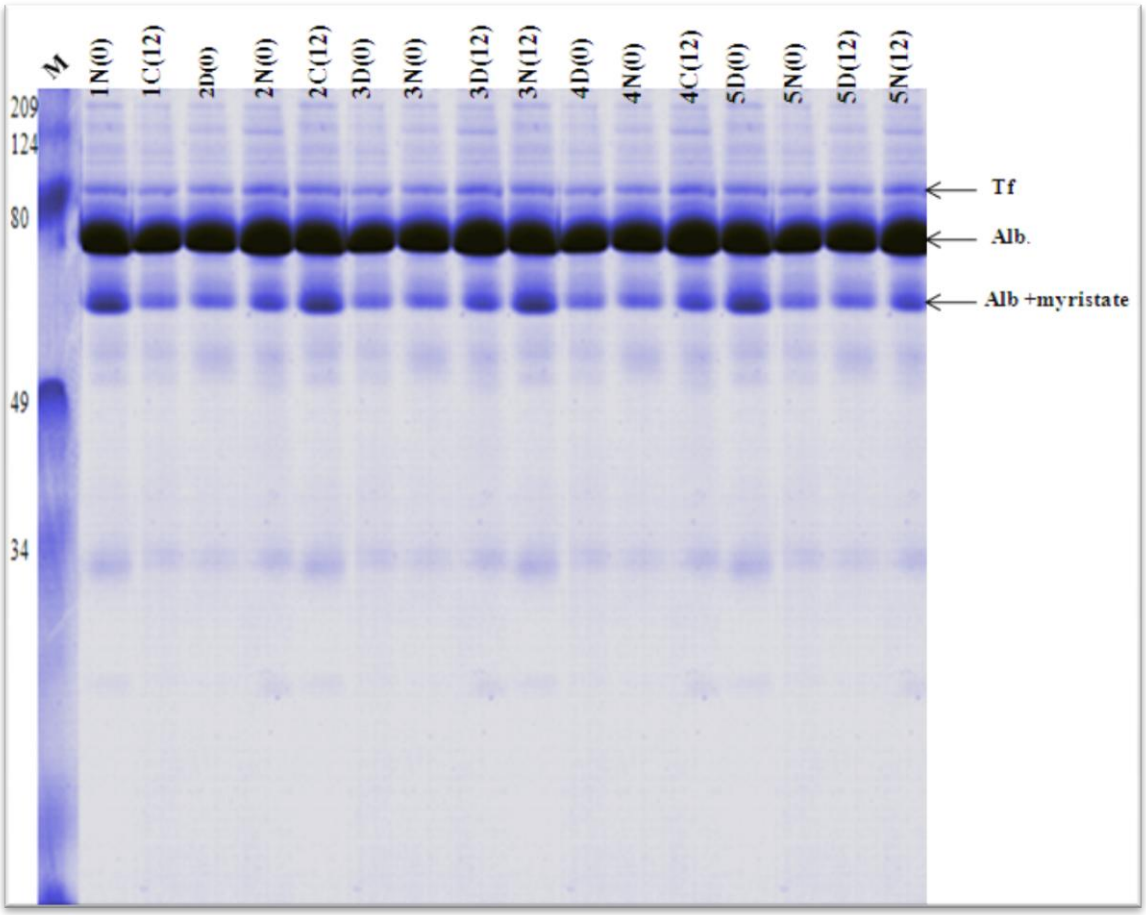

Figure 6.4 HPD protein profiles at the start of CAPD and after 12 months dialysis. Picture shows a 12% SDS PAGE gel profile of dialysate from the follow up CAPD patients (1-5) at the start of CAPD (0) and after 12 months; N= O.N and D= D.T, C=CCPD type HPD taken at 12 months.

6.2.3 Studying factors that might affect Staphylococcus growth in HPD.

6.2.3.1 HPD transferrin levels overtime.

Due to the importance of transferrin (Tf) as a source of iron for bacteria, Tf release in the follow up HPDs was also examined using an Elisa Kit. Figure 6.5 shows the differences in Tf levels between the patient HPDs. It is clear that there were significant differences in Tf released within the group (P value <0.05) and between the initial and 12 month dialysates, and that release of Tf into the HPD has increased.

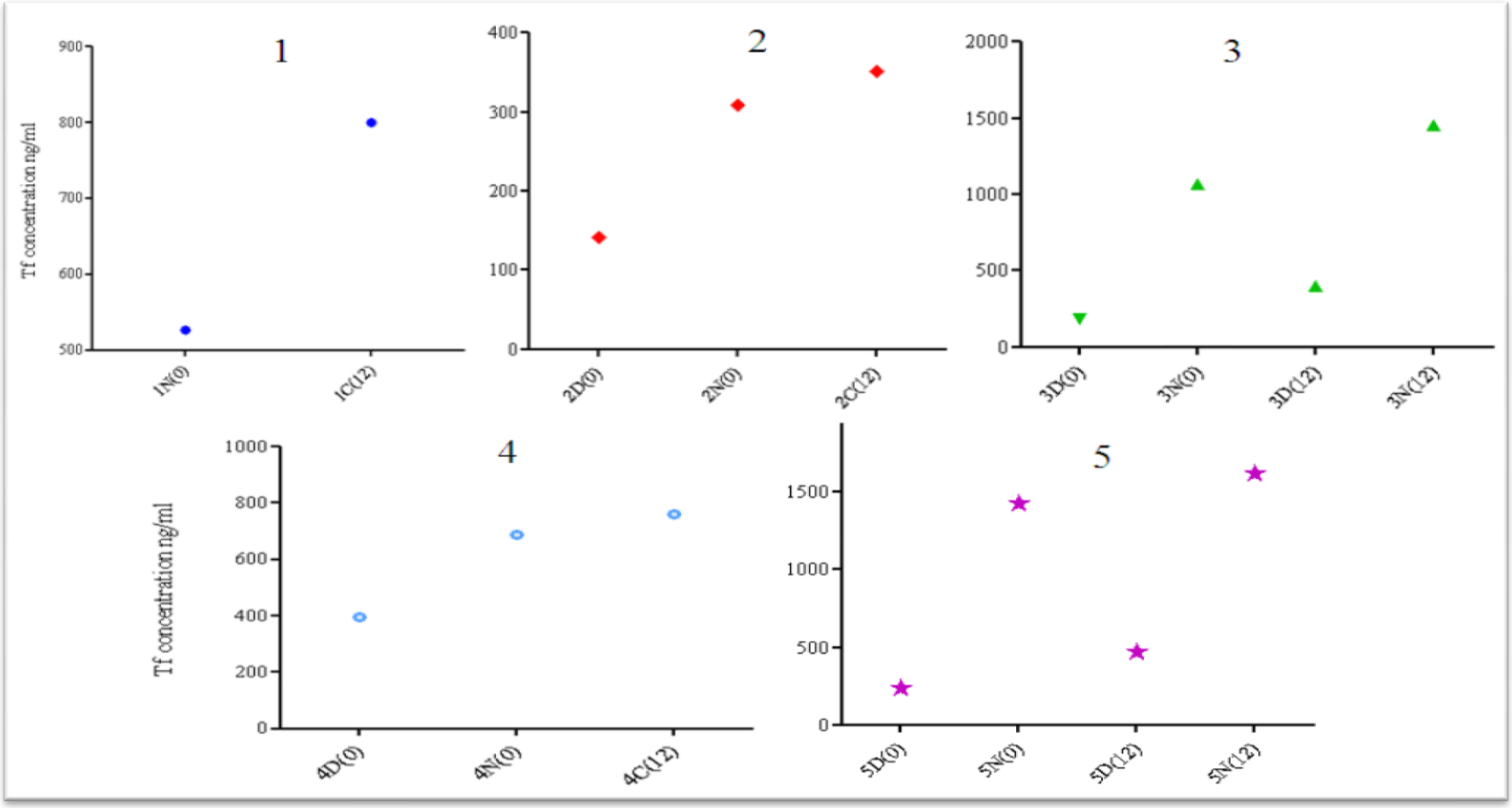

Figure 6.5 Transferrin levels at the start of CAPD and after 12 months dialysis. The numbers 1-5 represent patients 1-5; N = O.N, D = D.T HPD and C=CCPD. 0 and 12 represent HPD taken at the start of the CAPD and the follow up HPD at 12 months.

6.2.3.2 HPD pH over time

To determine if there were any change in the pH of the HPDs over time the pH of the patients initial and follow up HPD samples were measured 3 times and compared to the first time dialysis samples. Figure 6.6 shows there were no significant differences in the HPD pH between the first and 12 month dialysis, and between types of dialysis (CAPD vs CCPD).

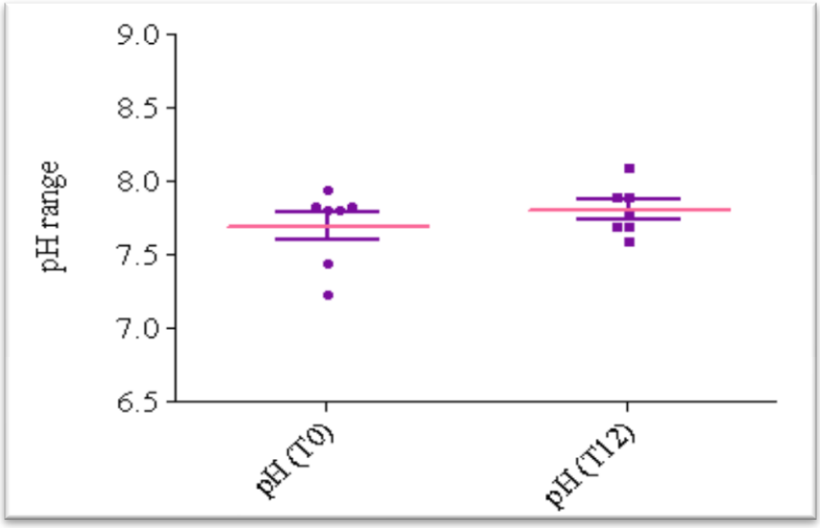

Figure 6.6 pH levels of HPD samples at the start of CAPD and after 12 months dialysis.

6.2.3.3 HPD glucose levels over time

Glucose levels of the follow up HPDs at the beginning and 12 months after the start of peritoneal dialysis are shown in Figure 6.7. Of the follow up patients, 3 of 5 (1, 2 and 4) were diabetics and were the group advised to change over to CCPD. Figure 6.7 shows the glucose changes overtime. It is clear that long time use of PD increases the glucose levels of the HPD for both diabetics and non diabetics.

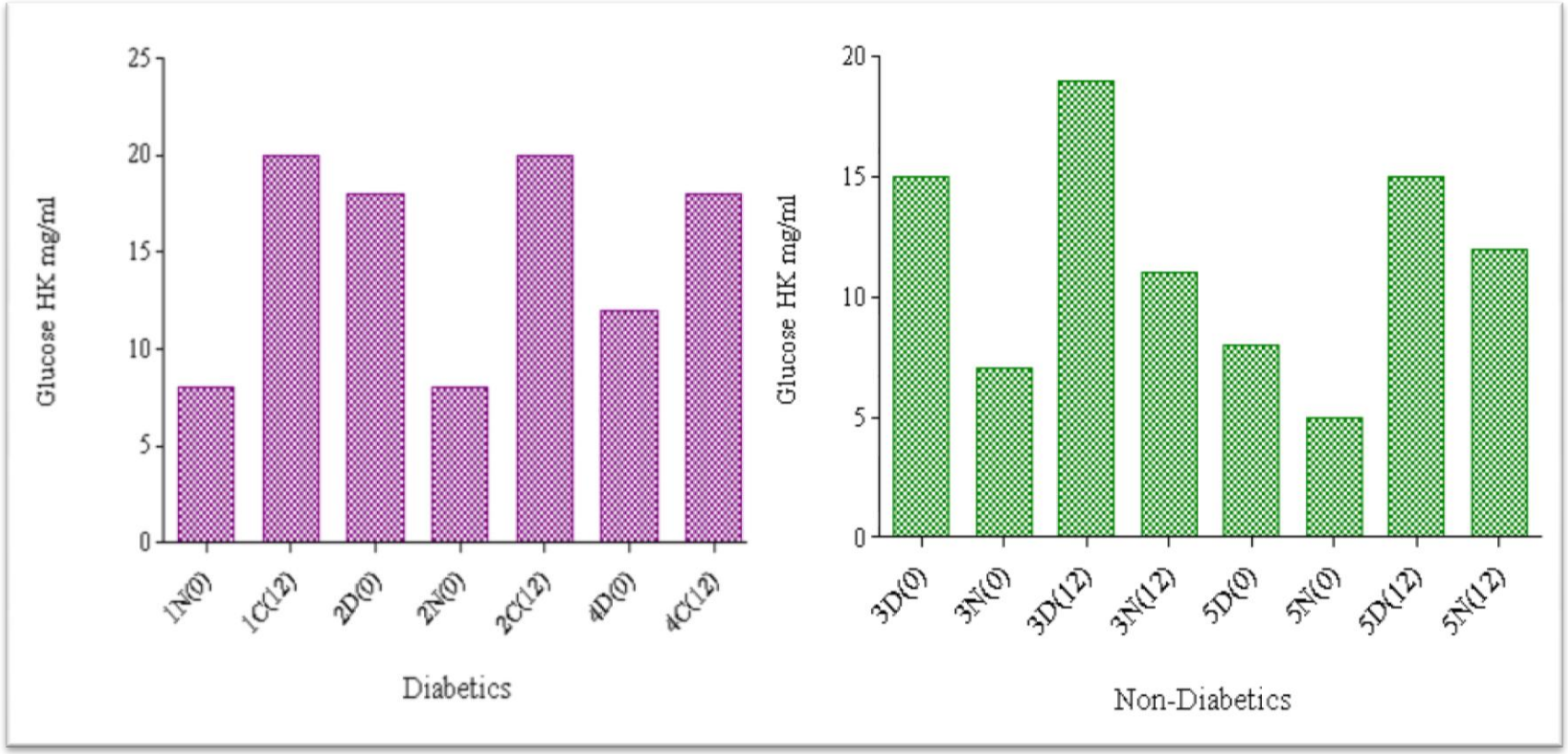

Figure 6.7 Changes in HPD glucose levels over time.The numbers 1-5 represent patients 1-5; N = O.N, D = D.T HPD and C=CCPD. T0 and T12 represent HPD taken at the start of the CAPD and the follow up HPD at 12 months.

6.2.3.4 HPD iron levels over time

Fe deficiency is a clinical problem in all forms of PD, and following changes in Fe levels gives a better understanding of the anaemia status of the patient over time. Since iron availability has been shown to be important for bacterial growth in HPD, monitoring Fe levels could possibly also report on the infection susceptibility of the patient. Total Fe levels were measured in duplicate using the ICPOES trace metal analysis technique and are shown at the start of CAPD and after 12 months in Figure 6.8. None of the follow up patients were taking iron supplements, which might explain why there was an overall decline in the free Fe levels. Fe levels becoming lower could be both positive and negative in terms of bacterial growth in HPD – lower availability might make it more bacteriostatic, but as iron limitation also increases bacterial virulence such as biofilm formation (Ratledge and Dover, 2000), any bacteria present might also become more pathogenic.

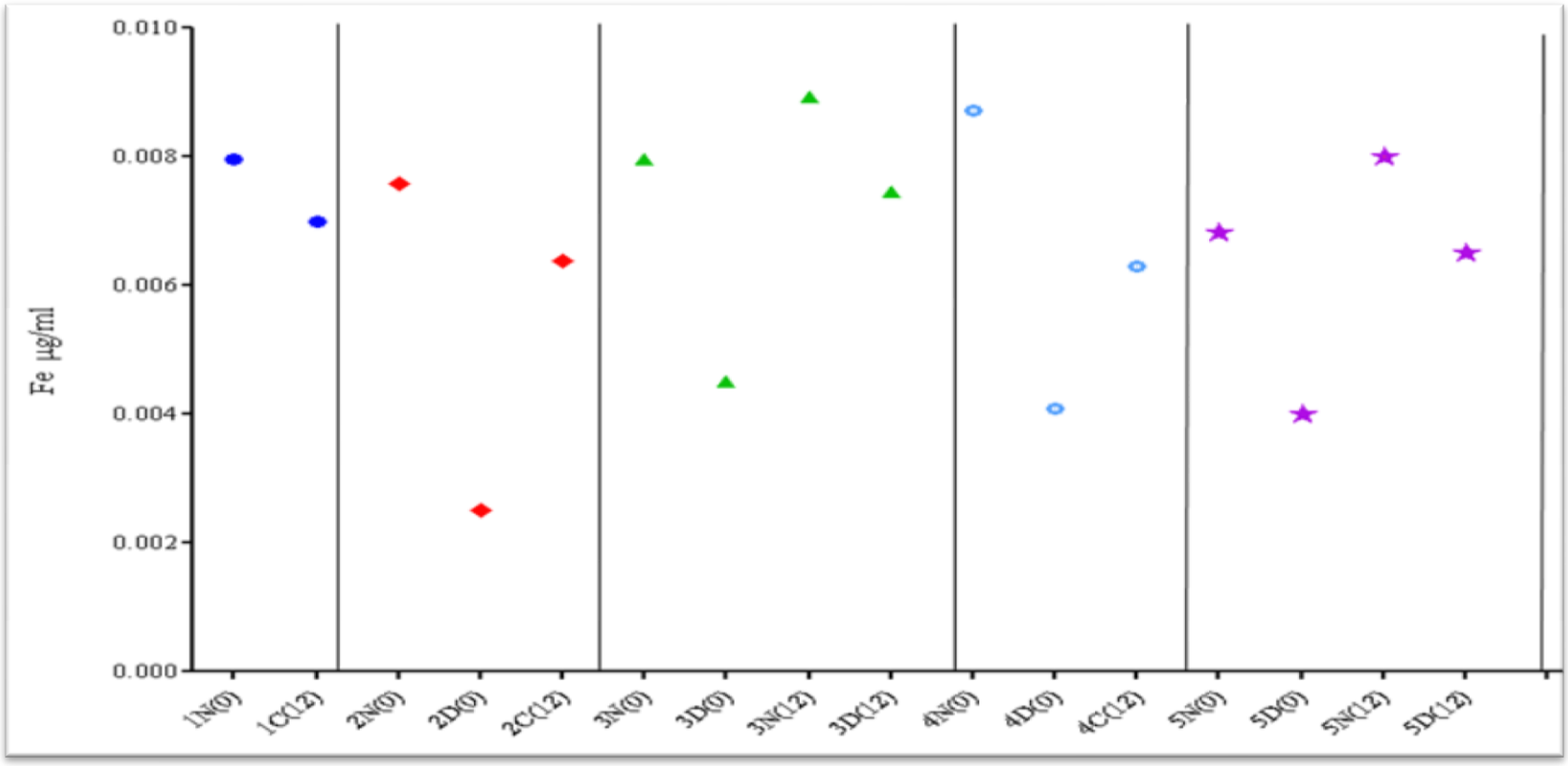

Figure 6.8 Fe levels at the start of CAPD and after 12 months peritoneal dialysis . The numbers 1-5 represent patients 1-5; N = O.N, D = D.T HPD and C=CCPD. (0) and (12) represent HPD taken at the start of the CAPD and the follow up HPD at 12 months

6.2.3.5 Changes in bacterial biofilm formation

The ability of the follow up HPDs to influence bacterial biofilm formation was compared with the original dialysates using the crystal violet attachment assay (Materials and Methods, section 2.2.3.2.2). The effects of the HPDs on *S. epidermidis* attachment is shown in Figure 6.9, and shows that the 12 month HPDs induced greater suface attachment than the first time for CAPD (P <0.0001). Figure 6.10 looks at the effects on attachment of addition of catecholamines and iron to the 12 month HPD from the follow up patients and shows that both factors increased the production of *S. epidermidis* biofilm.

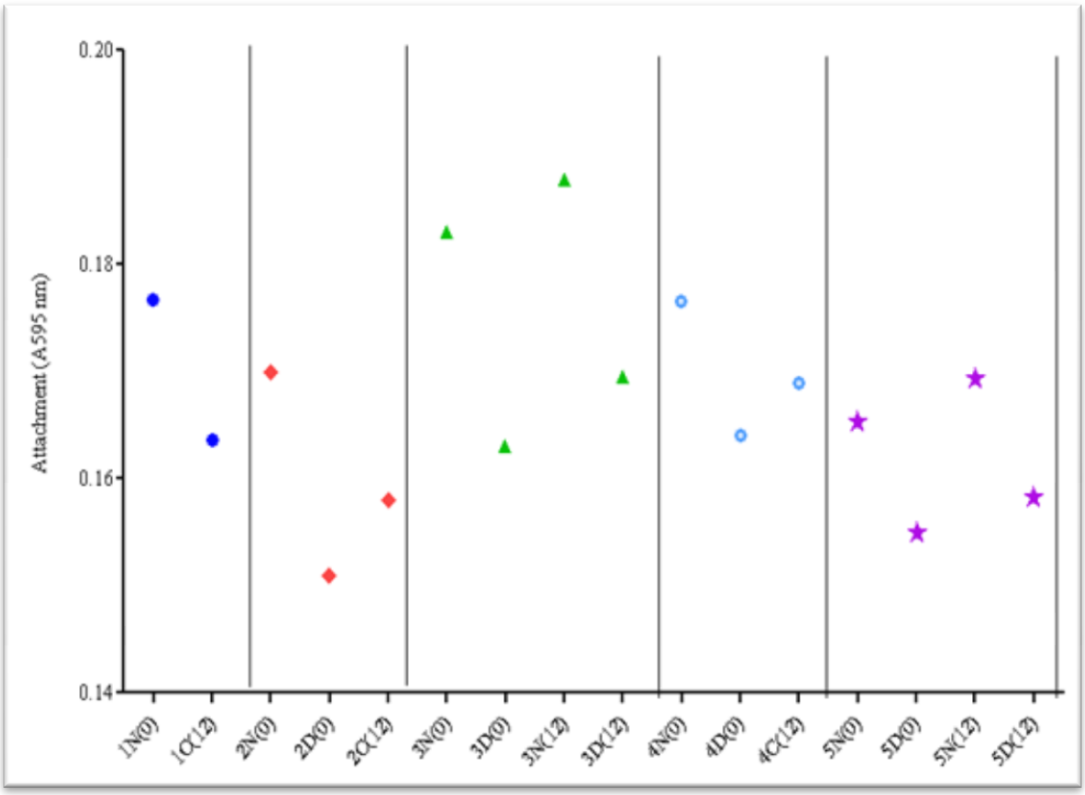

Figure 6.9 Influence of HPD on *S. epidermidis* biofilm formation at the start of CAPD and after 12 months dialysis. The numbers 1-5 represent patients 1-5; N = O.N, D = D.T HPD and C=CCPD. (0) and (12) represent HPD taken at the start of the CAPD and the follow up HPD at 12 months.

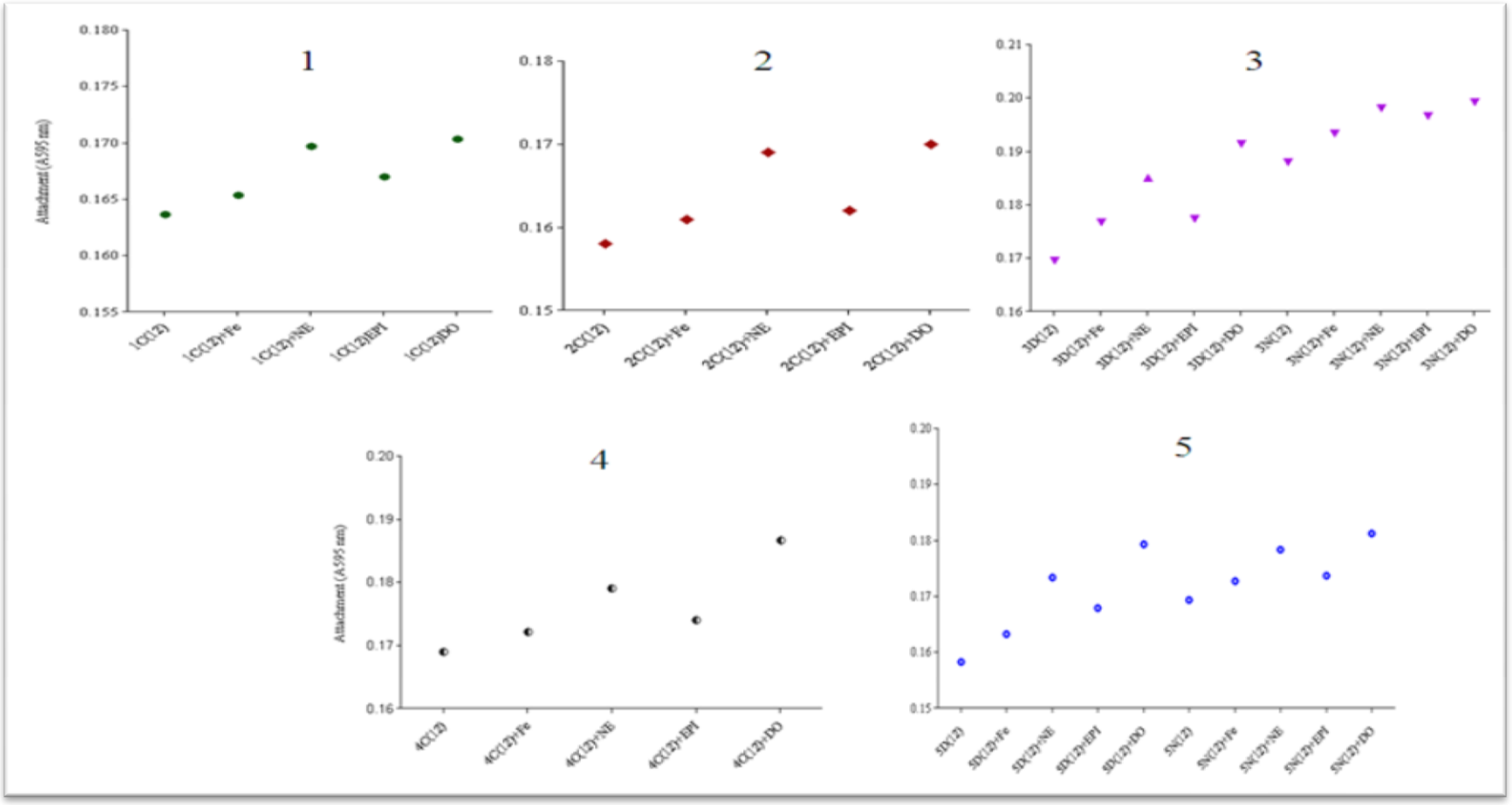

Figure 6.10 Effects of addition of catecholamines and iron to HPD on *S. epidermidis* biofilm formation after 12 months peritoneal dialysis. The numbers 1-5 represent patients 1-5; N = O.N, D = D.T HPD and C=CCPD. 12 represent HPD taken at the follow up HPD at 12 months.No additions (-ve), or 100µM additions of ferric nitrate (Fe),Norepinephrine (NE), Epinephrine (EPI) or Dopamine (DO).

6.2.4 Staphylococcal growth responses to follow up HPD

A comparison of growth responsiveness of *S. epidermidis* to the 5 PD patient follow up HPDs (T0 and T12 months) is shown in the time courses of growth in Figure 6.10. It can be seen that in un-supplemented HPD, compared with the initial HPD, the bacteria grew to a higher final optical density in the follow up HPDs. This indicates that the HPD has become less bacteriostatic over time. Figure 6.11, panels A and B show that addition of catecholamines or iron still increased growth of *S.epidermidis* in both D.T and O.N HPD. These results show that the stimulatory affect of the catecholamines and Fe had not change over time, and that after 12 months PD, the follow up patients HPD was still iron limited.

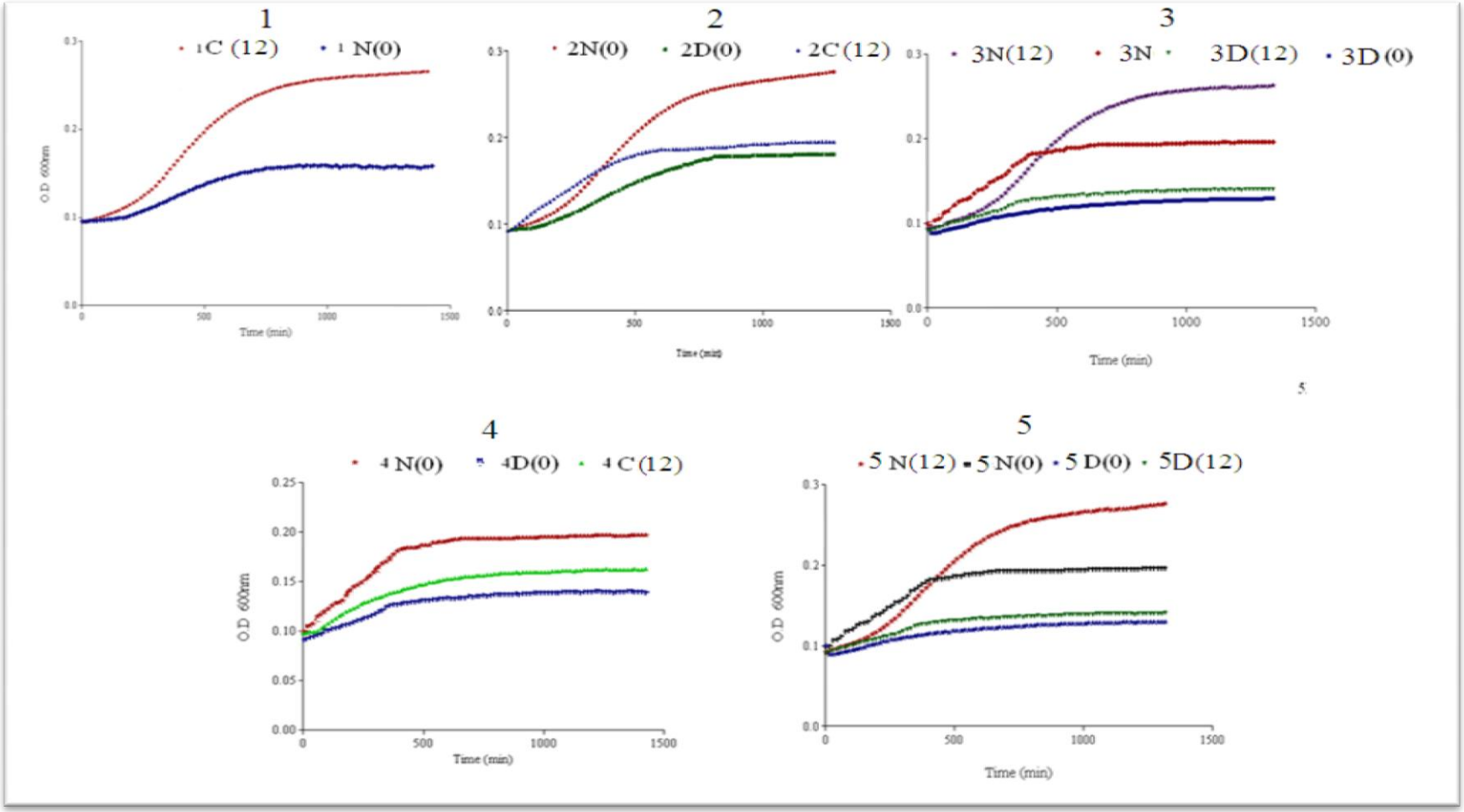

Figure 6.11 *S. epidermidis* growth in HPD at the start of CAPD and after 12 months dialysis for 5 patients with 16 dwell. 1= patient no1 with 1N (0) =O.N(0) and 1C (12) = CCPT(12) , 2= patients no 2 with 2D (0) =D.T(0) , 2N (0) =O.N(0) and 2C (12) = CCPT(12) , 3= patient no 3 with 3D(0) =D.T(0) , 3N(0)= O.N(0) , 3D(12) = D.T(12) and 3N(12) = O.N(12) , 4= patients no 4 with 4D (0) =D.T(0) , 4N (0) =O.N(0) and 4C (12) = CCPT(12), 5= patients no 5 with 5D (0) =D.T(0) , 5N (0) =O.N(0) , 5D(12) = D.T(12) and 5N(12)=O.N(12).

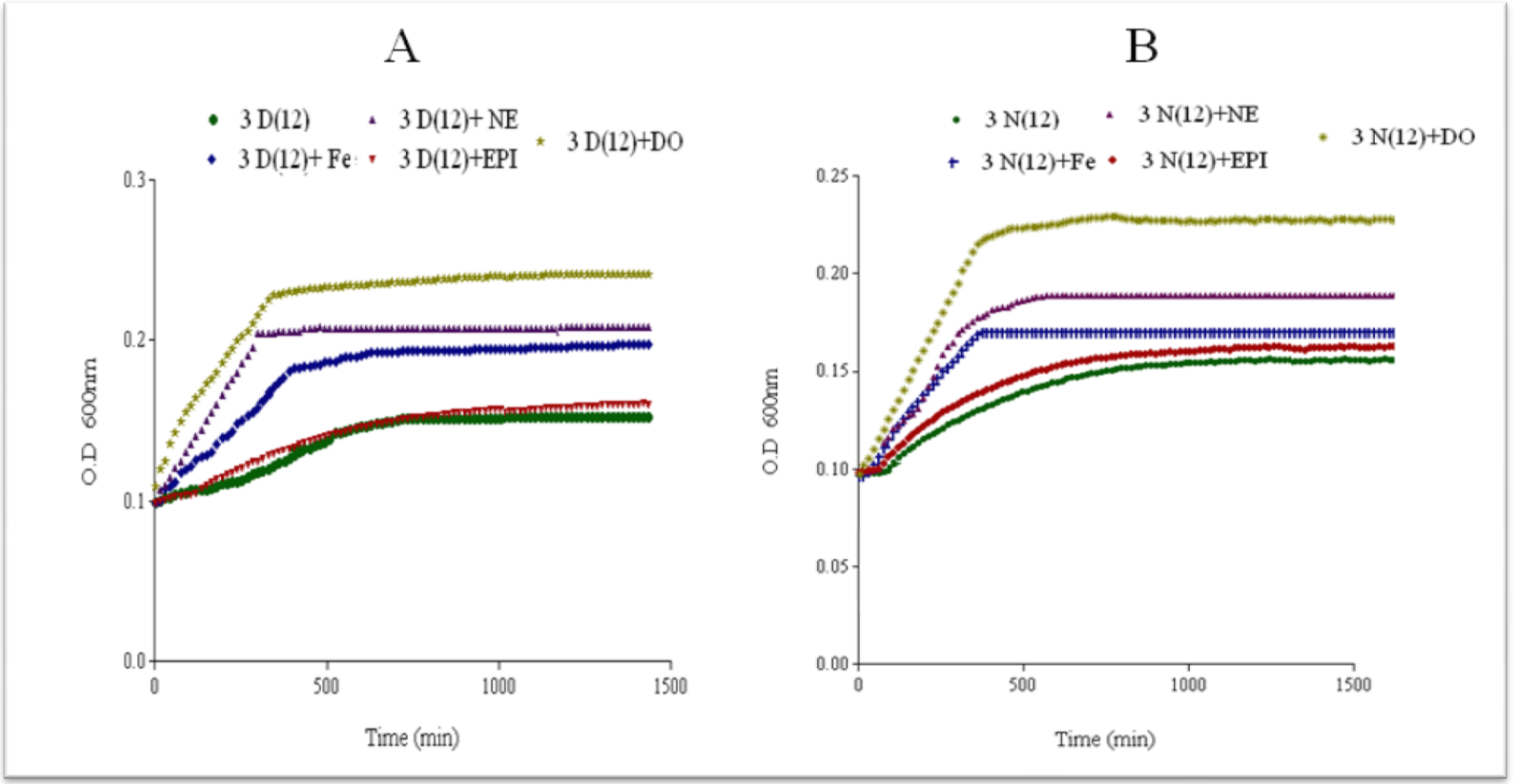

Figure 6.12 ***S. epidermidis*** **growth in HPD from patient 3 after 12 months** D.T **(**Panel A) or 12 months O.N (Panel B), Fe denotes the effect of adding 100µM ferric nitrate (Fe), NE, EPI or Do denote the effect of adding 100µM norepinephrine ,epinephrine or dopamine respectively.

6.3 Discussion

The intention of the comparative analysis of this chapter was to search for distinguishing features of patients HPD which might be prognostic marker(s) for development of infection, or future failure of the PD. An examination was made of the CAPD patients HPD status in terms of protein characterization and the ability of the HPDs to resist bacterial infection. Five initial CAPD patients provided after 12 months of dialysis follow up HPD samples.

Analysis of protein levels of the follow up patients HPDs showed protein losses had generally increased over the course of the 12 months PD, particularly for the CAPD patients. As was shown in Chapter 3, the CAPD follow up patients overnight PDS dwells had a greater net loss of protein than was found in the daytime dwells. This is in agreement with Westra *et al.* who found that protein losses during nightly cycling were higher than the daytime dwell protein loss (Westra et al., 2007). Poyrazoglu *et al*. also found a tendency for the protein levels of HPD to increase at the 1- year follow-up (Poyrazoglu et al., 2002). Dulancy *et al*. suggested that the quantity of protein lost is influenced by many factors, including the frequency and duration of the dialysis, composition of the PDS, peritoneal exchange area, serum protein concentration, and the general clinical status of the patient (Dulancy et al., 1984). In terms of the increased protein loss, Ho-dac-Pannekeet *et al.* reported that 57.3 % of PD patients showed a negative trend in net ultrafiltration between 0.3 months and 178 months post start of dialysis. (Ho-dac-Pannekeet et al., 1997). Also it has been shown that ultrafiltration failure increases with time spent on PD, presumably due to progressive damage to the

peritoneal membrane, causing greater 'leakiness' which might explained the protein loss increases observed in this and other studies (De Vriese et al., 2001).

The CCPD patients 1 and 4 showed a moderate reduction in protein loss compared to the original CAPD-produced HPD, possibly due to a reduction in the number of PDS exchanges occurring on the APD. Interestingly, Tjiong *et al*. reported that in their cohort of PD patients that there were no differences in overall protein loss (the sum of daytime and nightly APD dwells) between APD and CAPD patients (Tjiong et al., 2007). As with the initial (T0) CAPD-HPD protein concentration analyses, Table 6.2 showed that was still significant patient-patient variation in HPD protein loss within and between the follow up groups of CAPD and CCPD patients.

Three of the 5 follow up patients experienced at least one bout of peritonitis during their first 12 months of dialysis, and to analyse why this might have been so, protein profiles were compared between initial and follow up HPDs. Apart from differences in total protein concentrations, the profiles of normalised amounts of protein were generally similar between the start and 12 month dialysis points. As a result of this similarity, it was not felt necessary for re-sequencing of the proteins in the follow-up HPDs. In terms of other studies, Coles *et al*. showed that measuring IgG, C3 and albumin levels in HPDs was unsuccessful in predicting the risk of peritonitis, as protein loss generally increased with time (Coles et al., 1989). However, this study ignored the levels of transferrin in the dialysates. It has been shown in Chapter 3 and other reports (Freestone et al., 2000, 2003; Lyte et al., 2003, Sandrini et al., 2010) that Tf is very important as iron source for bacterial growth in host fluids such as blood, plasma and serum, and indeed HPD. So the Tf levels in the follow up HPDs were re-investigated., and the

result showed apparent increases over time. This increase in Tf could be correlated with the overall increase in protein loss.

Peritonitis can affect the exchange effectiveness of the peritoneal membrane. Although some studies have suggested that the peritoneal permeability returns to normal after treating the infection (Luo et al., 2000), other studies suggested that the peritoneal membrane may be affected longer term by peritonitis. These effects include poor fluid balance (Raja et al., 1984; Krediet et al., 1987), higher amounts of protein loss (Dulaney & Hatch, 1984), and lower serum albumin concentration (Young et al., 1983; Lindholm et al., 1989). Although, as mentioned before, 3 of this study's patients had a peritonitis episode, and increased protein result was observed, as indeed also levels of transferrin, but because of the small patient group size, it is hard to predict if the protein loss observed was a result of the peritonitis, or due to an overall effect of the damage caused by longer period of PD. More studies are needed to clarify the basis of this possible connection.

Glucose is a widely used osmotic agent but is also considered in some PD patients to contribute to metabolic disturbances during peritoneal exchanges (Lindholm & Norbeck, 1986; De Santo et al., 1979; Holmes & Shockley, 2000). Researchers have suggested that nearly 60-80 % (100-300 g/day) of the glucose in PDS is absorbed by the tissues in the peritoneal cavity (Holmes & Shockley, 2000). In this study, the second HPD biochemical parameter investigated in the follow on HPD samples was their glucose concentrations. The results showed an increase in the glucose levels of both diabetic and non diabetics patients. This suggests that less glucose is being absorbed by

the peritoneal membrane of these patients, which would be consistent with reduced efficiency of the exchange process.

The third HPD major biochemical parameter checked was total iron levels. Several studies have reported the iron deficiency in PD patients (Vychytil & Haag-weber 1999). As well as reporting on anaemia that might be present, because of the importance of iron as a nutrient for bacterial growth, HPD iron levels could be used to correlate with the infection susceptibility of the HPD. All of the patients in the follow up study had lower HPD Fe levels than when they started dialysis. The fact that both catecholamines, which chelate iron (Freestone et al., 2000, 2002, 2003; Sandrini et al., 2010) and free iron itself all increased growth in the follow up HPDs showed the 12 month dialysate, while supporting better growth than initial HPD samples, was still iron limited. None of the follow up PD patients was taking Fe supplements at the time of sampling, and of these 3 out of 5 had experienced peritonitis. However, despite lower levels of Fe, the follow up peritonitis patients all had higher transferrin levels, which of course can be used by any bacteria present in the HPD as a nutrient source, particularly in the presence of the catecholamines (Freestone et al., 2000, 2002, 2003; Sandrini et al., 2010). So, measuring Fe levels alone is probably not going to be a useful prognostic indicator for development of a future infection. But, measuring Tf levels, say using a simple dip stick methodology such as is used for glucose levels, has more promise and tests could easily be performed by the patient.

Biofilm information is an important part of the pathogenesis of bacteria such as *S. epidermidis*, and so a comparison examination was made of the follow up patients initial HPD and 12 month dialysates to induce biofilm formation. The result shows that

the 12 month HPDs supported more bacterial attachment, which is the initial and most important stage of biofilm formation. This suggests that there is a relationship between a time related increase in a component(s) of the HPD and induction of biofilm formation. Attachment of *S. epidermidis* to surfaces can be increased by the presence of deposited host proteins (fibrinogen etc) forming a matrix to which *S. epidermidis* cell wall associated proteins bind, thereby anchoring the bacterium (Rohde et al., 2010; Gotz, 2002; Mack et al., 2006; Rohde et al., 2006). So, increased protein release into the HPD could potentially provide a better protein coated attachment matrix. Alternately, since the results of this chapter also showed the *S. epidermidis* cultures grew better in the higher protein content follow up HPDs, higher final bacterial levels would also result in more *S. epidermidis* cells attaching. The reality could be a multifactor-process – more protein, more transferrin, more growth leading to greater attachment.

In summary, although the follow up PD group size was 5 patients, the results obtained from comparing their initial HPD with that produced 12 months later produce results, such as increases in protein levels, which agree with previous studies. In terms of finding a prognostic indicator, the growth and production of biofilm by *S.epidermidis* seems to show a positive correlation with protein concentration, with increased transferrin-complexed iron as also possibly part of the mechanism. So, monitoring for marked changes in protein levels, or levels of transferrin as a protein concentration marker, by a dip stick test so it could be carried out by patients as part of routine maintenance, is a possibility.

Chapter 7.General Discussion

7.1 Introduction

Chronic kidney disease is a progressive condition that results in significant morbidity and mortality. Lost kidney function can be replaced only by dialysis or by kidney transplantation. There are two types of dialysis: haemodialysis and peritoneal dialysis, the latter can be delivered in two principal ways: continuous ambulatory peritoneal dialysis (CAPD) and automated (machine-assisted) peritoneal dialysis (APD). Peritoneal dialysis is as effective as haemodialysis and has the advantage of both being easily delivered at home and more cost effective than haemodialysis. Peritoneal dialysis is also thought to be more suitable for patients with existing health conditions such as diabetes (Clase et al., 2004; Sepencer, 1998). Although CAPD is the simplest and cheapest form of PD, there are two major problems with this type of treatment: protein loss causing metabolic problems and infection leading to peritonitis. Peritoneal infection is the most serious acute clinical problem (Keane et al., 2000; Kim et al., 2004), causing significant morbidity and mortality (Fielding et al., 2002; Dalrymple & Go, 2008).

Peritonitis in patients on PD not only has significant short term effects on health, but also has a potentially long term impact on the efficiency of the entire dialysis process. Despite the frequency with which peritonitis can occur in PD patients (on average one episode every 18 months), and intensive patient education about the importance of aseptic technique during dwell exchanges, relatively little is understood about what predisposes patients to development of an infection. This project therefore used a novel approach which systematically analysed over time the peritoneal dialysate produced from 37 CAPD patient dwells for changes in its ability to withstand bacterial infection challenges. The hope was that factor(s) could be identified that might be useful to develop as prognostic indicators of infection risk.

7.2 Characterization of CAPD patient HPD

The first step of HPD characterization was comparing the total protein levels of HPD from the CAPD patients. The results in Chapter 3 showed that there was a significant difference between D.T and O.N dwells in terms of protein loss. The average total protein loss was 0.6 g/2l dwell for D.T samples and 2.58 g/2l for O.N dwells. This finding suggested that the longer dialysis dwell results in more protein loss than a short dwell, which is similar to that found in previous analyses (Cueto-Manzano et al., 2001). Considerable variation was found in terms of the total protein loss between the patients, although it was consistently found for all the HPDs analysed that overnight dwells resulted in greater protein loss than shorter day time dwells.

The protein profiles of the 14 sets of HPDs were investigated by SDS-PAGE, and the total numbers of proteins were found to show a high level of similarity. Sequencing the proteins showed that as expected they originated from the circulation (for example albumin, fibrin beta and some albumin complexes). Three proteins which had not been reported previously were also identified: ceruloplasmin, a copper binding protein, and albumin complexed with myristic and azapropazone, and albumin with myristic and tri-Iodobenzoic acid. Other major proteins in HPD included several important first line host defence proteins such as transferrin, IgG, complement C3, inter-alpha (globulin) inhibitor H1, and alpha-2-macroglobulin precursor. As well as the general metabolic effects of such protein loss, such as muscle wasting, loss of defence proteins could clearly have an additional impact on overall infection susceptibility. Since differences

were observed in the relative amounts of individual HPD proteins, variations in defence protein loss might also explain why some PD patients are more at risk of infection.

7.3 *S.epidermidis* growth in HPD

To cause peritonitis, bacteria must be able to grow in HPD. To investigate why some PD patients are more likely to develop peritonitis than others, the ability of *S. epidermidis* to grow in HPD was investigated in chapter 4. As with protein content, the bacteria grew in some patient HPDs better than others. Consistently, O.N dwells supported better growth than daytime dwells. Several sets of tests looked at HPD factors such as glucose availability and pH influences on growth, and found no clear influence. However, it was found that the protein concentration of the HPD did affect how well the *S. epidermidis* grew. In line with this, Fe availability of the HPD were investigated and it was found that addition of Fe increased growth of the bacteria, which indicated that the dialysates were all iron limited. Iron limitation in blood is caused by the presence of the iron binding protein transferrin (Ratledge & Dover, 2000). Western blot analysis using anti-Tf antibodies and a commercial Tf ELISA showed transferrin was present in all the dialysates. It was also found that the levels of Tf were related to total protein concentration; O.N HPD had more Tf than D.T dwells. Comparison of Tf levels between CAPD patients also showed differences. It was found that *S. epidermidis* directly bound Tf when growing in HPD, and that more Tf was bound when the bacteria grew in O.N dialysate. Since availability of iron is a major factor in the growth of *S. epidermidis*, differences in Tf levels could explain why the bacteria grew better in some HPDs than others.

The addition of catecholamine stress hormones adrenaline, noradrenaline and dopamine also significantly stimulated growth of *S. epidermidis* in HPD. It has previously been shown that catecholamines induce bacterial growth in blood and serum by enabling the bacteria to access the Tf iron (Lyte et al., 2003; Freestone et al., 2000, 2002, 2003, 2008). As Tf was present in HPD, this also seemed to be the explanation for the catecholamine growth stimulation of *S. epidermidis* seen in peritoneal dialysate. In normal kidney function, catecholamines are excreted in the urine and kept separate from Tf, while in PD both are eliminated into the same fluid. This is potentially an infection risk, as catecholamines remove iron which bacteria can then take up for growth. We hypothesised that dialysate catecholamine levels might also be an influence on how well the bacteria grew in the HPDs. Unfortunately, although it was tried, it was not possible to detect catecholamines in the CAPD patient HPDs. This was probably due to degradation since they are unstable during storage and tend to decompose, especially at higher pH values such as occur in HPD (Raggi et al., 1999; Kushnir et al., 2002).

7.4 Effects of growth in HPD on *S.epidermidis* virulence

Since the outcome of a bacterial infection is determined by both the number of bacteria present and the expression of virulence factors, analysis was carried out in chapter 5 to investigate if the patients HPDs also affected *S. epidermidis* virulence. The effect of growth in HPD on surface attachment, which is the initial stage of biofilm formation, was investigated, as was the production of haemolytic activity. Growth in HPD increased *S. epidermidis* attachment compared with PDS. Also more attachment was seen with O.N HPD than with D.T samples, which again might be related to protein

concentration (HPD proteins could be deposited on the plastic surface of the bacterial culture plate providing a matrix for *S. epidermidis* attachment) (Gotz, 2002; Rohde et al., 2010; Mack et al., 2006a; Rohde et al., 2006).

In terms of haemolysin production, growth in O.N HPD stimulated much more activity than did culturing the *S. epidermidis* in D.T dialysates. All of the CAPD patient overnight dialysates induced high level haemolytic activity production, while only a few D.T patients HPDs did. Addition of catecholamines to the D.T HPD increased *S. epidermidis* haemolysin expression to the levels produced in the overnight dialysates. Catecholamines levels increase during stress, and stress hormone release is associate with increased infections (Freestone et al, 2008). Catecholamine stress hormones have also been shown to increase bacterial virulence, including biofilm formation (Lyte et al., 2003, Freestone et al., 2008), as they also did for *S. epidermidis* biofilm formation and haemolysin production. It is therefore unfortunate that it was not possible to measure catecholamine levels in the HPDs, and this has to be a priority for future work.

7.5 Can the risk factors for peritonitis be predicted for new CAPD patients?

Identifying the infection risk factors for peritonitis in patients starting out on CAPD may help to tailor future treatment and training to reduce the incidence of peritoneal infections (Kotsanas et al., 2007). The risk factors for peritonitis previously reported include age, race diabetes, higher blood mass index, staphylococcal nasal carriage, PD bag connections and low serum albumin (McDonald et al., 2004; Bordes et al., 1995; Kotsanas et al., 2007). On average patients can expect one episode of peritonitis every

18 months, and in chapter 6 we examined the characteristics of HPD from patients who had been on CAPD for a year. The aim was to investigate if protein elimination profiles changed with the longer duration of peritoneal dialysis, in order to determine if biomarkers that might be prognostic for infection susceptibility could be identified.

Only 5 of the initial 14 patients were still maintained on CAPD, and so the follow up sample group size was limited to 5. Of this group, 3 patients had experienced at least one bout of peritonitis, 2 of whom were also diabetic. Interestingly, it had been found in chapter 4 that diabetic patients had higher than average levels of protein in their HPD, and that *S. epidermidis* grew particularly well in their HPDs. Almost all (4/5) of the follow up patients showed up to a 10- fold increase in HPD protein loss. *S. epidermidis* growth assays and biofilm attachment tests all showed significant increases relative to the original HPD sample.

These follow up patient HPD results, combined with the findings from chapters 3, 4 and 5 suggest that a higher dialysate protein concentration, with increased transferrin-complexed iron, is the common feature of those HPDs that better support *S. epidermidis* growth and biofilm formation. So, monitoring for changes in protein levels, or specifically levels of transferrin, ideally by development of a self administered dialysate dip stick test, could in the future form part of routine surveillance in patients on PD to identify individuals at greatest risk of peritonitis.

7.6 Future work

This project has shown for the first time that significant variation exists in how bacteria respond to PD patient dialysates. The protein concentration of the HPD seems to be an influential factor in how well the dialysate resists infection. So, a follow up study, involving many more patients in which periodic analysis of protein levels, possibly using transferrin as a marker since its presence seems to be important for growth, could be helpful in identifyng patients at risk of infection. A rather more specialist but very revealing test would be to periodically directly measure how well the HPD supported bacterial growth.

In the case of haemolysin production, other factors such as the presence of catecholamines or other patient factor(s) not identified so far could also be an important influence on infection susceptibility, and so measuring the catecholamine levels in HPD is a major future priority. Creating HPD molecular profiles using mass spectroscopy analysis might point to the identity of any low molecular weight factors that could influence a bacterial pathogen. Carrying out global gene expression analyses using microarrays would also be worthwhile, in order to fully characterise the effects of HPD on *S. epidermidis* virulence.

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Appendix 1

Publication completed during the course of this thesis

Chapter 8: Staphylococci, Catecholamine Inotropes and Hospital-Acquired Infections, Primrose PF , Noura Al-dayan, and Mark Lyte.

Microbial Endocrinology , 2010,151-166.

Appendix 2

Protein Sequence

1. Inter-alpha (globulin) inhibitor H1

```
  1 MDGAMGPRGL LLCMYLVSLL ILQAMPALGS ATGRSKSSEK RQAVDTAVDG
 51 VFIRSLKVNC KVTSRFAHYV VTSQVVNTAN EAREVAFDLE IPKTAFISDF
101 AVTADGNAFI GDIKDKVTAW KQYRKAAISG ENAGLVRASG RTMEQFTIHL
151 TVNPQSKVTF QLTYEEVLKR NHMQYEIVIK VKPKQLVHHF EIDVDIFEPQ
201 GISKLDAQAS FLPKELAAQT IKKSFSGKKG HVLFRPTVSQ QQSCPTCSTS
251 LLNGHFKVTY DVSRDKICDL LVANNHFAHF FAPQNLTNMN KNVVFVIDIS
301 GSMRGQKVKQ TKEALLKILG DMQPGDYFDL VLFGTRVQSW KGSLVQASEA
351 NLQAAQDFVR GFSLDEATNL NGGLLRGIEI LNQVQESLPE LSNHASILIM
401 LTDGDPTEGV TDRSQILKNV RNAIRGRFPL YNLGFGHNVD FNFLEVMSME
451 NNGRAQRIYE DHDATQQLQG FYSQVAKPLL VDVDLQYPQD AVLALTQNHH
501 KQYYEGSEIV VAGRIADNKQ SSFKADVQAH GEGQEFSITC LVDEEEMKKL
551 LRERGHMLEN HVERLWAYLT IQELLAKRMK VDREERANLS SQALQMSLDY
601 GFVTPLTSMS IRGMADQDGL KPTIDKPSED SPPLEMLGPR RTFVLSALQP
651 SPTHSSSNTQ RLPDRVTGVD TDPHFIIHVP QKEDTLCFNI NEEPGVILSL
701 VQDPNTGFSV NGQLIGNKAR SPGQHDGTYF GRLGIANPAT DFQLEVTPQN
751 ITLNPGFGGP VFSWRDQAVL RQDGVVVTIN KKRNLVVSVD DGGTFEVVLH
801 RVWKGSSVHQ DFLGFYVLDS HRMSARTHGL LGQFFHPIGF EVSDIHPGSD
851 PTKPDATMVV RNRRLTVTRG LQKDYSKDPW HGAEVSCWFI HNNGAGLIDG
```

2. Human serum albumin in a complex with myristic acid and tri-iodobenzoic acid.

```
  1 DAHKSEVAHR FKDLGEENFK ALVLIAFAQY LQQCPFEDHV KLVNEVTEFA
 51 KTCVADESAE NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE
101 CFLQHKDDNP NLPRLVRPEV DVMCTAFHDN EETFLKKYLY EIARRHPYFY
151 APELLFFAKR YKAAFTECCQ AADKAACLLP KLDELRDEGK ASSAKQRLKC
201 ASLQKFGERA FKAWAVARLS QRFPKAEFAE VSKLVTDLTK VHTECCHGDL
251 LECADDRADL AKYICENQDS ISSKLKECCE KPLLEKSHCI AEVENDEMPA
301 DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVVLLLRLA
351 KTYETTLEKC CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE
401 YKFQNALLVR YTKKVPQVST PTLVEVSRNL GKVGSKCCKH PEAKRMPCAE
451 DYLSVVLNQL CVLHEKTPVS DRVTKCCTES LVNRRPCFSA LEVDETYVPK
501 EFNAETFTFH ADICTLSEKE RQIKKQTALV ELVKHKPKAT KEQLKAVMDD
551 FAAFVEKCCK ADDKETCFAE EGKKLVAASQ AALGL
```

3. Alpha-2-macroglobulin precursor (Alpha-2-M)

```
   1 PPAPSFCNMG KNKLLHPSLV LLLLVLLPTD ASVSGKPQYM VLVPSLLHTE
  51 TTEKGCVLLS YLNETVTVSA SLESVRGNRS LFTDLEAEND VLHCVAFAVP
 101 KSSSNEEVMF LTVQVKGPTQ EFKKRTTVMV KNEDSLVFVQ TDKSIYKPGQ
 151 TVKFRVVSMD ENFHPLNELI PLVYIQDPKG NRIAQWQSFQ LEGGLKQFSF
 201 PLSSEPFQGS YKVVVQKKSG GRTEHPFTVE EFVLPKFEVQ VTVPKIITIL
 251 EEEMNVSVCG LYTYGKPVPG HVTVSICRKY SDASDCHGED SQAFCEKFSG
 301 QLNSHGCFYQ QVKTKVFQLK RKEYEMKLHT EAQIQEEGTV VELTGRQSSE
 351 ITRTITKLSF VKVDSHFRQG IPFFGQVRLV DGKGVPIPNK VIFIRGNEAN
 401 YYSNATTDEH GLVQFSINTT NVMGTSLTVR VNYKDRSPCY GYQWVSEEHE
 451 EAHHTAYLVF SPSKSFVHLE PMSHELPCGH TQTVQAHYIL NGGTLLGLKK
 501 LSFYYLIMAK GGIVRTGTHG LLVKQEDMKG HFSISIPVKS DIAPVARLLI
 551 YAVLPTGDVI GDSAKYDVEN CLANKVDLSF SPSQSLPASH AHLRVTAAPQ
 601 SVCALRAVDQ SVLLMKPDAE LSASSVYNLL PEKDLTGFPG PLNDQDDEDC
 651 INRHNVYING ITYTPVSSTN EKDMYSFLED MGLKAFTNSK IRKPKMCPQL
 701 QQYEMHGPEG LRVGFYESDV MGRGHARLVH VEEPHTETVR KYFPETWIWD
 751 LVVVNSAGVA EVGVTVPDTI TEWKAGAFCL SEDAGLGISS TASLRAFQPF
 801 FVELTMPYSV IRGEAFTLKA TVLNYLPKCI RVSVQLEASP AFLAVPVEKE
 851 QVPHCICANG RQTVSWAVTP KSLGNVNFTV SAEALESQEL CGTEVPSVPE
 901 HGRKDTVIKP LLVEPEGLEK ETTFNSLLCP SGGEVSEELS LKLPPNVVEE
 951 SARASVSVLG DILGSAMQNT QNLLQMPYGC GEQNMVLFAP NIYVLDYLNE
1001 TQQLTPEIKS KAIGYLNTGY QRQLNYKHYD GSYSTFGERY GRNQGNTWLT
1051 AFVLKTFAQA RAYIFIDEAH ITQALIWLSQ RQKDNGCFRS SGSLLNNAIK
1101 GGVEDEVTLS AYITIALLEI PLTVTHPVVR NALFCLESAW KTAQEGDHGS
1151 HVYTKALLAY AFALAGNQDK RKEVLKSLNE EAVKKDNSVH WERPQKPKAP
1201 VGHFYEPQAP SAEVEMTSYV LLAYLTAQPA PTSEDLTSAT NIVKWITKQQ
1251 NAQGGFSSTQ DTVVALHALS KYGAATFTRT GKAAQVTIQS SGTFSSKFQV
1301 DNNNRLLLQQ VSLPELPGEY SMKVTGEGCV YLQTSLKYNI LPEKEEFPFA
1351 LGVQTLPQTC DEPKAHTSFQ ISLSVSYTGS RSASNMAIVD VKMVSGFIPL
1401 KPTVKMLERS NHVSRTEVSS NHVLIYLDKV SNQTLSLFFT VLQDVPVRDL
1451 KPAIVKVYDY YETDEFAIAE YNAPCSKDLG NA
```

4. Ceruloplasmin

```
   1 MKILILGIFL FLCSTPAWAK EKHYYIGIIE TTWDYASDHG EKKLISVDTE
  51 HSNIYLQNGP DRIGRLYKKA LYLQYTDETF RTTIEKPVWL GFLGPIIKAE
 101 TGDKVYVHLK NLASRPYTFH SHGITYYKEH EGAIYPDNTT DFQRADDKVY
 151 PGEQYTYMLL ATEEQSPGEG DGNCVTRIYH SHIDAPKDIA SGLIGPLIIC
 201 KKDSLDKEKE KHIDREFVVM FSVVDENFSW YLEDNIKTYC SEPEKVDKDN
 251 EDFQESNRMY SVNGYTFGSL PGLSMCAEDR VKWYLFGMGN EVDVHAAFFH
 301 GQALTNKNYR IDTINLFPAT LFDAYMVAQN PGEWMLSCQN LNHLKAGLQA
 351 FFQVQECNKS SSKDNIRGKH VRHYYIAAEE IIWNYAPSGI DIFTKENLTA
 401 PGSDSAVFFE QGTTRIGGSY KKLVYREYTD ASFTNRKERG PEEEHLGILG
 451 PVIWAEVGDT IRVTFHNKGA YPLSIEPIGV RFNKNNEGTY YSPNYNPQSR
 501 SVPPSASHVA PTETFTYEWT VPKEVGPTNA DPVCLAKMYY SAVDPTKDIF
 551 TGLIGPMKIC KKGSLHANGR QKDVDKEFYL FPTVFDENES LLLEDNIRMF
 601 TTAPDQVDKE DEDFQESNKM HSMNGFMYGN QPGLTMCKGD SVVWYLFSAG
 651 NEADVHGIYF SGNTYLWRGE RRDTANLFPQ TSLTLHMWPD TEGTFNVECL
 701 TTDHYTGGMK QKYTVNQCRR QSEDSTFYLG ERTYYIAAVE VEWDYSPQRE
 751 WEKELHHLQE QNVSNAFLDK GEFYIGSKYK KVVYRQYTDS TFRVPVERKA
 801 EEEHLGILGP QLHADVGDKV KIIFKNMATR PYSIHAHGVQ TESSTVTPTL
 851 PGETLTYVWK IPERSGAGTE DSACIPWAYY STVDQVKDLY SGLIGPLIVC
 901 RRPYLKVFNP RRKLEFALLF LVFDENESWY LDDNIKTYSD HPEKVNKDDE
 951 EFIESNKMHA INGRMFGNLQ GLTMHVGDEV NWYLMGMGNE IDLHTVHFHG
1001 HSFQYK
```

5. Transferrin

```
   1 MRLAVGALLV CAVLGLCLAV PDKTVRWCAV SEHEATKCQS FRDHMKSVIP
  51 SDGPSVACVK KASYLDCIRA IAANEADAVT LDAGLVYDAY LAPNNLKPVV
 101 AEFYGSKEDP QTFYYAVAVV KKDSGFQMNQ LRGKKSCHTG LGRSAGWNIP
 151 IGLLYCDLPE PRKPLEKAVA NFFSGSCAPC ADGTDFPQLC QLCPGCGCST
 201 LNQYFGYSGA FKCLKDGAGD VAFVKHSTIF ENLANKADRD QYELLCLDNT
 251 RKPVDEYKDC HLAQVPSHTV VARSMGGKED LIWELLNQAQ EHFGKDKSKE
 301 FQLFSSPHGK DLLFKDSAHG FLKVPPRMDA KMYLGYEYVT AIRNLREGTC
 351 PEAPTDECKP VKWCALSHHE RLKCDEWSVN SVGKIECVSA ETTEDCIAKI
 401 MNGEADAMSL DGGFVYIAGK CGLVPVLAEN YNKSDNCEDT PEAGYFAVAV
 451 VKKSASDLTW DNLKGKKSCH TAVGRTAGWN IPMGLLYNKI NHCRFDEFFS
 501 EGCAPGSKKD SSLCKLCMGS GLNLCEPNNK EGYYGYTGAF RCLVEKGDVA
 551 FVKHQTVPQN TGGKNPDPWA KNLNEKDYEL LCLDGTRKPV EEYANCHLAR
 601 APNHAVVTRK DKEACVHKIL RQQQHLFGSN VTDCSGNFCL FRSETKDLLF
 651 RDDTVCLAKL HDRNTYEKYL GEEYVKAVGN LRKCSTSSLL EACTFRRP
```

6. Albumin

```
  1 MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA
 51 FAQYLQQCPF EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT
101 VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPRLV RPEVDVMCTA
151 FHDNEETFLK KYLYEIARRH PYFYAPELLF FAKRYKAAFT ECCQAADKAA
201 CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV ARLSQRFPKA
251 EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
301 ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF
351 LGMFLYEYAR RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE
401 FKPLVEEPQN LIKQNCELFK QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV
451 SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV LNQLCVLHEK TPVSDRVTKC
501 CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL SEKERQIKKQ
551 TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
601 AASQAALGL
```

7 . Complement Component C3 precursor

```
  1 VQLTEKRMDK VGKYPKELRK CCEDGMRENP MRFSCQRRTR FISLGEACKK
 51 VFLDCCNYIT ELRRQHARAS HLGLARSNLD EDIIAEENIV SRSEFPESWL
101 WNVEDLKEPP KNGISTKLMN IFLKDSITTW EILAVSMSDK KGICVADPFE
151 VTVMQDFFID LRLPYSVVRN EQVEIRAVLY NYRQNQELKV RVELLHNPAF
201 CSLATTKRRH QQTVTIPPKS SLSVPYVIVP LKTGLQEVEV KAAVYHHFIS
251 DGVRKSLKVV PEGIRMNKTV AVRTLDPERL GREGVQKEDI PPADLSDQVP
301 DTESETRILL QGTPVAQMTE DAVDAERLKH LIVTPSGCGE QNMIGMTPTV
351 IAVHYLDETE QWEKFGLEKR QGALELIKKG YTQQLAFRQP SSAFAAFVKR
401 APSTWLTAYV VKVFSLAVNL IAIDSQVLCG AVKWLILEKQ KPDGVFQEDA
451 PVIHQEMIGG LRNNNEKDMA LTAFVLISLQ EAKDICEEQV NSLPGSITKA
501 GDFLEANYMN LQRSYTVAIA GYALAQMGRL KGPLLNKFLT TAKDKNRWED
551 PGKQLYNVEA TSYALLALLQ LKDFDFVPPV VRWLNEQRYY GGGYGSTQAT
601 FMVFQALAQY QKDAPDHQEL NLDVSLQLPS RSSKITHRIH WESASLLRSE
651 ETKENEGFTV TAEGKGQGTL SVVTMYHAKA KDQLTCNKFD LKVTIKPAPE
701 TEKRPQDAKN TMILEICTRY RGDQDATMSI LDISMMTGFA PDTDDLKQLA
751 NGVDRYISKY ELDKAFSDRN TLIIYLDKVS HSEDDCLAFK VHQYFNVELI
801 QPGAVKVYAY YNLEESCTRF YHPEKEDGKL NKLCRDELCR CAEENCFIQK
851 SDDKVTLEER LDKACEPGVD YVYKTRLVKV QLSNDFDEYI MAIEQTIKSG
901 SDEVQVGQQR TFISPIKCRE ALKLEEKKHY LMWGLSSDFW GEKPNLSYII
951 GKDTWVEHWP EEDECQDEEN QKQCQDLGAF TESMVVFGCP N
```

8. Fibrin beta

```
  1 GHRPLDKKRE EAPSLRPAPP PISGGGYRAR PAKAAATQKK VERKAPDAGG
 51 CLHADPDLGV LCPTGCQLQE ALLQQERPIR NSVDELNNNV EAVQSTSSSS
101 QFYMYLLKDL WQKRQKQVKD NENVVNEYSS ELEKHQLYID ETVNSNIPTN
151 LRVLRSILEN LRSKIQKLES DVSAQMEYCR TPCTVSCNIP VVSGKECEEI
201 IRKGGETSEM YLIQPDSSVK PYRVYCDMNT ENGGWTVIQN RQDGSVDFGR
251 KWDPYKQGFG NVATNTDGKN YCGLPGEYWL GNDKISQLTR MGPTELLIEM
301 EDWKGDKVKA HYGGFTVQNE ANKYQISVNK YRGTAGNALM DGASQLMGEN
351 RTMTIHNGMF FSTYDRDNDG WLTSDPRKQC SKEDGGGWWY NRCHAANPNG
401 RYYWGGQYTW DMAKHGTDDG VVWMNWKGSW YSMRKMSMKI RPFFPQQ
```

9. Human Serum Albumin in complex With Myristate and Azapropazone

```
  1 DAHKSEVAHR FKDLGEENFK ALVLIAFAQY LQQCPFEDHV KLVNEVTEFA
 51 KTCVADESAE NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE
101 CFLQHKDDNP NLPRLVRPEV DVMCTAFHDN EETFLKKYLY EIARRHPYFY
151 APELLFFAKR YKAAFTECCQ AADKAACLLP KLDELRDEGK ASSAKQRLKC
201 ASLQKFGERA FKAWAVARLS QRFPKAEFAE VSKLVTDLTK VHTECCHGDL
251 LECADDRADL AKYICENQDS ISSKLKECCE KPLLEKSHCI AEVENDEMPA
301 DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVVLLLRLA
351 KTYETTLEKC CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE
401 YKFQNALLVR YTKKVPQVST PTLVEVSRNL GKVGSKCCKH PEAKRMPCAE
451 DYLSVVLNQL CVLHEKTPVS DRVTKCCTES LVNRRPCFSA LEVDETYVPK
501 EFNAETFTFH ADICTLSEKE EQIKKQTALV ELVKHKPKAT KEQLKAVMDD
551 FAAFVEKCCK ADDKETCFAE EGKKLVAASQ AALGL
```

10. IgG Kappa chain

```
  1 DIEMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA
 51 ASSRATGIPD RFSGSGSGTD FTLTISRLEP EDFAVYYCQQ YGSSPWTFGQ
101 GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
151 DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
201 LSSPVTKSFN RGEC
```

Appendix 3

Sample ID	Age	Current Status	Sex	Diabetes	Malignancy	Taking immunosuppressants	Taking oral iron	Taking beta blocker	Taking alpha blocker
1	39	HD	F	N	N	Y (PREDNISOLONE)	N	N	N
2	53	TPLT	F	Y	N	N	N	N	Y
3	57	TPLT	F	N	N	N	N	N	N
4	**67**	**CCPD**	**M**	**Y**	**N**	**N**	**N**	**N**	**Y**
5	69	CCPD	F	N	N	N	Y	N	N
6	60	CCPD	M	N	N	N	N	N	N
7	71	CAPD	M	N	N	N	N	Y	Y
8	**58**	**CCPD**	**F**	**Y**	**N**	**N**	**N**	**N**	**N**
9	61	TPLT	M	N	N	N	N	Y	N
10	**54**	**CAPD**	**F**	**N**	**N**	**N**	**N**	**Y**	**N**
11	67	D	F	N	N	N	N	N	N
12	69	HD	F	N	N	N	Y	Y	N
13	**68**	**CCPD**	**M**	**Y**	**N**	**N**	**N**	**Y**	**N**
14	**51**	**CAPD**	**M**	**N**	**N**	**N**	**N**	**Y**	**N**

The additional clinical data for the patients used in this study, the highleted one is the follow up samples