Contributory factors in the development of infectious peritonitis in peritoneal dialysis patients

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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2017

Abstract

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Peritoneal dialysis (PD) is the simplest and most economical therapeutic technique for renal failure patients. However, the percentage patients receiving PD in the UK has decreased since 2000 because of its potential complications, which primarily include protein loss and risk of infectious peritonitis. The latter issue in particular is a clinically significant condition contributing to the high mortality and morbidity rates observed in PD patients. Although some of the risk factors for PD-associated peritonitis have been identified, it is still unclear why certain PD patients are more susceptible than others to infection. The components of peritoneal dialysis fluid (PDF) were therefore examined to identify protein and hormonal factors that might increase PD-patient susceptibility to infection. Comprehensive characterisation studies were performed on initial and follow-up PDF samples collected from nine kidney failure patients. The data showed that staphylococci behaved differently in different PDF samples. Proteomic analysis identified an association between serum transferrin and infection risk, as transferrin in PDF was discovered to be significantly more iron-saturated than transferrin in the blood. Further, use of radioactive iron-labelled transferrin showed it could act as a direct iron source for the growth of peritonitis-causing bacteria. In addition, the catecholamine stress hormones noradrenaline and adrenaline were shown for the first time to be present in PDF and to be involved in modulating the growth of peritonitis-causing bacteria. Comparison studies between non-infected and infected PDF samples confirmed that the release of proteins, particularly transferrin, and catecholamines into the PDF were significantly higher during infection. Transferrin and catecholamines are therefore key indicators signaling the potential for bacterial growth in PDF. A test that detects increased levels of transferrin and catecholamines may therefore have value in predicting the likelihood of infection and thereby guide appropriate prophylactic treatment.

Dedicated to all people living with peritoneal dialysis across the world

Acknowledgements

"*All praise is due to Allah, the Lord of the Worlds*"

Neither words nor actions can fully express my gratitude to all those who supported me during this challenging scientific journey, but here is my best attempt. First and foremost, I would like to thank my first supervisor, Dr. Primrose Freestone, for her invaluable assistance and guidance throughout the planning and writing of my PhD thesis. She is an excellent supervisor and I am truly lucky to have known her both professionally and personally. Also, I would like to thank my second supervisor, Dr. Jonathan Barratt, for his clinical focus advice and crucial contribution in providing me the patient's peritoneal dialysis samples, which were the backbone of this study. Primrose and Jonathan, I am very grateful to you both in every possible way.

I am also very thankful to my sponsors, King Saud bin Abdul-Aziz University for Health Sciences in Riyadh, KSA and the Saudi Arabian Cultural Bureau in London, UK, for providing the funds that enabled me to complete my PhD successfully.

I am very grateful to my progress review panel members, Dr. Cordula Stover and Dr. Alan Bevington, for their advice, support and feedback—it was much appreciated. Many thanks to Dr. Sara Sandrini and Dr. Simon Byrne for all of their help and advice during this study. I would like also to thank Dr. Andrew Bottrill and Ms. Shairbanu Ashra from the Protein Nucleic Acid Chemistry Laboratory at the University of Leicester for their excellent work in protein sequencing and their assistance with data collection and analysis. Special thanks to Dr. Maria Viskaduraki from the Bioinformatics and Biostatistics Analysis Support Hub at the University of Leicester for her great support and advice in statistical analysis. A big thanks to all of the staff in the department of Infection, Immunity and Inflammation who helped me indirectly in the accomplishment of this research, in particular to Ms. Alison Brown, Ms. Liz Le-Butt, Dr. Hannah Brewin, Dr. Susan Kapadia, Ms. Avril Saunders and Ms. Sushila Rambhai.

A special thanks to all of my laboratory friends: Kholoud, Ohoud, Wafa'a, Dina, Mashael, Giannis, Abdullah and all the others whom I have not mentioned here. Finally, I am very grateful for the limitless support of my brother, Badran, without whom I would have neither started nor finished this PhD project. I would like to express my love and appreciation to my mother, father, sisters and friends (especially Faten, Amal, Taghreed, Balqees, Munirah and Meyad) in KSA for their continuous love and encouragement through this challenging study period.

With love,

Marwah

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List of abbreviations

Å	Angstrom
Adr	Adrenaline
APD	Automated peritoneal dialysis
API	Analytical profile index
APS	Ammonium persulphate
BSA	Bovine serum albumin
CAs	Catecholamines
CAPD	Continuous ambulatory peritoneal dialysis
CCPD	Continuous cycling peritoneal dialysis
CFU	Colony-forming unit
CKD	Chronic kidney disease
cm	Centimetre
kDa	Kilodalton
dL	Decilitre
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ELISA	Enzyme linked immunosorbent assay
ESKD	End stage kidney disease
Fe	Iron
Fe^{+2}	Ferrous iron
Fe^{+3}	Ferric iron
g	Gram
GFR	Glomerular filtration rate
HD	Haemodialysis
HU	Haemolytic unit
IgG	Immunoglobulin G
Kg	Kilogram
L	Litre
LC-MS	Liquid chromatography-mass spectrometry
M	Molar
mA	Milliampere
m^2	Meter squared
MALDI-TOF MS	Matrix assisted laser desorption/ionization-time of flight mass spectrometry
mg	Milligram
ml	Millilitre
µl	Microlitre
mmol	Millimole
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
M.W	Molecular weight
NE	Noradrenaline

NICE	The national institute for health and care excellence
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PD	Peritoneal dialysis
PDF	Peritoneal dialysis fluid
PDS	Peritoneal dialysis solution
pg	Picogram
pmp	Per million population
psi	Pound-force per square inch
PVDF	Polyvinyliden fluoride
RBC	Red blood cell
RNA	Ribonucleic acid
RRT	Renal replacement therapy
RT	Room temperature
SDS	Sodium dodecyl sulphate
TEMED	Tetramethylethylenediamine
Tf	Transferrin
TNFα	Tumour necrosis factor alpha
TSA	Tryptone soya agar
TSB	Tryptone soya broth
USRDS	United States renal data system
WBC	White blood cell

CHAPTER 1 Introduction

1.1 The human kidneys

1.1.1 Anatomy and physiology

The kidneys are the clearance system of the human body. Kidney dysfunction, if it is not treated, can lead to a rapid accumulation of wastes and result in death within just a few days. The kidneys are a pair of organs located posterolateral of abdominal wall in the retroperitoneal space (Koeppen & Stanton, 2012; Hall, 2015). The normal length, width and thickness of an adult kidney are approximately 11 centimetres (cm), 6 cm and 3 cm, respectively (Koeppen & Stanton, 2012). Adult kidneys weigh between 115 grams (g) and 170 g, which is less than 0.5% of total body weight (Hall, 2015; Koeppen & Stanton, 2012). Figure 1.1 shows the anatomical features of the kidney. The kidneys are bean-shaped organs that are composed of two major structures: the cortex, which represents the outer component and comprises one-third of the kidney and the medulla, which represents the inner part and forms the other two-thirds of the kidney (Koeppen & Stanton, 2012). Together, the cortex and the medulla of an adult kidney contain about 1.2 million nephrons, which are the functional unit of the kidney, as well as blood vessels, nerves and lymphatics (Koeppen & Stanton, 2012). As illustrated in Figure 1.1 b and c, a single nephron is composed of a glomerulus and a tubule system (Koeppen & Stanton, 2012).

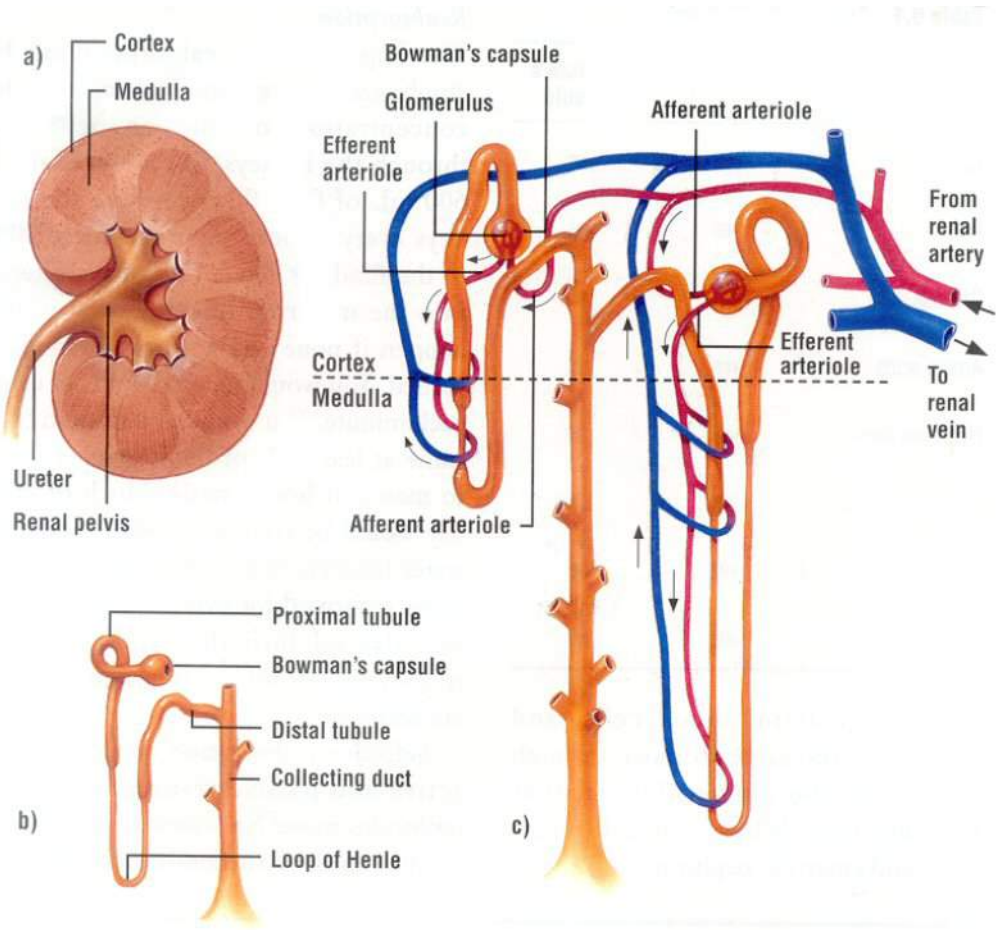


Figure 1.1 Anatomy of a normal kidney.
a) A cut surface of the kidney, demonstrating its main parts, b) The microscopic anatomy of the nephron illustrating the glomerulus, which is surrounded by the Bowman's capsule and the tubule system and c) The microscopic structure of the nephron indicating the location of the glomerulus in the cortex and the tubule system distributed in the cortex and the medulla. The arrows demonstrate blood flow through a series of branches of the renal veins and arteries. The figure was adapted from http://www.kidney.com.

There are three basic renal functions. The first involves filtering blood from the metabolic end products (Koeppen & Stanton, 2012). The glomerulus, which is a small ball of capillaries, is responsible for this function through a process called glomerular filtration (Koeppen & Stanton, 2012). Because the metabolic end products are toxic to the human body, they must be eliminated as soon as possible after they are produced (Hall, 2015). A particularly important example of these toxic products is creatinine, which originates from creatine metabolism in skeletal muscle (Koeppen & Stanton, 2012). Other toxic products are urea and uric acid, which are end products of protein catabolism and uric acid metabolism, respectively (Koeppen & Stanton, 2012; Hall, 2015). After the glomerular filtration process is completed, the second important renal function takes place, as the filtered blood passes through the tubule system (Dudek, 2007). The function of the glomerulus is closely linked to that of the tubule system, which consists of the

proximal tubule, then the descending and ascending parts of the loop of Henle and finally the distal tubule (Dudek, 2007), Figure 1.1 b. The proximal tubule of the nephron is able to reabsorb glucose and proteins, including the serum iron-holding protein transferrin, from tubular fluid to the plasma (Bazzi et al., 2015; Dudek, 2007; Ishibashi, 1994; Storm et al., 2013). The proximal tubule is also responsible for the third basic function of the kidneys, which is the secretion of organic anions and organic cations, including the catecholamines noradrenaline, adrenaline and dopamine, from the plasma to the tubular fluid (Dudek, 2007; Hall, 2015). The tubule system is responsible for changing the volume and constitution of the glomerular filtrate through a transport process along the entire system that ultimately leads to the formation of urine (Dudek, 2007; Hall, 2015). The kidneys, then, participate in a number of vital regulatory mechanisms in the human body. They are responsible for homeostatic functions through the maintenance of body fluids, water, electrolytes and the acid-base balance in the circulation (Hall, 2015; Koeppen & Stanton, 2012). The kidneys play a significant role in the process of regulating arterial blood pressure as well (Koeppen & Stanton, 2012; Hall, 2015). In addition to their significant contribution to the urinary system, the kidneys also belong to the endocrine system. They produce erythropoietin hormone, which stimulates the synthesis of erythrocytes in the bone marrow (Hall, 2015; Koeppen & Stanton, 2012). People who have severe kidney disease or are on dialysis can become anaemic because of a reduction in their erythropoietin production. Finally, the kidneys also produce calcitriol hormone, which enhances the absorption of calcium in the intestines (Hall, 2015; Koeppen & Stanton, 2012).

1.1.2 Clinical assessment of kidney functions

The glomerular filtration rate (GFR) is used as a diagnostic tool to evaluate kidney function. The GFR calculation is mainly based on creatinine clearance (Barratt *et al.*, 2008; Dudek, 2007; Guyton & Hall, 2006; Lai, 2009; Schwartz & Work, 2009). The glomerular capillaries filter creatinine, which to a first approximation is neither reabsorbed nor secreted by the tubule system of the nephrons (Koeppen & Stanton, 2012; Dudek, 2007). Thus, the creatinine clearance provides an estimation of the GFR (Dudek, 2007; Koeppen & Stanton, 2012). The normal range of the GFR is from approximately 80 to 120 millilitres per minute per 1.73 meters squared (ml/min/1.73 m^2) (Barratt *et al.*, 2008).

1.2 Chronic kidney disease (CKD)

CKD is a worldwide health problem that necessitates detection, diagnosis and treatment in its earliest stages (Levey & Coresh, 2012). The identification of CKD is based on the reduction of kidney functions; that is, the GFR is less than 60 ml/min/1.73 m^2 for ≥ three consecutive months (Clarkson *et al.*, 2010; Dudek, 2007; Lai, 2009; Levey & Coresh, 2012b). The Kidney Disease Outcomes Quality Initiative (Eknoyan *et al.*, 2013) places CKD into six categories according to the level of the GFR. A GFR value of less than 15 ml/min/1.73 m^2 is considered to be a critical biomarker for end stage kidney disease (ESKD; also termed kidney failure), and at this stage, renal replacement therapy (RRT) must be considered (Levey & Coresh, 2012). Another significant endogenous indicator of the presence of CKD is the concentration of albumin in the urine (albuminuria) (Eknoyan *et al.*, 2013) (Table 1.1). There are a variety of established clinical factors, which promote the risk of CKD development. Diabetes mellitus and hypertension are the number one conditions, which increase the risk of CKD occurrence (Clarkson *et al.*, 2010; Dudek, 2007; Lai, 2009; Couchoud *et al.*, 2015). Other etiological conditions include glomerulonephritis, infective or obstructive nephropathies, cystic or congenital disease, older age, obesity, congestive heart failure, chronic systemic infections, recurrent acute kidney injury and metabolic syndrome (Clarkson *et al.*, 2010; Dudek, 2007; Lai, 2009; Levey & Coresh, 2012). CKD is associated with clinical complications. Cardiovascular disease is the primary cause of mortality in patients with CKD (Barratt *et al.*, 2008; Clarkson *et al.*, 2010; Lai, 2009). Infection is another significant cause of death in ESKD patients (Levy *et al.*, 2015). Anaemia (a reduction in erythrocyte mass and haemoglobin) is an essential feature of CKD due to erythropoietin deficiency. Other noticeable consequences include neurological dysfunction and mineral bone disease (Clarkson *et al.*, 2010; Levey & Coresh, 2012; Levy *et al.*, 2015). Endocrine abnormalities are also involved in CKD; for example, catecholamine hormones are elevated in CKD due to decreased degradation or a decrement in neuronal uptake (Barratt *et al.*, 2008). ESKD is fatal unless it is managed by RRT–either kidney dialysis or kidney transplantation (Barratt *et al.*, 2008).

Table 1.1 Classification of CKD by GFR and albuminuria according to the Kidney Disease Outcomes Quality Initiative.
Kidney Disease: Improving Global Outcomes published the classification of CKD (Eknoyan *et al.*, 2013).

Prognosis of CKD by GFR and albuminuria categories				**Description and range of persistent albuminuria (mg/g)***		
				1	**2**	**3**
				Normal to moderately high	Moderately high	Severely high
				< 30	30 - 300	> 300
Description and range of GFR (ml/min/1.73 m^2)	**1**	High or normal GFR	> 90			
	2	Mildly low GFR	60 - 89			
	3A	Moderately low GFR	45 - 59			
	3B	Moderately to severely low GFR	30 - 44			
	4	Severely low GFR	15 - 29			
	5	End stage kidney disease	< 15			

Key: Green: low risk (if no other indicators of CKD); Blue: moderately high risk; Orange: high risk; Red: very high risk. *mg/g refers to milligrams per gram.

1.3 End stage kidney disease (ESKD)

1.3.1 Management of ESKD

Patients who are predicted to experience total or nearly total loss of their kidney function are normally pre-prepared for RRT (Lai, 2009). Advance preparation for RRT is critical for many reasons. First, it allows kidney failure patients to consider which RRT modality will be the most convenient (Lai, 2009; Barratt *et al.*, 2008). A second advantage of early

preparation for RRT is to establish vascular access (for haemodialysis) or peritoneal access (for peritoneal dialysis), because the access point requires time to mature before the dialysis commences (Barratt *et al.*, 2008). Above all, the availability of the RRT modality should be taken into account when a decision is made (Barratt *et al.*, 2008; Clarkson *et al.*, 2010; Lai, 2009).

1.3.2 Epidemiology of ESKD

Figure 1.2 represents the statistics of ESKD patients who were treated by dialysis or transplantation in 2013 in various countries (Saran *et al.*, 2016). According to the United States Renal Data System (USRDS) (Saran *et al.*, 2016) there was in 2013 a noticeable variation in the incidence rates of treated ESKD patients between different countries. The annual data report of USRDS stated that the highest incidence rates of treated ESKD were in Taiwan, the Jalisco region of Mexico and the US (458, 421 and 363 per million population [pmp], respectively) in 2013 (Saran *et al.*, 2016). The RRT incidence rate in the UK is similar to that in Australia, the Netherlands, Sweden and Denmark. However, a significant variation has been found in the RRT incidence rate in the UK as compared to Taiwan, the Jalisco region of Mexico and the US. For instance, the incidence rate of 363 pmp in the US is three times the rate of the UK (113 pmp) in 2013 (Saran *et al.*, 2016) (Figure 1.2 A). The RRT prevalence rate at the end of 2013 also showed considerable variation when the UK was compared to Taiwan, Japan and the US (Figure 1.2 B). In the UK, there were 905 patients pmp receiving RRT, whereas the prevalence rate in Taiwan, Japan, and the US (3,138, 2,411 and 2,043 pmp, respectively) was more than twice that of the UK at the end of 2013 (Saran *et al.*, 2016).

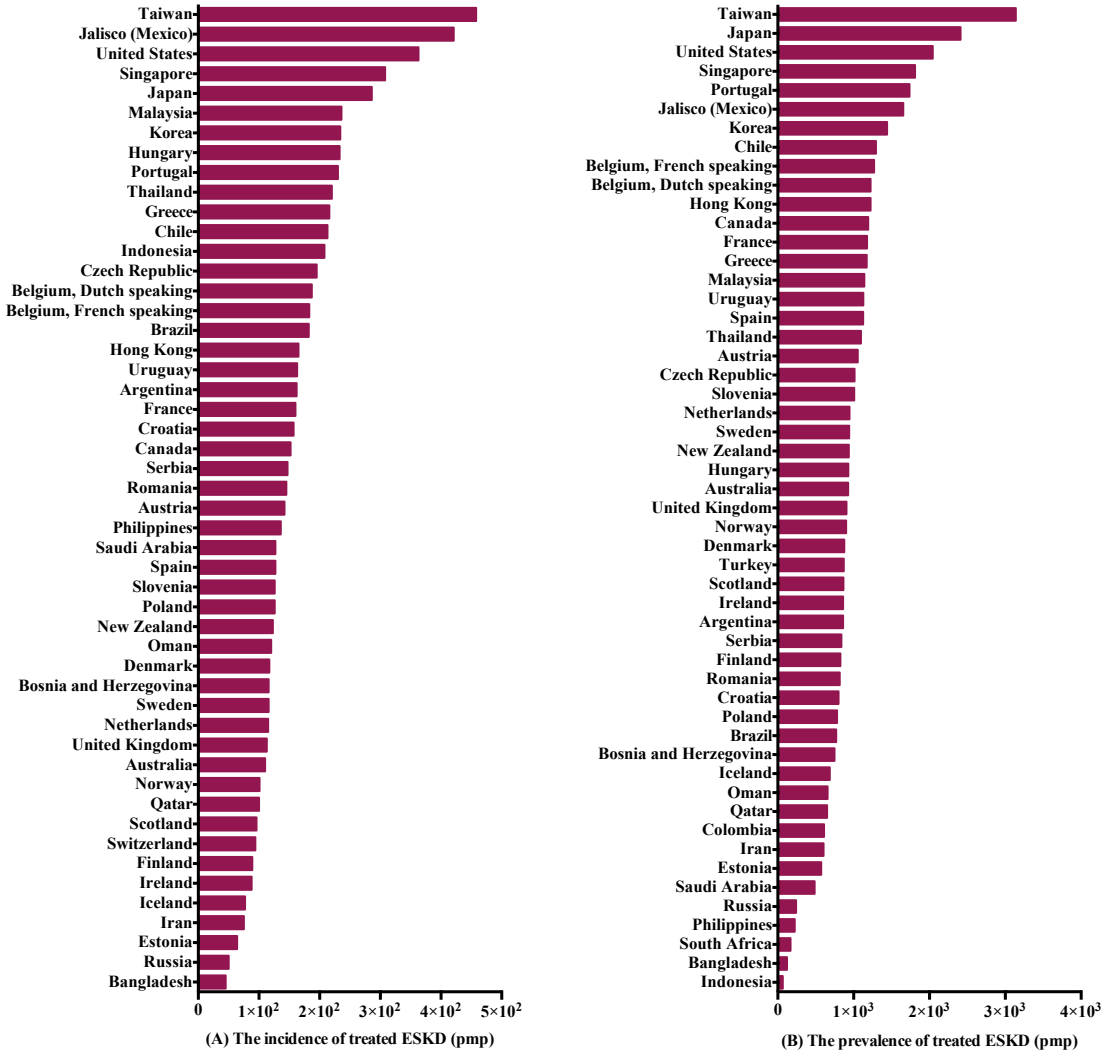


Figure 1.2 The incidence and prevalence of treated ESKD in patients/pmp in different countries in 2013.

The data were from the United States Renal Data System (Saran *et al.*, 2016). (A) The incidence rate of treated ESKD in 2013. The Belgian data excludes patients younger than 20 years old. The Indonesian data shows the West Java region. Twenty-two regions were included in the French data, and 18 of 19 regions for the Spanish data. (B) The prevalence rate of treated ESKD at the end of 2013. The Japanese and Indonesian data excludes transplantation patients. The Belgian data includes patients older than 20 years old. The Indonesian data demonstrates the West Java region. The data for 18 of 19 regions for Spain and 22 regions for France is included.

The recent United Kingdom Renal Registry report (MacNeill *et al.*, 2016) shows the percentage of different RRT modalities (kidney transplantation, haemodialysis and peritoneal dialysis) in patients on a yearly basis from 1999 to 2014 in the UK. Generally speaking, the trends in the RRT modality vary significantly. The proportion of kidney transplantation is the highest in the UK. Since 2006, the proportion of UK kidney transplantation has risen gradually, from 45% in 2006 to 52.8% in 2014 (MacNeill *et al.*, 2016). As shown in Figure 1.3, the second most common modality for treating kidney failure is haemodialysis. In contrast, the percentage of peritoneal dialysis patients has

decreased slowly but surely since 2000. As reported by MacNeill *et al.* (2016), the actual number of ESKD patients on peritoneal dialysis decreased between 2004 and 2014 (from 5,185 patients in 2004 to 3,638 in 2014) in the UK. In addition to the decrease in the peritoneal dialysis patient number, the median time spent in using the peritoneal dialysis modality has declined from two years in 2009 to 1.6 years in 2014 (MacNeill *et al.*, 2016). A reduction in peritoneal dialysis utilisation over that period might be associated with attention paid to peritoneal dialysis complications and/or the availability of kidney transplantation.

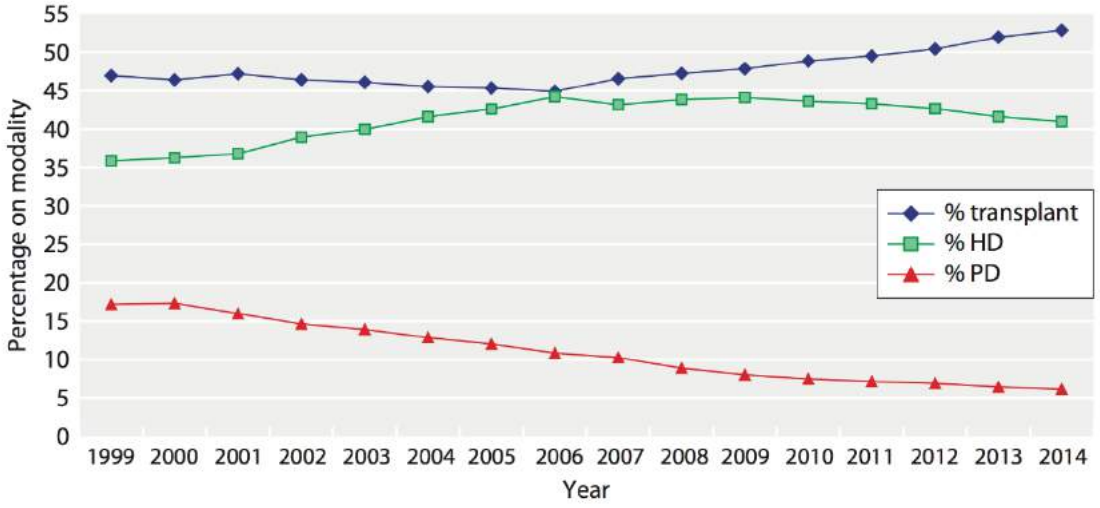


Figure 1.3 Trends in the RRT modality in RRT patients in the UK from 1999 to 2014.
Key: transplant: kidney transplantation; HD: Haemodialysis; PD: Peritoneal Dialysis; %: percent. The figure was adapted from MacNeill *et al.* (2016).

1.4 Treatment modalities for ESKD

1.4.1 Kidney transplantation

Kidney transplantation is the most optimal therapeutic approach for ESKD patients (Clarkson *et al.*, 2010; Barnett & Mamode, 2011). It is an effective treatment that provides an improved quality of life, a greater chance of patient survival and a lower hospitalisation rate compared to kidney dialysis (Barnett & Mamode, 2011; Clarkson *et al.*, 2010; Vathsala, 2005). Both kidney donors and recipients (patients) need a kidney function assessment prior to the transplantation process (Barnett & Mamode, 2011; Clarkson *et al.*, 2010). There are two types of transplantation based on the source of the donor kidney: (1) living donor transplantation, and (2) deceased donor transplantation, either after

circulatory death or brain death (Barnett & Mamode, 2011; Clarkson *et al.*, 2010). Immunosuppressive agents are essential for the life of the donated kidney allograft and to avoid rejection in the patient during the transplantation process (Clarkson *et al.*, 2010). Due to the use of these immunosuppressive factors, the risk of infectious disease is high after the transplantation process, particularly wound infections, urinary and respiratory tract infections (Barnett & Mamode, 2011). Other serious potential outcomes of transplantation are myocardial infarction, bleeding and thrombosis (Barnett & Mamode, 2011; Clarkson *et al.*, 2010). Above all, although complicated to perform kidney transplantation is cost-effective, the available number of donors is limited in comparison to the growing number of ESKD patients (Vathsala, 2005).

1.4.2 Haemodialysis (HD)

HD is the principal treatment for ESKD patients. It is based on using a haemodialyser (HD machine) with two components: a dialysis delivery system and an extracorporeal circuit (blood tubing) (Elliott, 2000). These two constituents contain separate blood and dialysate circuits that are separated by thin, semipermeable membranes (Clarkson *et al.*, 2010; Elliott, 2000) (Figure 1.4).

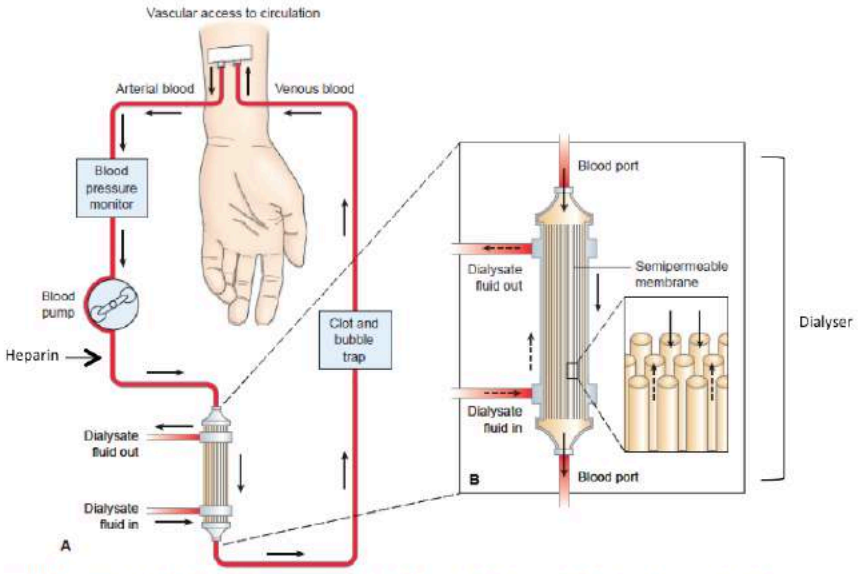


Figure 1.4 Basic principle of the haemodialysis circuit.
A) Arterial blood is pumped into B) a haemodialyser, where it flows through semipermeable membranes. The dialysate flows in around the semipermeable membranes, where the waste products of the blood diffuse through the membranes into the dialysate. Afterwards, the filtered blood returns to the patient via venous tubing. The Figure was modified from http://www.haemodialysis-system.html.

The technical procedure of HD is initiated by pumping a patient's blood from an arterial line to a haemodialyser. Afterwards, the patient's blood flows across capillary tubes situated in the haemodialyser (Levy *et al.*, 2015). At this stage, small molecular weight solutes (but not large molecules such as proteins) exchange takes place between the patient's blood and the dialysate (purified water with electrolytes and buffer) across the semipermeable membrane (Levy *et al.*, 2015). Because the dialysate does not include waste products such as urea and creatinine, these will diffuse from the blood into the dialysate (Levy *et al.*, 2015). Then, the patient's blood is returned to the patient under the effect of positive pressure via venous tubing (Clarkson *et al.*, 2010; Elliott, 2000; Lai, 2009). Although it is a well-established therapeutic procedure, there are some contraindications related to this technique. These are summarised in Table 1.2. The HD modality is responsible for various complications associated with vascular access such as thrombosis and infection (Clarkson *et al.*, 2010). Other consequences are related to the gastrointestinal tract include nausea and vomiting (Elliott, 2000). Technical problems include clotting in the extracorporeal blood, air embolisms and blood leakage into the dialysate (Clarkson *et al.*, 2010; Elliott, 2000).

Table 1.2 Contraindications to haemodialysis.
Absolute and relative contraindications were reviewed from Barratt *et al.* (2008), Lai (2009) and Levy *et al.* (2015).

Absolute contraindications	Relative contraindications
• Thrombosis in central veins (no vascular access possible) • Serious angina • Hypotensive heart failure	• Serious vascular disease (e.g. absent pulses in arms) • Active diabetic retinopathy • Needle phobia • Long distance from haemodialysis unit • Body shape (concern about appearance of arteriovenous fistula)

1.4.3 Peritoneal dialysis (PD)

1.4.3.1 Historical development of PD

A long scientific journey has led to the way in which PD is used today. The original idea of PD may go back to as early as the 1740s, when an English surgeon, Christopher Warwick, tried to treat a 50-year-old woman who had severe ascites (fluid accumulation in the peritoneal cavity) (Gokal *et al.*, 2013). Warwick infused a mixture of Bristol water and claret wine into the woman's peritoneal cavity through a leather pipe, resulting in the woman's recovery from the illness in a period of weeks (Gokal *et al.*, 2013). Nearly 200

years after Warwick's scientific discovery, George Ganter, a German clinical investigator, attempted to use PD to treat uraemia in rabbits and pigs (Gokal *et al.*, 2013). Soon after that, interest in PD extended to using the technique in human beings (Gokal *et al.*, 2013). In 1960 in the US, Richard Rubin performed the first successful application of PD as a treatment for a kidney failure patient (Gokal *et al.*, 2013). In 1975, PD was also used as an alternative therapy to HD when there was difficulty in establishing an arteriovenous fistula in a proposed HD patient (Gokal *et al.*, 2013). A few years later, the concept of using instruments for PD originated, which led to the development of automated peritoneal dialysis (Gokal *et al.*, 2013). Since that time, the PD procedures and techniques have further evolved, and substantial improvements and enhancements have been made (NICE clinical guideline, 2011).

1.4.3.2 The peritoneum as a dialysis membrane

The peritoneum is a membrane that underlies the peritoneal cavity and covers most of the viscera. Anatomically, the peritoneal membrane is composed of two layers separated by a basement membrane. The first layer consists of simple squamous epithelial cells known as the mesothelium, whereas the second layer contains connective tissue (Levy *et al.*, 2015). There are blood vessels distributed throughout the peritoneal membrane, and its normal function is to deliver nutrients to the tissues and organs present within the peritoneal cavity and to remove wastes from them (Barratt *et al.*, 2008; Levy *et al.*, 2015). The permeability of the peritoneal membrane depends on the presence of the three-pore model of peritoneal capillary transport. These are: (1) ultrapores (the smallest pores; the average radius is 3 - 5 angstroms [Å]), which are responsible for transporting water molecules only; (2) small pores (average radius 40 - 50 Å), which transport micromolecules such as urea and creatinine; and (3) large pores (average radius > 150 Å), which are present in low numbers and transport large molecules such as albumin (Barratt *et al.*, 2008; Levy *et al.*, 2015). The vascularity of the peritoneal membrane is more important than the peritoneal membrane's surface area (1.7 to 2 m^2 in adults) (Barratt *et al.*, 2008; Clarkson *et al.*, 2010; Flessner, 1991). In other words, the distribution of capillaries in the peritoneal membrane varies among PD patients, and for this reason, the effectiveness of PD varies (Clarkson *et al.*, 2010). The vascularity distribution and the permeability of the peritoneal membrane are both key factors that make the peritoneum an ideal dialysis membrane for water, solutes and electrolyte exchange (Barratt *et al.*, 2008; Clarkson *et al.*, 2010; Flessner, 1991).

1.4.3.3 The principle of PD

The removal of waste products and excess water from the body takes place in the peritoneal cavity by aseptically infusing peritoneal dialysis solution (PDS). Afterwards, the transport of solutes and fluid occurs across the peritoneal membrane between the PDS and the distributed peritoneal capillaries (Levy *et al.*, 2015) (Figure 1.5). The exchange of solutes and fluids is based on triple chemo-physical forces: osmosis (ultrafiltration), diffusion and convection (Levy *et al.*, 2015). Fluid exchange is a process, which occurs by osmosis, where the osmotic gradient is generated by the presence of an osmotic agent (mainly glucose) in PDS, whereas solute exchange exists by diffusion and convection (Table 1.3). The diffusion average across the peritoneal membrane depends on three different variables; that is, the physical size of the solute, the electrical charge of the solute and its concentration (Bersenas, 2011). For instance, urea diffuses across the peritoneal membrane more rapidly than creatinine, because urea has a smaller size (molecular weight [M.W] 60 Dalton [Da]) than creatinine (M.W 113 Da) (Bersenas, 2011). The peritoneal membrane indeed works as a filter membrane, an alternative to the kidney, and allows the removal of metabolites, waste products and excess fluid from the body.

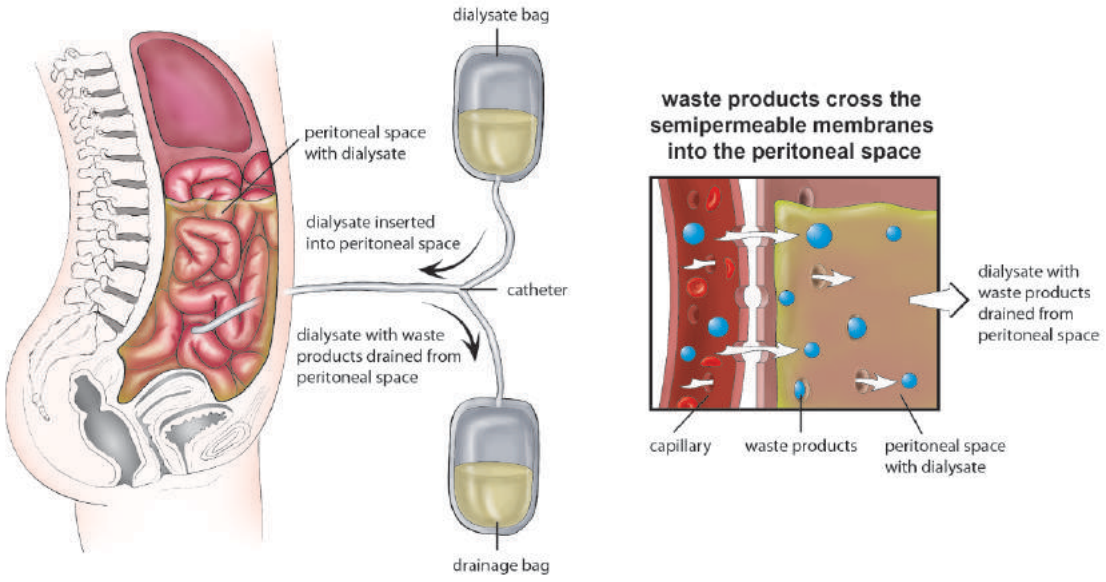


Figure 1.5 The principle of peritoneal dialysis.
The dialysate bag contains the peritoneal dialysis solution, which is drained into the abdominal cavity via an implanted catheter. After dialysis, the peritoneal dialysis fluid contains waste metabolites and extracted water. It is then drained out to the drainage bag and discarded. The figure was adapted from http://www.biomedicalvisuals.com.

Table 1.3 A summary of the different processes, which drive peritoneal dialysis.
The principle of chemo-physical activities was re-adjusted from Levy *et al.* (2015).

Chemo-physical activity	Principle
1. Osmosis (ultrafiltration)	Water moves from an area with low osmolar concentration (capillaries) into an area with high osmolar concentration (peritoneal cavity) across the peritoneal membrane.
2. Diffusion	Solutes in a high concentration fluid (blood) diffuse across the peritoneal membrane into a low concentration fluid (peritoneal dialysis solution).
3. Convection	Moving solutes against a pressure gradient drags solute molecules along.

1.4.3.4 The access of PD

The establishment of permanent PD access is a necessary and key procedure, which must be performed when PD is implemented as RRT. This is performed by introducing a plastic tube, known as a catheter, through which the PDS can be infused into and drained out of the peritoneal cavity (Akoh & Hakim, 2001; Levy *et al.*, 2009). Basically, the PD catheter consists of three segments classified according to the place of implementation: (1) the intraperitoneal section located inside the peritoneal cavity; (2) the external fragment, which is visible because it is placed outside the human body and which connects to the PDS bag; and (3) the tunnelled part that is positioned between the subcutaneous tissue and the abdominal wall (Akoh & Hakim, 2001; Levy *et al.*, 2009). The tunnel section of the catheter increases the distance that the bacteria have to move from the skin to the peritoneal membrane (Levy *et al.*, 2015). Most catheters are designed with double cuffs, the subcutaneous cuff and the deep cuff, to fix the catheter in its correct position and to block bacterial migration through the tunnel (Levy *et al.*, 2015; Levy *et al.*, 2009) (Figure 1.6). The PD catheters are mainly composed of smooth silicon rubber (silastic), which is soft, flexible and inert, and is designed to minimise surrounding tissue damage. It does not include harmful medical plasticisers (Levy *et al.*, 2015). The most frequently-used catheter for PD patients is the double-cuff Tenckhoff catheter, which was designed by Tenckhoff in 1970 (Akoh & Hakim, 2001; Lai, 2009; Thodis *et al.*, 2005) (Figure 1.7). Catheter-related infections (peritonitis, tunnelled infection and exit-site infection) are considered to be one of the primary complications of PD catheters and a major reason to terminate PD and transfer to HD (Barratt *et al.*, 2008; Levy *et al.*, 2015).

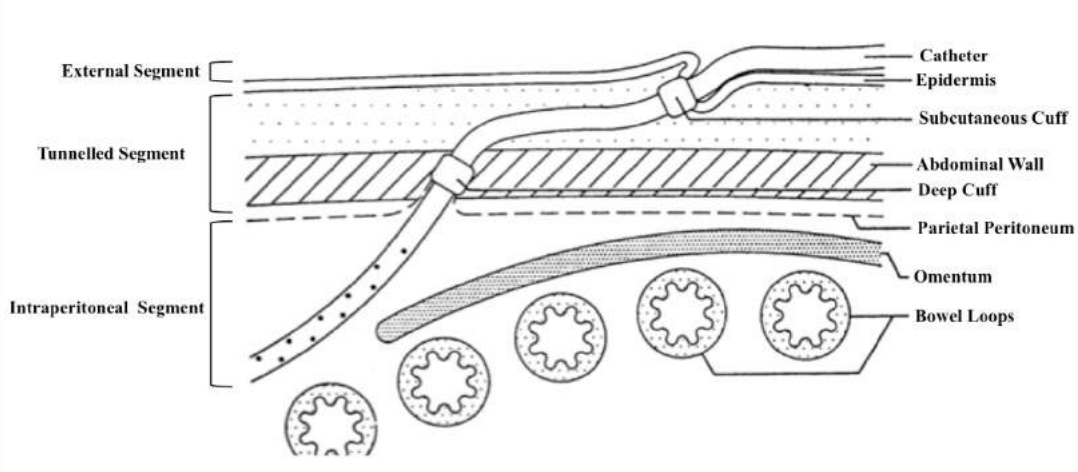


Figure 1.6 Diagram depicting the PD catheter implementation.
The figure demonstrates the three basic segments of the PD catheter, and the location of the two cuffs. The figure was adapted from Ash & Janle (1993).

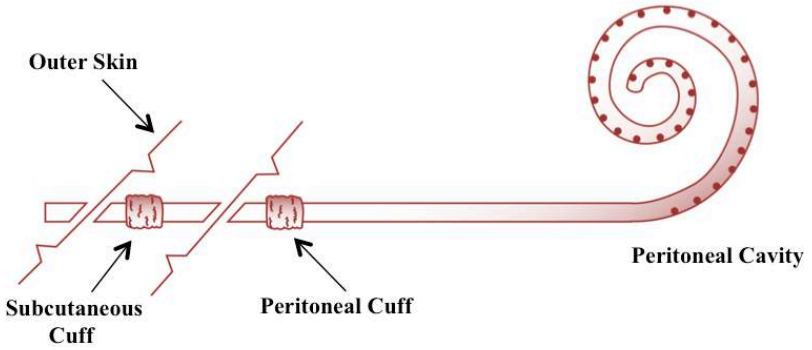


Figure 1.7 Diagram of the curled Tenckhoff PD catheter.
The figure illustrates the placement of the two cuffs and a curved intraperitoneal end, which has many perforations to allow the inflow and outflow of fluid. The figure was adapted from Bersenas (2011).

1.4.3.5 The techniques of PD

1.4.3.5.1 Continuous ambulatory peritoneal dialysis (CAPD)

CAPD is a manual PD technique that mainly involves four exchanges of the peritoneal dialysis fluid (PDF) per day by applying two litres of PDS to each exchange. Gravity is used to fill and empty the peritoneal space. As shown previously in Figure 1.5, the PDS is drained into the peritoneal cavity via a catheter. During this stage, solute clearance and excess water removal takes place between the PDS and the peritoneal membrane. After four to six hours (dwelling time), PDF will drain out via the catheter into a drainage bag (Barratt *et al.*, 2008; Clarkson *et al.*, 2010; Ellam & Wilkie, 2011; Lai, 2009; Munib, 2004). The final exchange remains inside the patient's peritoneal cavity overnight and is

drained out on the morning of the next day (Barratt *et al.*, 2008; Clarkson *et al.*, 2010; Ellam & Wilkie, 2011; Lai, 2009; Munib, 2004). The current study focuses mainly on PDF samples that were taken from CAPD patients.

1.4.3.5.2 Automated peritoneal dialysis (APD)

APD utilises an automated dialysis cycling machine. The instrument is connected to the PD patient's catheter just before the patient goes to sleep. While the patient sleeps, the cycler machine automatically performs repeated dialysis exchanges (Barratt *et al.*, 2008; Lai, 2009; Levy *et al.*, 2015; Munib, 2004). There are four different modes of APD, according to whether the dialysis fluid remains in the peritoneal cavity or extra manual exchanges are performed during the day (Barratt *et al.*, 2008; Lai, 2009; Levy *et al.*, 2015; Munib, 2004). The main differences between them are stated in Table 1.4.

Table 1.4 A comparison of different modes of APD.
The principle of APD modalities was summarised from Barratt *et al.* (2008) and Munib (2004).

APD modality	Principle
1. Continuous cycling peritoneal dialysis (CCPD)	• Four to six dialysis exchanges are performed in a continuous and automatic manner overnight. • The last exchange remains in the patient's peritoneal cavity during the day-time until the next overnight exchange is commenced.
2. Optimised cycling peritoneal dialysis (OCPD)	• In addition to the overnight dialysis exchanges, two further exchanges are performed during the day-time. • It is performed to enhance ultrafiltration during the day or to promote solute clearance.
3. Nocturnal cycling peritoneal dialysis (NIPD)	• The PD machine performs exchanges at night with an empty peritoneal cavity during the day-time.
4. Tidal APD	• The cycler machine is scheduled to leave a specific amount of the previous fluid in the peritoneal cavity before the next exchange takes place, so the cavity is never empty.

1.4.3.6 PD solutions

Peritoneal dialysis solution (PDS) is an aqueous solution, which has three basic components: an osmotic agent to produce osmotic drive for ultrafiltration, a buffer to correct metabolic acidosis and electrolytes (Barratt *et al.*, 2008; Chan *et al.*, 2003) (Table 1.5).

Table 1.5 A summary of the standard composition of the peritoneal dialysis solution.
The table was modified from Barratt *et al.* (2008).

PDS constituents	Solute type and concentration
1. Osmotic agent	• Glucose (anhydrous); 1.36 to 3.86 grams per decilitre (g/dL) or • Amino acids; 1.1 g/dL or • Icodextrin; 7.5 g/dL
2. Buffer	• Lactate; 35 to 40 millimoles per litre (mmol/L) or • Lactate/bicarbonate; 25/10 to 15 mmol/L or • Bicarbonate; 34 to 39 mmol/L
3. Electrolyte	• Sodium; 132 mmol/L, • Calcium; 1.0 to 1.75 mmol/L, • Magnesium; 0.25 to 0.75 mmol/L and • Chloride; 95 to 102 mmol/L

The most commonly-used osmotic agent in PDS is glucose, because it is effective, safe, low-cost and easily metabolised (Sitter & Sauter, 2005). The glucose, however, is quickly absorbed by the peritoneum (150-300 grams per day [g/day]), which leads to a fall in osmosis effectiveness over extended exchange periods. The peritoneal glucose absorption also leads to difficulty in blood glucose control in diabetic PD patients and can cause multiple metabolic complications such as hyperglycaemia, hyperinsulinaemia, hyperlipidaemia and weight gain (Barratt *et al.*, 2008; Hain & Kessel, 1987; Levy *et al.*, 2015). Furthermore, as a consequence of glucose heating during autoclaving of PDS, glucose degradation products are produced, which have a contributory role in peritoneal membrane failure (Sitter & Sauter, 2005; Witowski *et al.*, 2003). The glucose-based PDS are produced with an acidic pH to avoid caramelisation of the glucose. As a result, the acidic PDS causes patient discomfort during solution inflow (Hain & Kessel, 1987; Levy *et al.*, 2015). In order to minimise the metabolic and biocompatibility adverse effects of glucose, there are several alternatives to glucose-based solutions:

Amino acids can also be used as osmotic agents to substitute for glucose in PDS. Actually, 1.1% of amino acid PDS solution produces an ultrafiltration rate equivalent to 1.36% glucose PDS solution (Barratt *et al.*, 2008; Levy *et al.*, 2015). It also provides PD patients

with approximately 20 to 25% of their daily protein requirements; thus, it is useful for malnourished PD patients (Hain & Kessel, 1987). More importantly, amino acid solutions have a higher physiological pH than glucose-based solutions. Therefore, peritoneal membrane integrity is better preserved with amino acid-based solutions than with glucose (Chan *et al.*, 2003). Despite valuable advantages of amino acid solutions in PD, their high cost limits their widespread usage (Hain & Kessel, 1987). Another limitation is that the amino acid PDS solution is usually performed only once per day, because more exchanges can result in the development of uremia and acidosis (Barratt *et al.*, 2008; Hain & Kessel, 1987). Icodextrin, a large glucose polymer (average M.W 17,000 Da), can be used as an osmotic agent in PDS. In comparison with glucose, icodextrin achieves more sustained, positive net ultrafiltration rates over long exchange periods (Hain & Kessel, 1987; Levy *et al.*, 2015). In addition, it causes lower deterioration in the peritoneal membrane function than glucose. However, it can cause hypersensitivity reactions and sterile peritonitis with abundant mononuclear cells in the PDF (Hain & Kessel, 1987; Levy *et al.*, 2015).

1.4.3.7 Why use PD first?

The fundamental reason to initiate RRT with PD is that it can offer higher quality survival than HD in the first two years of dialysis therapy (Chaudhary *et al.*, 2011; Ellam & Wilkie, 2011; Han *et al.*, 2008). PD is superior to HD in terms of preservation of residual renal function. Moreover, PD is the simplest and most economical therapeutic technique for ESKD patients (Chaudhary *et al.*, 2011; Ellam & Wilkie, 2011; Zorzanello, 2004). It is a home-based therapy that provides more independence and flexibility in terms of the patient's lifestyle than complex machine-based HD (Chaudhary *et al.*, 2011; Ellam & Wilkie, 2011; Han *et al.*, 2008; Zorzanello, 2004). There are other reasons to offer PD as the initial choice modality; these are mentioned in Table 1.6. On the whole, the strengths of PD outweigh those of HD.

Table 1.6 A comparison of PD and HD.
The advantages of PD and HD were reviewed from Chaudhary *et al.* (2011), Goldstein *et al.* (2013), Levy *et al.*, (2015), Munib (2004) and Zorzanello (2004).

Dialysis modality	Advantages
PD	• Promotes patient freedom • Lower cost • Preserves vascular access sites • Higher clearance rate for large molecular weight molecules • Less chance for hepatitis B and C transmission • Safer for patients with heart problems (e.g. severe ischemic heart disease) • Improved quality of life
HD	• Long-term survival technique • Convenient for patients who cannot perform dialysis by themselves

1.5 Complications associated with PD

Although there are significant advantages of PD over HD, the percentage of PD patients in the UK has decreased since 2000 (Figure 1.3). The most likely reason for the underutilisation of PD is its potential complications, particularly infectious peritonitis (Chaudhary *et al.*, 2011; Ellam & Wilkie, 2011; Zarrinkalam *et al.*, 2001). PD complications can lead to its termination and a transfer to HD. The investigation of PD complications is therefore critical for several reasons. First of all, physicians need to identify risk factors, which could promote the development of complications in some patients as opposed to others. Second, understanding the underlying mechanisms of those complications will help in outlining management strategies to minimise their risk. More importantly, an investigation of complications particularly associated with infection will help to maximise the patients' time with the PD technique, as in some patients, the PD modality is the *only* option for them to replace the function of their damaged kidneys. Thus, this will improve the odds for the patients' survival as well as improve their quality of life. The most common drawbacks of the PD technique are illustrated in Figure 1.8. The two main complications are the chronic loss of protein into the peritoneal dialysate and infectious peritonitis.

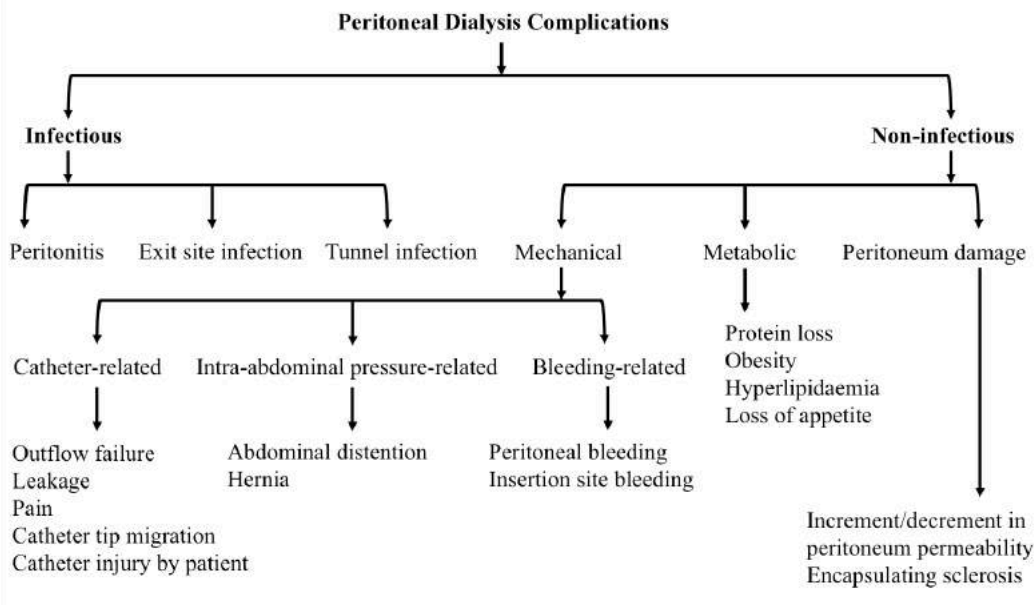


Figure 1.8 The infectious and non-infectious complications of PD.
The complications were assembled from Chaudhary *et al.* (2011), Ellam & Wilkie (2011), Leblanc *et al.* (2001), Kim *et al.* (2015), Levy *et al.* (2015) and Munib (2004).

1.5.1 Protein loss during PD

One of the principal functions of the kidneys is to reabsorb proteins from the glomerular filtrate into the blood (Section 1.1.1). However, when kidney failure exists and PD is used as a RRT, the loss of protein into the PDF becomes one of the obvious limitations of PD (Blumenkrantz *et al.*, 1981). Consequently, the PDF becomes a medium rich in complex proteins (Blumenkrantz *et al.*, 1981a). It has been reported (Blumenkrantz *et al.*, 1981; Dulaney & Hatch, 1984) that PD patients lose a substantial number of proteins during the PD exchange. The range of proteins lost into the PDF of PD patients is between 5 and 15 g/day; however, this amount increases when peritonitis occurs (Blumenkrantz *et al.*, 1981; Barratt *et al.*, 2008). The most abundant protein lost is albumin, which accounts for 50 to 79% of the total protein lost, whereas immunoglobulin G (IgG) represents the second most abundant protein lost, at 5 to 28% of the total (Blumenkrantz *et al.*, 1981; Dulaney & Hatch, 1984; Levy *et al.*, 2015; Barratt *et al.*, 2008). The loss of protein into the PDF can result in poor outcomes for some PD patients. In order to compensate for the lost proteins and to preserve the nitrogen balance, chronic PD patients should have a 60% higher dietary protein intake (around 1.2 grams of protein per kilogram of body weight per day [g/kg/day]) than the healthy population (Blumenkrantz *et al.*, 1981; Westra *et al.*, 2007). There are several PD studies which have performed comprehensive proteomics analyses to identify the proteins lost into the PDF (Cuccurullo *et al.*, 2011; Lin *et al.*,

2008; Wen *et al.*, 2013; Krediet, 2011; Raaijmakers *et al.*, 2008). Raaijmakers *et al.* (2008) classified these proteins into eight groups according to their biological functions (Table 1.7).

Table 1.7 The classification of proteins lost into the PDF based on the study by Raaijmakers *et al.* (2008).

Classification	Example	Reference
1. Acute phase proteins	Alpha-1 antitrypsin, transferrin, ceruloplasmin, transthyretin, retinol-binding protein, albumin and immunoglobulins.	Tyan *et al.* (2013), Oliveira *et al.* (2014), Wen *et al.* (2013), Sritippayawan *et al.* (2007) and Dulaney & Hatch (1984)
2. Extracellular matrix proteins	Collagen.	Dulaney & Hatch (1984)
3. Coagulation factor proteins	Fibrinogen and plasminogen.	Pattinson *et al.* (1981) and Oliveira *et al.* (2014)
4. Complement factor proteins	C3 and C4.	Wen *et al.* (2013) and Blumenkrantz *et al.* (1981)
5. Apolipoproteins	Apolipoprotein A and apolipoprotein B.	Dulaney & Hatch (1984) and Wen *et al.* (2013)
6. Enzymes	Acid phosphatase.	Dulaney & Hatch (1984)
7. Hormones	Parathyroid hormone.	Dulaney & Hatch (1984)
8. Vitamin binding proteins	Vitamin D-binding protein.	Oliveira *et al.* (2014)

Various classes of proteins are produced locally (from an intraperitoneal source), or close to the peritoneal cavity itself, or from the blood serum (Dulaney & Hatch, 1984). For example, a series of proteases, acid hydroxylases and active complement components are generated from neutrophils and activated macrophages, which are endogenous to peritoneal fluid (Dulaney & Hatch, 1984). What are the variables that influence protein

loss into the PDF? Basically, there is sustained inter-patient variability in protein losses due to differences in the permeability characteristics of the peritoneum, the glucose concentration in the PDS, the PD adequacy, the residual renal function, the presence of peritonitis and the patient's nutritional status (Blumenkrantz *et al.*, 1981; Krediet, 2011). A study conducted by Cuccurullo *et al.* (2011) investigated the effect of different concentrations of glucose in PDS on the proteomics profile of PDF derived from PD patients. Cuccurullo *et al*. (2011) have found that four different types of proteins were under-expressed when the highest glucose concentration PDS was used (4.25%, as opposed to 1.5% and 2.5% glucose). The proteins identified were alpha-1-antitrypsin, fibrinogen beta chain, transthyretin and apolipoprotein (Cuccurullo *et al.*, 2011). Other factors are shown to affect protein accumulation in the PDF, including the serum protein concentration and the patient's overall clinical status (Dulaney & Hatch, 1984). Comparable studies were performed to investigate differences in the protein profile of PDF derived from normal CAPD patients compared to PDF collected from CAPD patients who had peritonitis (Krediet, 2011; Lin *et al.*, 2008; Carozzi *et al.*, 1990). Interestingly, beta-2-microglobulin protein was present in higher concentrations in the peritonitis PDF, as compared to normal PDF. Thus, the beta-2-microglobulin protein was suggested to be a biological marker for CAPD peritonitis (Lin *et al.*, 2008). However, prospective studies are needed to establish predictive endogenous biomarkers that could anticipate PD peritonitis. The cause and effect association between protein loss and the risk of peritonitis needs to be established; therefore, this is an objective of the current study.

1.5.2 PD-associated peritonitis

Despite continuous improvements and innovations in patient education, in the PD connecting system, in the dialysis procedures and in prophylaxis, peritonitis is still the Achilles' heel of PD (Davenport, 2009; Sweny *et al.*, 2003). Peritonitis is an inflammation of the peritoneal membrane, which is most often caused by an infectious agent. It is a clinically significant condition considered to be a leading complication of the PD technique. It is also a contributing factor to the high mortality and morbidity rates of PD patients. Peritonitis initiates peritoneal membrane failure, thereby leading to PD termination and the switch to HD (Ellam & Wilkie, 2011; Zarrinkalam *et al.*, 2001; Kerschbaum *et al.*, 2012; Li, 2001). One of the main catastrophic peritonitis

complications is repeated episodes of peritonitis, which results in long-term ultrafiltration failure and requires switching to HD. As a result of repeated courses of antibiotics, fungal peritonitis may develop and worsen a patient's clinical health status (Zarrinkalam *et al.*, 2001). The innate immune system plays an important role in fighting the episode of peritonitis through a number of effective immune defence mechanisms, including peritoneal white blood cells (mostly macrophages), antibodies, complement factors, opsonins and defensins (endogenous antimicrobial peptides) (Zarrinkalam *et al.*, 2001). During an episode of peritonitis, these innate immune tools are promoted by the release of soluble proinflammatory factors (e.g. tumour necrosis factor alpha [TNFα]), leukotrienes (e.g. interleukin-8), and an influx of leukocytes (mainly neutrophils) from the blood (Zarrinkalam *et al.*, 2001). A significant variation in the rate of peritonitis around the world in the period between 2006 and 2010 has been documented (Piraino *et al.*, 2011). As illustrated in Table 1.8, the peritonitis rate in the US was three times lower (0.24 episodes per year) than that in the UK (0.82 episodes per year) in 2009 (Piraino *et al.*, 2011). The reasons behind the differences between countries may include the variability in recording peritonitis rates and differences in infection prevention policies. Differences in the size of the patient populations and variations in patient training and education programmes may also factor into the equation (Piraino *et al.*, 2011).

Table 1.8 Worldwide peritonitis rates.
The table was modified from Piraino *et al.* (2011).

Country	Year	Number of patient population	Number of episodes per year	Reference
Japan	2006	139	0.22	Nakamoto *et al.* (2006)
Canada	2008	312	0.33	Fang *et al.* (2008)
US	2009	137	0.24	Qamar *et al.* (2009)
Portugal	2006	312	0.39	Rodrigues *et al.* (2006)
China	2008	496	0.20	Fang *et al.* (2008)
Taiwan	2008	100	0.06	Chen *et al.* (2008)
UK	2009	1,904	0.82	Davenport (2009)
Austria	2009	332	0.24	Kopriva-Altfahrt *et al.* (2009)
Brazil	2009	680	0.74	Moraes *et al.* (2009)
Spain	2009	641	0.38	Fontan *et al.* (2009)
Netherlands	2009	205	0.60	Ruger *et al.* (2011)
France	2010	1,631	0.36	Castrale *et al.* (2010)
Australia	2010	4,675	0.60	Jarvis *et al.* (2010)
Qatar	2010	241	0.24	Shigidi *et al.* (2010)

1.5.2.1 Clinical signs and symptoms of peritonitis

Abdominal pain is considered to be a vital clinical feature of peritonitis in 80% of PD patients; however, in some patients, it does not present as a symptom until the fluid in the dialysis bag has been turbid for many days (Levy *et al.*, 2015). Cloudy dialysis fluid is a principal sign of acute peritonitis; thus, PD patients should be trained to report cloudy fluid on drainage as soon as they see it (Barratt *et al.*, 2008; Levy *et al.*, 2015). Other important characteristics of peritonitis include fever, nausea and diarrhoea in 50%, 30% and 7 to 10% of PD patients, respectively (Levy *et al.*, 2015). In some cases, fibrin is formed and results in catheter blockage, which leads to poor drainage of the dialysis fluid. Loss of ultrafiltration is also a relevant sign of peritonitis and may persist even after successful treatment of a peritonitis episode (Barratt *et al.*, 2008; Levy *et al.*, 2015).

1.5.2.2 Diagnosis of peritonitis

After the appearance of the signs and symptoms of peritonitis, an investigation is normally rapidly launched into determining the causative microorganism. Samples of the turbid PDF are usually sent to a medical microbiology laboratory to allow microscopic examination of the PDF (Levy *et al.*, 2015). The first obvious feature is the high number of white blood cells (WBCs; leukocytes): > 100 WBC/µl with ≥ 50% neutrophils (Barratt *et al.*, 2008; Ellam & Wilkie, 2011). If bacterial cells appear under the microscope, Gram staining must be performed to start the identification of the causative microorganism (Levy *et al.*, 2015). Afterwards, culturing the PDF using an enrichment medium to identify the microorganism at the species level and that will help to determine which class of antibiotics will be the most effective in treating the peritonitis (Levy *et al.*, 2015).

1.5.2.3 Aetiology of peritonitis

There are two principal routes for peritonitis: (1) from normal flora that reside on the skin (skin contaminants) via the dialysis catheter; and (2) from within the peritoneum itself via the intestine (Levy *et al.*, 2015). The skin contaminants are infectious agents, which are introduced accidently at the time of connection or by an infected exit-site (Levy *et al.*, 2015). The most common normal skin flora that can cause peritonitis are *Staphylococcus epidermidis* (*S.epidermidis*) and *Staphylococcus aureus* (*S.aureus*). Staphylococci are the most common bacteria to infect clinical devices (e.g. PD catheters), which penetrate the skin. *Pseudomonas species* can also cause peritonitis via skin contamination, and is mainly associated with exit-site infection (Levy *et al.*, 2015; Ellam & Wilkie, 2011; Castrale *et al.*, 2010; Barretti *et al.*, 2009; Li, 2001). Intestinal bacteria, including Gram-negative rods, which are introduced into the peritoneum directly from the intestine, can cause peritonitis. In other words, any pathological condition that enhances bowel permeability (e.g. episodes of diarrhoea and diverticulitis) or a bowel perforation (e.g. appendicitis) can cause peritonitis in PD patients (Levy *et al.*, 2015; Goldstein *et al.*, 2013). Fungal infections, in particular the *Candida species*, can cause peritonitis following long courses of antibiotics. *Mycobacterium tuberculosis* can also cause peritonitis in immunocompromised PD patients due to reduced cellular immunity (Kaplan *et al.*, 1993; Goldstein *et al.*, 2013; Levy *et al.*, 2015).

Many studies have been conducted to identify the causative microbiological agents responsible for peritonitis during PD treatment. Coagulase-negative staphylococci (mainly *S.epidermidis*) and *S.aureus* were the bacteria most commonly responsible for

peritonitis (Castrale *et al.*, 2010; Thirugnanasambathan *et al.*, 2012; Kofteridis *et al.*, 2010; Pajek *et al.*, 2011; Vikrant *et al.*, 2013); therefore, these two bacteria were test microbes in the current study. In terms of the analysis of the microbiological profile of PDF during peritonitis, Kofteridis *et al.* (2010) have found that 42% of the episodes of peritonitis were caused by Gram-positive bacteria, whereas 19% of the episodes were caused by Gram-negative bacteria. In addition, polymicrobial peritonitis and fungal peritonitis were found in 5% and 4% of the episodes, respectively, whereas 30% of the PDF cultures were negative (Kofteridis *et al.*, 2010). The microbiological profile of the PDF from the Kofteridis *et al.* (2010) study is illustrated in Table 1.9. *S.epidermidis* and *S.aureus* were the most common Gram-positive bacteria, identified in 48 and 20 cases, respectively. In contrast, the predominant Gram-negative bacterium was *Escherichia coli*, which was isolated in eight cases, followed by *Pseudomonas aeruginosa*, *Enterobacter species* and *Klebsiella species* (Kofteridis *et al.*, 2010).

Table 1.9 The microbiological profile of peritoneal dialysis fluid from PD patients, who had peritonitis.
The table was adapted from Kofteridis *et al.* (2010).

Infectious agent	Number of episodes
Gram-positive bacteria	**104**
Staphylococcus epidermidis	48
Staphylococcus aureus	20
Corynebacterium species	8
Enterococcus species	6
Streptococcus mitis	6
Staphylococcus hominis	5
Other *Staphylococcus, Streptococcus* and *Propionibacterium species*	11
Gram-negative bacteria	**46**
Escherichia coli	8
Pseudomonas aeruginosa	7
Enterobacter species	7
Klebsiella species	7
Acinetobacter species	4
Other *Pseudomonas, Neisseria, Serratia, Proteus* and *Haemophilus species*	13
Fungi	**11**
Candida albicans	5
Candida tropicalis	3
Candida inconspicua	2
Paecilomyces species	1
Polymicrobial	**13**
Culture-negative	**73**

1.5.2.4 Pathogens involved in PD peritonitis

1.5.2.4.1 *Staphylococcus epidermidis*

S.epidermidis is a Gram-positive, non-motile, coccus bacterium arranged in grape-like clusters (Baker *et al.*, 2011). It is coagulase-negative and commonly resides on the human skin and in mucus membranes as normal flora in all healthy individuals (Dasgupta, 2002; Otto, 2009). However, *S.epidermidis* becomes an opportunistic pathogen in immunocompromised patients or when translocated from its original niche (skin and mucus membranes) to a new place (the peritoneal cavity, in the case of PD) (Otto, 2009; Otto, 2008). *S.epidermidis* has been shown (Dasgupta, 2002; Otto, 2009) to be the most predominant bacterium responsible for infection on indwelling medical devices, such as PD catheters; therefore, *S.epidermidis* is considered to be the most common bacteriological agent, which causes PD-related peritonitis. The virulence of this bacterium depends mainly on its ability to produce biofilm on plastic surfaces (e.g. PD catheters), which makes the extermination of the infection difficult, and in some cases, requires catheter removal (Dasgupta, 2002; Otto, 2009). Hence, biofilm production becomes a central concern for PD patients. The term *biofilm* refers to the accumulation of staphylococcal cells on plastic surfaces, a process that enables bacteria to be resistant to antibiotics as well as to the host's immune system (Dasgupta, 2002; Otto, 2009) (Figures 1.9 and 1.10). Although the major virulence factor for *S.epidermidis* is biofilm formation, there are other virulence factors involved in *S.epidermidis* pathogenesis (Table 1.10).

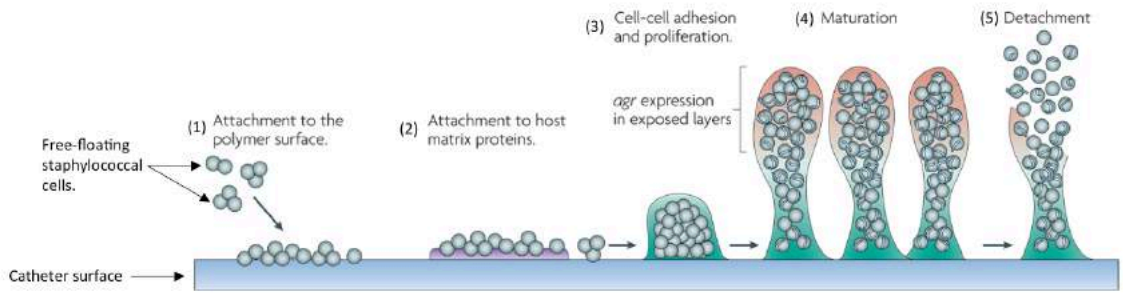


Figure 1.9 A diagrammatic representation of biofilm development for *S.epidermidis* on the PD catheter surface.
The biofilm formation initiates with direct attachment of free-floating (planktonic) bacteria to the catheter surface, followed by attachment to the host matrix proteins such as fibrinogen or fibronectin. After the initial attachment, the staphylococcal cells adhere to the catheter surface, produce molecular signals, and proliferate to produce staphylococcal microcolonies. Then, the mature biofilm formed by intracellular aggregation and biofilm-structuring forces generates the three-dimensional appearance of mature biofilm. Finally, the detachment step is important to allow the dissemination of *S.epidermidis* to other colonisation niches. The *agr* refers to the quorum sensing signals system, which possibly has a role in biofilm maturation, structuring and detachment. The figure was adapted from Otto (2009).

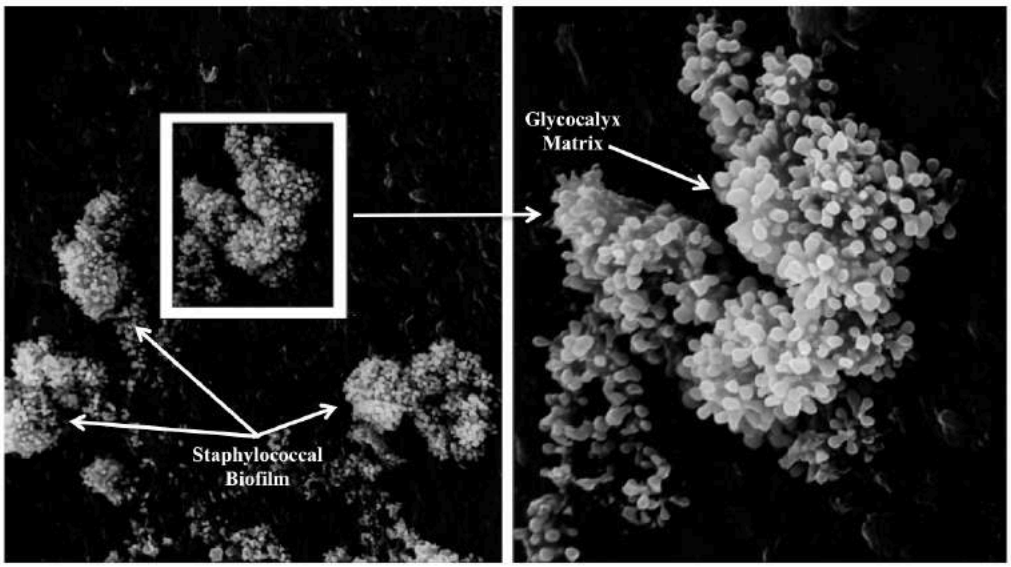


Figure 1.10 A scanning electron microscopy image shows the *S.epidermidis* biofilm on the outer surface of a PD catheter.
The image on the right shows magnification of the biofilm bacteria with the sticky glycocalyx matrix that covers microcolonies. The figure was modified from Dasgupta (2002).

Table 1.10 Virulence factors of *S.epidermidis.*
The table was re-adjusted from Otto (2009).

	Virulence factor	Gene	Biological role	Reference
Toxins	Phenol-soluble modulins (PSMs)	*psmα*, *psmδ*, *psmβ1*, *psmβ2* and *psmε*	Proinflammatory cytolysins	Yao *et al.* (2005)
Exoenzymes	Cysteine protease staphopain B (SspB)	*sspB*	Tissue damage	Dubin *et al.* (2001)
	Metalloprotease or elastase (SepA)	*sepA*	Lipase maturation and tissue damage	Teufel & Gotz (1993)
	Lipase GehC and GehD	*gehC* and *gehD*	Persistence in host fatty acid secretions	Simons *et al.* (1998)
Other factors	Staphyloferrin A	*sfna*	Iron acquisition (siderophore)	Lindsay *et al.* (1994) and Cotton *et al.* (2009)
	SitA, SitB and SitC	*sitA*, *sitB* and *sitC*	Iron importers	Cockayne *et al.* (1998)

1.5.2.4.2 *Staphylococcus aureus*

S.aureus is similar to *S.epidermidis* in terms of its Gram reaction, cellular shape and arrangement (Baker *et al.*, 2011). However, *S.aureus* is coagulase-positive, and it is part of the normal human microbiota found in the nasal cavity in around 19% to 55.1% of the general population (Kluytmans *et al.*, 1997). *S.aureus* is the next most common bacteria, after *S.epidermidis*, which causes PD peritonitis. However, PD-related *S.aureus* peritonitis is responsible for a higher patient hospitalisation rate, a higher catheter removal rate and a higher mortality rate (Barretti *et al.*, 2012). PD patients, who are carriers for *S.aureus* are at higher risk for *S.aureus* infections than others; thus, the carriage of *S.aureus* is an essential risk factor for infection development. *S.aureus* strains, which have the *mec*A gene cause more severe infections than others, because the *mec*A gene produces resistance to beta-lactam antibiotics, including methicillin (Barretti *et al.*, 2012; Kluytmans *et al.*, 1997; Govindarajulu *et al.*, 2010). Methicillin-resistant *S.aureus* (MRSA) results in a poorer outcome for patients having peritonitis than does methicillin-sensitive *S.aureus* (MSSA) (Barretti *et al.*, 2012; Kluytmans *et al.*, 1997; Govindarajulu *et al.*, 2010). In addition to its capacity for biofilm formation, *S.aureus* has the ability to produce a number of virulence factors that play a key role in *S.aureus* pathogenesis. Barretti *et al.* (2012) have isolated specific *S.aureus*-secreted virulence factors including exoenzymes and toxins from patients who were experiencing *S.aureus* peritonitis episodes (Table 1.11). Among all of the pathogenic factors produced, beta-haemolysin most negatively affected the peritonitis outcome, which suggests beta-haemolysin may play a pathogenic role in PD-associated *S.aureus* peritonitis (Barretti *et al.*, 2012).

Table 1.11 The secreted pathogenic factors of *S.aureus* isolated from PD-related peritonitis, and the action of these factors.
The table was shaped from Barretti *et al.* (2012).

	Virulence factor	Gene	Biological action	Reference
Exoenzymes	Alpha-haemolysin	*hla*	• Toxic effect on a wide range of host cells. • Dermonecrotic and neurotoxic. • Forms pores in the membrane of a variety of cell types, resulting in membrane permeability damage.	Dinges *et al.* (2000)
	Beta-Haemolysin (Sphingomyelinase)	*hlb*	• Lyses eukaryotic cells, in particular sheep erythrocytes; therefore, produces beta-haemolytic activity on blood agar. • Enhances *S.aureus* growth in the murine mammary glands. • Hydrolyses sphingomyelin of the cell membranes.	Dinges *et al.* (2000) and Barretti *et al.* (2012)
	Lipase	*lip*	• Produced during infection and causes functional impairment in immune cells (e.g. macrophages). • Hydrolyses triacylglycerol substrate.	Jaeger *et al.* (1994)
	Lecithinase	*plcB*	• Lipolytic activity.	Matos *et al.* (1995)
	Deoxyribonuclease	*dnase*	• Hydrolyses deoxyribonucleic acids.	Kerro Dego *et al.* (2002)
	Thermonuclease	*nuc*	• Catalyses the hydrolysis of nucleic acids (DNA and RNA).	Barry *et al.* (1973)
Toxins	Enterotoxin A (SEA) Enterotoxin B (SEB) Enterotoxin C (SEC)	*sea* *seb* *sec*	• Superantigenic, cause gastroenteritis and resistant to heat and pepsin digestion.	Dinges *et al.* (2000)
	Toxic shock syndrome toxin-1	*tst*	• Superantigenic and lethal properties.	(Dinges *et al.*, 2000)

1.6 What predisposes PD patients to the development of peritoneal infections?

There are a considerable number of patient risk factors for PD-associated peritonitis reported in the literature, including having an insufficient educational level, having a low socioeconomic status, using the connect PD system, using CAPD versus APD and being female (Kerschbaum *et al.*, 2012; Oygar *et al.*, 2011; Farias *et al.*, 1994; Rodriguez-Carmona *et al.*, 1999). PD is a home-based therapy (as compared to haemodialysis); therefore, the educational levels of the patients and their socioeconomic status may have an influence on their PD outcomes (Kerschbaum *et al.*, 2012; Chern *et al.*, 2013). It has been reported by Chern *et al.* (2013) that a lower level of education is a strong predictor of peritonitis. Moreover, a study conducted by Chow *et al.* (2005) demonstrated that low socioeconomic status and limited education were closely related to an increased rate of PD peritonitis. The mean peritonitis-free time for PD patients who were not on social security assistance was much higher (16.4 months) than for those who were on assistance (2.7 months) (Chow *et al.*, 2005).

Another factor that has been considered is the disconnect bag system reduces the rate of CAPD peritonitis. The migration of skin microflora, such as *S.epidermidis* and *S.aureus*, via the PD catheter to the peritoneal cavity can occur during the fluid exchange process (Daly *et al.*, 2001). Therefore, the design of the PD system that is utilised can have an impact on the likelihood of peritonitis (Daly *et al.*, 2001). Many studies have been performed to evaluate the rate of peritonitis when the disconnect system is used, as compared to the standard connect system (Daly *et al.*, 2001; Burkart *et al.*, 1992; Canadian, 1989; Owen *et al.*, 1992). The standard connect system means that when the PD exchange is finished, the bag is drained and rolled up, and it remains connected to the patient's catheter until the next exchange is due (Daly *et al.*, 2001). However, in the disconnect system, the patient disconnects from the dialysis bag between exchanges (Daly *et al.*, 2001). When the next exchange is due, the disconnect system is flushed with fresh PDS, which is drained into the drainage bag. This process allows the bacteria to be washed away into the drainage bag before starting PD (Daly *et al.*, 2001). Accordingly, the use of the connect system was statistically and clinically associated with a significantly higher rate of peritonitis, as compared with the rate of peritonitis when the disconnect system was used (Daly *et al.*, 2001).

It has also been suggested by Rodriguez-Carmona *et al.* (1999) that APD may be associated with a lower peritonitis rate than CAPD because of the fewer number of connections and exchanges involved. Rodriguez-Carmona *et al.* (1999) reported that the rate of peritonitis in CAPD patients was increased by 0.2 episodes per patient year, as compared to APD patients. A study conducted by Kotsanas *et al.* (2007) also indicated that females had a higher percentage of peritonitis caused by gram-negative rods, including *Escherichia coli*, *Klebsiella species*, *Proteus species* and *Pseudomonas*. The study's authors have reported for the first time that gender is an independent risk factor for peritonitis, with women being almost twice as susceptible to developing peritonitis as men are (Kotsanas *et al.*, 2007). This tendency might be due to ascending infections from the urogenital tract, which are caused by gram-negative rods. In contrast, equality between males and females has been observed in the incidence of peritonitis caused by gram-positive cocci (Kotsanas *et al.*, 2007).

Despite the identifying of the risk factors for PD-associated peritonitis and policies to reduce the risk of infection, its occurrence is still a problem related with significant morbidity and mortality rates. This means that there are undiscovered prognostic factors, possibly endogenous host factors, which may play a role in the establishment of PD-related peritonitis. The investigation of host risk factors could provide novel insights into the pathogenesis of PD peritonitis, as well as insights into new prevention approaches. Interestingly, previous work performed by Noura Al-Dyan (2011) in our laboratory at the University of Leicester, Leicester, UK, demonstrated an association between the protein concentration in the PDF and the stimulation of *S.epidermidis* growth and virulence, including biofilm formation (Al-Dyan, PhD thesis). By following the progress of the PD technique in five patients over an 18-month period, she found that higher protein concentrations were detected in the patients' PDF compared with the levels present at the beginning of their dialysis. Staphylococcal growth and biofilm formation were found to be highest in the PDF samples having a higher protein load. Her findings spurred me to investigate the association between the identities of the proteins in the PDF and behaviour of bacteria causing peritonitis. The Al-Dayan study was unclear whether the total PDF protein load, or specific proteins were responsible for stimulating microbial growth and virulence.

Several studies (Dasgupta *et al.*, 1986; Oasgupta *et al.*, 1987; Gorman *et al.*, 1994) have demonstrated that PD catheters coated with microbial biofilm were removed from patients who were experiencing peritonitis. However, Pihl *et al.* (2013) found that

bacteria were present on the PD catheters removed from the PD patients who were *not* experiencing peritonitis. In this study, bacteria were detected on 12 of the 15 PD catheters taken from patients who did not have clinical signs and symptoms of peritonitis, with *S.epidermidis* being the most predominant bacterium isolated (Pihl *et al.*, 2013). *S.epidermidis* has been shown to be one of the most common causes of peritonitis (Section 1.5.2.3), but it can exist in a balanced commensal-type relationship with the host's immune system, without giving rise to clinical signs of infection (Cameron, 1995; Eisenberg *et al.*, 1987; Pihl *et al.*, 2013). A change in the balance between *S.epidermidis* and the host's immune system may increase the likelihood of peritonitis development (Pihl *et al.*, 2013). The mechanism responsible for *S.epidermidis* switching from pre-infectious microbial agent to infectious microbial agent is unclear, and might be associated with host-related factors such as the proteins released into the PDF.

Evidence of an association between protein loss into the PDF and the risk of peritonitis is limited. Previous publications (Modun *et al.*, 1994; Smith *et al.*, 1991; Wilcox *et al.*, 1990) have reported that the PDF works as a stimulating factor for the growth of *S.aureus* and *S.epidermidis*. Dong *et al*. (2013) have shown that the protein leakage into the PDF is an independent predictor for the risk of peritonitis. However, whether the types of the protein lost are more critical than the quantity of the protein lost is still unknown (Dong *et al.*, 2013). In other words, either the protein load of the PDF, or some specific proteins released into the PDF, act to influence bacterial growth and virulence, and so infection risk. It has been shown by Modun *et al.* (1994) and Williams *et al.* (1995) that bacteria do not grow well in PDS, however, after the PD exchange, the PDF is rich in serum proteins (including the iron-holding protein transferrin), which have been shown to support bacterial growth (Modun *et al.*, 1994; Williams *et al.*, 1995).

1.6.1 Transferrin, iron and infection

Transferrin is key iron-holding member of the family of glycoproteins found in the plasma and ranging in M.W from 76 to 81 kDa (Lacey *et al.*, 2001; Parkkinen *et al.*, 2002; Modun *et al.*, 1994). The normal transferrin concentration in the blood is from 2 g/L to 3 g/L (Gomme *et al.*, 2005). It is synthesised mainly in the liver. Structurally, transferrin consists of a single polypeptide chain containing 679 amino acid residues connected to each other by 19 disulfide bridges. Transferrin is arranged into two homologous lobes (the *N*-lobe and *C*-lobe), and each lobe has a high affinity single ferric iron (Fe^{+3}) binding

site; thus, transferrin binds two atoms of Fe^{+3} per molecule of protein (Lacey *et al.*, 2001; Parkkinen *et al.*, 2002; Gomme *et al.*, 2005). Transferrin can exist in four different isoforms according to the number of Fe^{+3} bound to it. These isoforms are differic transferrin isoform, *N*-terminal monoferric transferrin isoform, *C*-terminal monoferric transferrin isoform and apo-transferrin isoform (Kitsati *et al.*, 2015; Evans & Williams, 1980; Von Bonsdorff *et al.*, 2002) (Table 1.12).

Table 1.12 A comparison of different transferrin isoforms (Von Bonsdorff *et al.*, 2002).

Name	Abbreviation	Feature
Differic transferrin or holo-transferrin isoform	Tf-Fe_2 or Holo-Tf	Transferrin molecule with two ferric iron atoms.
N-terminal monoferric transferrin isoform	Tf-Fe_N	Monoferric transferrin with single ferric iron atom in the *N*-lobe.
C-terminal monoferric transferrin isoform	Tf-Fe_C	Monoferric transferrin with one ferric iron atom in the *C*-lobe.
Apo-transferrin isoform	Apo-Tf	Transferrin molecule without ferric iron atom.

Transferrin is a multi-tasking protein responsible for many physiological functions in the human body. It is a key part of the innate immune system because it makes extracellular body fluids bacteriostatic due to its ability to bind free ferric iron, thus inhibiting the growth of bacteria (Afzali & Goldsmith, 2004; Gomme *et al.*, 2005). Transferrin is usually partially saturated with ferric iron to maintain the magnitude of free iron at around 10^{-18} M, which is too low to enable microbial growth (Gomme *et al.*, 2005; Modun *et al.*, 1994; Parkkinen *et al.*, 2002). However, in the presence of catecholamine stress hormones (noradrenaline, adrenaline, dopamine and their metabolites), transferrin becomes a bacterial source of iron and can lead to bacterial growth induction in blood or plasma. These factors have been shown to act together to significantly stimulate bacterial growth and virulence (Freestone *et al.*, 2000; Sandrini *et al.*, 2010).

1.6.2 Catecholamine stress hormones

Catecholamine stress hormones are a group of tyrosine-derived effectors, which are chemically characterised by having a catechol (a benzene ring with two adjacent hydroxyl groups) and an opposing amine side chain (Freestone *et al.*, 2008). The biosynthetic pathway of catecholamines is demonstrated in Figure 1.11. In addition to their involvement in the mammalian nervous system function, the catecholamines, in particular

noradrenaline, have an effect on microbial growth and virulence (Freestone *et al.*, 1999; Lyte & Freestone, 2009; Sandrini *et al.*, 2015). Iron is a very essential element for bacterial growth and virulence. One of the iron uptake systems of bacteria operates by secreting siderophores, low-molecular weight secreted molecules with a high affinity for Fe^{+3}. However, in the presence of transferrin, the siderophores are usually unable to retrieve host Fe^{+3} (Freestone *et al.*, 2008; Freestone *et al.*, 2000; Sandrini *et al.*, 2010). It has been shown that catecholamine stress hormones (noradrenaline, adrenaline and dopamine) can mediate the Fe^{+3} removal from transferrin. In other words, the catechol part of the catecholamine stress hormones can complex with the Fe^{+3} sequestered by transferrin and make it accessible to bacteria (Freestone *et al.*, 2008; Freestone *et al.*, 2000; Sandrini *et al.*, 2010). Because catecholamines will be present in PDF and will act on transferrin to make it a bacterial nutrient source, one objective of the current study is to measure the levels of catecholamines in the PDF. This will allow investigating the hypothesis that the levels of catecholamine stress hormones and iron-binding proteins (such as transferrin) in PDF may increase patient susceptibility to peritoneal infection by stimulating bacterial growth. To the best of my knowledge, no studies have looked at the elimination of catecholamines into PDF, so these investigations will allow the determination of whether catecholamines release is a risk factor for the development of infectious peritonitis. The current study aims to investigate (in more detail) the mechanisms at work in terms of increased susceptibility to a peritoneal infection (i.e. whether the total PDF protein load, or specific proteins, such as transferrin, are responsible for bacterial growth enhancement). If the stimulatory factor turns out to be transferrin, this could lead to a simple dipstick prognostic test, which could identify those patients who have become more susceptible to developing a peritoneal infection.

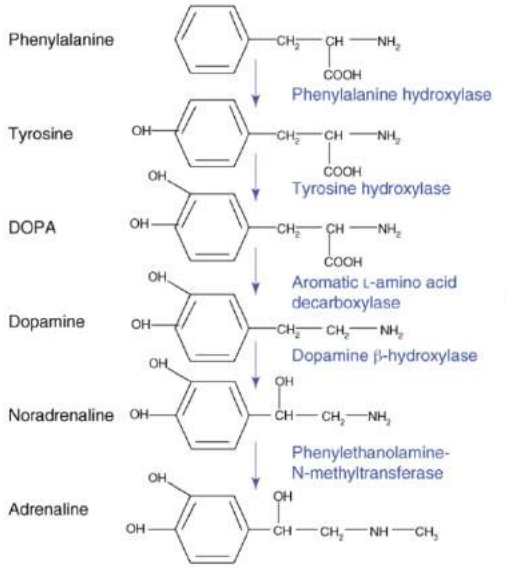


Figure 1.11 The catecholamine chemical structure and endogenous biosynthetic pathways in mammals.
Catecholamines are chemical compounds derived from the amino acid tyrosine (commonly from dietary food sources) and containing a benzene ring with two adjacent hydroxyl groups and an amine side chain. Key: DOPA refers to 3,4-dihydroxyphenylalanine. The figure was adapted from Freestone *et al.* (2008).

1.7 Aims and objectives

Although some of the risk factors for PD-associated peritonitis have been identified, it is still unclear why some PD patients are more susceptible to infection than others. The central aim of the present study is therefore to widen our knowledge and understanding of the host-associated components present within PDF, which may play a key role in stimulating bacterial growth and virulence, and so explain differences between rates of patient infections. A step further will be taken to establish the mechanism between host-related factors and the risk of PD peritonitis. To achieve these goals, a comparative analysis needs to be performed between the PDF (which contains protein molecules and other factors) and PDS (which contains glucose and electrolytes). Furthermore, the differences between the normal non-infected PDF (derived from a patient who does not suffer from peritonitis) and the infected PDF (derived from the same patient who later contracted infectious peritonitis) are also important factors to the study. Therefore, the specific objectives of my study are:

- To comprehensively characterise the PDF from a range of CAPD or APD patients who are new to PD therapy. PDF samples will be collected at the start of their dialysis and at different times during the PD time-course.
- To conduct a follow-up study on a daily basis of APD patient from the beginning of dialysis until termination from APD and the switch to HD (a unique study).
- To accomplish overall protein and hormonal profiling of PDF samples and identification of proteins released into the PDF.
- To investigate how PDF may affect *S.aureus* and *S.epidermidis* growth and virulence.

CHAPTER 2 Materials and Methods

2.1 Materials

2.1.1 PD patients and their peritoneal dialysis fluids

PD patients who were experiencing kidney failure and who frequented the PD unit at Leicester General Hospital were included in this study. I greatly appreciate their donation of their peritoneal dialysis fluids (PDFs) to this study. There were nine PD patients enrolled in the study. The large-scale clinical data from the participating PD patients is summarised in Table 2.1. More than half of these patients had undergone CAPD; hence, they supplied day-time PDFs. Patient 6 underwent APD; therefore, she provided night-time PDFs. On the other hand, Patient 1 underwent CAPD, and later on, was switched to APD; thus, day-time and night-time PDFs were obtained. Approximately 2 L of the PDF were collected from each patient at each visit, which were then used for *in vitro* investigations. In total, there were 20 PDFs obtained from a range of nine PD patients at the beginning of their dialysis and at different points in time. To avoid protein deterioration and microbial contamination, the PDFs were aliquoted under sterile conditions into sterile tubes and kept at -20°C until they were analysed.

Table 2.1 A summary of the clinical characteristics for the nine PD patients enrolled in the current study.

Patient ID	Gender	Age	Cause of kidney failure	PD modality
1	M	78	Idiopathic	CAPD & APD
2	F	51	Idiopathic	CAPD
3	F	40	Renal cortical necrosis	CAPD
4	F	63	Lithium toxicity	CAPD
5	F	63	Idiopathic	CAPD
6	F	54	Hypertensive nephrosclerosis	APD
7	M	82	Idiopathic	CAPD
8	F	46	Systemic lupus erythematosus	CAPD
9	F	24	Idiopathic	CAPD

Key: M: Male; F: Female. CAPD and APD refer to continuous ambulatory peritoneal dialysis and automated peritoneal dialysis, respectively. Idiopathic means that the reason for kidney failure is unknown. Apart from Patients 6 and 8, none of the PD patients were diabetic.

2.1.2 Ethical approval

The study protocol was reviewed and approved by the Leicestershire Local Research Ethics Committee One in Leicester, UK. Permission for obtaining and describing data was approved. Information related to PD patients was anonymised and de-identified prior to analysis. Each PD patient enrolled in the study read and signed the consent form before I began analysing the PDFs. A copy of the ethical approval letter and of the consent form are provided in Appendix II and Appendix III, respectively.

2.1.3 PD solutions

There are four different kinds of PD solutions used by PD patients and in analysis studies. The solutions for this study were kindly provided by Dr. Jonathan Barratt, Leicester General Hospital. PD solutions mainly consist of different concentrations of glucose as osmotic agent. Additionally, they contain electrolytes (magnesium, chloride, calcium and sodium) and lactate or bicarbonate as a buffer. The pH value of the lactate buffered solutions and the bicarbonate buffered solutions is around 5.5 and 7.4, respectively. The actual solute composition of the various types of PD solutions is illustrated in Table 2.2 (as according to the manufacturers' manuals). The PD solutions were stored at room temperature (RT) until used. For PD patients, the PD solutions were warmed to 37°C prior to being used for dialysis, whereas for investigative studies, they were used at RT.

Table 2.2 The solute composition of the PD solutions utilised.

Component	Dianeal PD4 glucose 1.36%	Bicavera glucose 1.5%	Dianeal PD4 glucose 2.27%	Dianeal PD4 glucose 3.86%
Glucose monohydrate (g/L)	13.6	15	22.7	38.6
Sodium chloride (g/L)	5.4	5.7	5.4	5.4
Calcium chloride dehydrate (g/L)	0.184	0.2	0.184	0.184
Magnesium chloride hexahydrate (g/L)	0.051	0.1	0.051	0.051
Sodium lactate (g/L)	4.5	0.0	4.5	5.4
Sodium bicarbonate (g/L)	0.0	2.9	0.0	0.0
pH	5.5	7.4	5.5	5.5

Baxter Healthcare Ltd., UK manufactured the Dianeal PD4 glucose solutions. Fresenius Medical Care Ltd., Germany produced the Bicavera glucose solution.

2.1.4 Bacterial strains

There are three bacterial strains that were studied during the course of this study: *Staphylococcus aureus* strain T288354, *Staphylococcus epidermidis* strain Tü3298, and *Staphylococcus epidermidis* strain RP62A. Dr. Richard Haigh, Department of Genetics, University of Leicester kindly provided these.

2.1.5 Bacterial culture media

Two types of growth media were prepared: tryptone soya broth (TSB) and tryptone soya agar (TSA). They were purchased from Oxoid, UK. The culture media were prepared according to the manufacturer's instructions (Table 2.3).

Table 2.3 Preparation of the bacterial growth media.

Media	Preparation
Tryptone soya broth (TSB)	3 g of TSB medium were mixed with 100 ml of distilled water.
Tryptone soya agar (TSA)	4 g of TSA medium were added to 100 ml of distilled water.

2.1.6 Sterilisation of bacterial culture media

The bacterial culture media were prepared, as described in Table 2.3, and then sterilised by autoclaving at 121°C, 15 psi (pound-force per square inch) for 15 min. After sterilisation, broth (liquid) media were kept at RT until used, whereas agar media were cooled to around 50°C, poured into sterile plastic Petri dishes at ~20 ml per Petri dish, and kept at 4°C until utilised.

2.1.7 Maintenance and preservation of bacterial culture strains

A single colony from each of the original cultures of *S.aureus* or *S.epidermidis* was inoculated in TSB and incubated at 37°C in a shaking incubator for around 18 hours. Next, the broth cultures were incorporated with 50% sterile glycerol (Baker *et al.*, 2011), then aliquoted into sterile Eppendorf tubes and stored at -80°C. The bacterial working cultures were prepared from -80°C stocks every three to four weeks.

2.1.8 Proteins

All of the proteins used in the current study were purchased from Sigma-Aldrich, UK. Three types of proteins were used for transferrin investigations: human holo-transferrin (catalogue number T4132), human partially saturated transferrin (catalogue number T-3309) and human apo-transferrin (catalogue number T1147). An anti-transferrin antibody developed in goat (product number T2027) was used as a primary antibody and an anti-goat IgG peroxidase antibody produced in rabbit (product number A5420) was used as a secondary antibody in Western blot techniques.

2.1.9 Protein molecular weight estimation markers

One type of protein marker was used to monitor protein movement on SDS-PAGE and to assist in estimating the proteins' molecular weights: Precision Plus Protein^{TM}, All Blue Standards, Bio-Rad, UK (catalogue number: 161-0373).

2.1.10 Iron chelating agents

There were two types of iron chelating agents analysed for their abilities to acquire free iron present within PDF: human apo-transferrin and deferoxamine mesylate salt. They were purchased from Sigma-Aldrich, UK (product number T1147 and D9533 for the human apo-transferrin and deferoxamine mesylate salt, respectively).

2.1.11 Polyacrylamide gel electrophoresis

2.1.11.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gel consists of two gels: a stacking gel and a resolving gel. These were prepared following the protocol of Sambrook *et al.* (1989). The stacking gel was used at 5% concentration, whereas three different concentrations of resolving gel were used, at 8%, 12% and 15%, to differentiate between the low and high molecular weight proteins (Table 2.4).

Table 2.4 SDS-polyacrylamide gels recipe.

Constituent	5% Stacking gel	8% Resolving gel	12% Resolving gel	15% Resolving gel
Distilled water	0.68 ml	2.3 ml	1.6 ml	1.1 ml
30% Acrylamide mix	0.17 ml	1.3 ml	2.0 ml	2.5 ml
1 M Tris-HCl pH 6.8	0.13 ml	0.0	0.0	0.0
1.5 M Tris-HCl pH 8.8	0.0	1.3 ml	1.3 ml	1.3 ml
10% SDS	0.01 ml	0.05 ml	0.05 ml	0.05 ml
10% APS	0.01 ml	0.05 ml	0.05 ml	0.05 ml
TEMED	0.001 ml	0.003 ml	0.002 ml	0.002 ml
Total volume	**1 ml**	**5 ml**	**5 ml**	**5 ml**

Key: HCl: hydrochloric acid; SDS: sodium dodecyl sulphate; APS: ammonium persulfate; TEMED: tetramethylethylenediamine.

Unless otherwise mentioned, all chemicals were purchased from Sigma, UK; Fisher Scientific, UK; Bio-Rad, UK; or GE Healthcare, UK. Preparation of SDS-PAGE buffers and solutions was as follows:

- **1 M Tris-HCl pH 6.8:** 121.14 g of Tris base were dissolved in 1 L of distilled water to make the buffer, and adjusted the pH to 6.8 by using concentrated HCl.
- **1.5 M Tris-HCl pH 8.8:** 181.71 g of Tris base were liquefied in 1 L of distilled water to prepare the buffer, and adapted the pH to 8.8 by using concentrated HCl.
- **1 M Tris-HCl pH 7.5:** 121.14 g of Tris base were dissolved in 1 L of distilled water to make the buffer, and modified the pH to 7.5 by using concentrated HCl.
- **10% (w/v) SDS:** 100 g of SDS were liquefied in 1 L of distilled water.
- **2 Times concentrated (2x) SDS-gel protein sample loading buffer:** 4.5 ml distilled water, 0.5 ml 1 M Tris-HCl pH 6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 0.05% dithiothreitol (DTT) and 0.2 ml 0.05% bromophenol blue were mixed together.
- **5 Times concentrated (5x) SDS-PAGE running buffer:** 15 g Tris base, 72 g glycine and 5 g SDS were dissolved in 1 L of distilled water.
- **Coomassie Brilliant Blue SDS-PAGE staining solution:** 0.25 g Coomassie brilliant blue R250 stain, 250 ml methanol, 100 ml glacial acetic acid and 650 ml distilled water were incorporated together.

- **SDS-gel de-staining solution:** 250 ml methanol, 100 ml glacial acetic acid and 650 ml distilled water were mixed with each other.

2.1.11.2 Urea polyacrylamide gel electrophoresis (Urea-PAGE)

Urea polyacrylamide gel contains resolving gel only; no stacker was included. It was formulated following the methodology of Evans & Williams (1980), Table 2.5.

Table 2.5 Urea polyacrylamide gel recipe.

Component	6% Urea resolving gel
Urea	7.3 g
Distilled water	9.6 ml
30% Acrylamide mix	2.7 ml
20x Tris-borate EDTA buffer	1.0 ml
10% Ammonium persulfate	0.01 ml
TEMED	0.03 ml
Total volume	20 ml

Preparation of urea-PAGE buffers and solutions was as follows:

- **Twenty times concentrated (20x) Tris-borate EDTA buffer (TBE buffer):** 100 mM Tris base with a pH of 8.4, 20 mM boric acid and 1.6 mM ethylenediamine tetra acetic acid (EDTA) were mixed together.
- **Two Times concentrated (2x) urea gel protein sample loading buffer:** 0.01% bromophenol blue, 10% glycerol and 100 mM Tris-HCl with a pH of 7.5 were incorporated with each other.
- **Coomassie Brilliant Blue urea gel staining solution:** 40% methanol, 10% glacial acetic acid and 0.05% Coomassie Brilliant Blue were added together.
- **Urea gel de-staining solution:** 7.5% methanol, 5% glacial acetic acid and 87.5% distilled water were mixed together.

2.1.11.3 Polyacrylamide gels staining

SDS-PAGE gels and urea polyacrylamide gels were washed with nanopure water and incubated overnight with the corresponding staining solution. Next, the gels were washed to remove excess stain and re-incubated with the appropriate de-staining solution for one to two hours. After that, the gels were photographed and kept in nanopure water at 4°C until further analysis was performed.

2.1.12 Western blot buffers and solutions

Western blotting solutions and buffers were prepared according to Sambrook *et al.* (1989).

- **Western blotting (running/transfer) buffer:** 5.9 g Tris base, 2.9 g glycine, 100 ml methanol and 3.4 ml of 10% SDS with ice cold distilled water up to 1 L.
- **Western blot washing buffer:** 150 mM sodium chloride (NaCl), 10 mM Tris-HCl with a pH of 7.5 and 0.05% tween-20 (detergent solution) with distilled water up to 1 L.
- **5 M NaCl:** 292.2 g of NaCl dissolved in 1 L of distilled water.
- **Western blot blocking buffer:** 5% of bovine serum albumin in blot washing buffer. The blocking buffer was freshly prepared and cooled on ice prior to use.

2.2 Methods

2.2.1 PDF characterisation methods

2.2.1.1 Growth kinetics of staphylococci in PDS or PDF

The growth assays were performed following a method described by Sandrini *et al.* (2014). The growth of staphylococci over time was measured using the Multiskan Go Spectrophotometer (Thermo Fisher Scientific). The spectrophotometer measures the optical density (OD) of a bacterial culture at regular intervals. The time course of growth assay was initiated by inoculating 1:100 of an overnight culture of staphylococci into TSB medium at 37°C in a shaking incubator for ~18 hours. An ~10^7 CFU/ml inoculum of the culture was inoculated into the PDS or PDF. Then, 150 µl from each inoculated PDS or PDF was transferred into a 96-well plate. The plate was inserted in the spectrophotometer, which was adjusted to the programme illustrated in Table 2.6. After 96 readings (24 hours total incubation), the OD values were exported into a Prism file and the data were used to create staphylococcal growth curves.

Table 2.6 Protocol used in the Multiskan Go Spectrophotometer.

Temperature	37°C
Kinetic interval	00:15:00
Shaking on	00:14:55
Shaking off	00:00:05
Wavelength	600 nm
Readings	96

2.2.1.2 pH level evaluation of PDF

The pH values of the PDFs were evaluated using a pH meter (3510 pH Meter, Jenway). According to the manufacturer's instruction manual, the pH meter was initially calibrated using three different standard pH solutions (pH of 4.0, 7.0 and 10.0). Afterwards, the pH probe was immersed into a sufficient quantity of a PDF. The pH reading was measured in triplicate, and the mean of the three readings determined the final pH value.

2.2.1.3 Determination of glucose concentration in PDF

The glucose concentration in the PDFs was determined using a glucose (hexokinase) assay kit, from Sigma, UK (product code GAHK-20). The kit is based on the enzymatic determination of glucose through a series of biochemical reactions. The glucose in the PDF is phosphorylated, and the yield product is oxidised to NAD^+ (nicotinamide adenine

dinucleotide). The equimolar quantity of NAD^+ is reduced to NADH (NAD + hydrogen). The absorbance of NADH is directly proportional to the glucose concentration. Following the manufacturer's instructions, the PDFs were diluted first with deionised water to be within the concentration limits of the assay (0.05–5 mg of glucose/ml). The solutions in Table 2.7 were prepared in cuvette test tubes, mixed and incubated for 15 min at RT before reading.

Table 2.7 Sample preparation for glucose concentration measurement.

Tube	Glucose assay reagent	Sample volume	Deionised water	Final volume
A) Sample blank	0.0	0.01 ml of glucose standard	1 ml	1.01 ml
B) Reagent blank	1 ml	0.0	0.01 ml	1.01 ml
C) Test	1 ml	0.01 ml of diluted PDF	0.0	1.01 ml

By using the Varian Cary® 50 UV-Visible Scanning Spectrophotometer, the absorbance values were read at 340 nm versus deionised water, and then used in the following formula to determine the glucose concentration:

$$\text{Glucose concentration (mg/ml)} = \frac{(\Delta A)(TV)(F)(180.2)}{(6.22)(1)(1{,}000)(SV)}$$

ΔA (delta absorbance) = $A_{Test} - A_{Total\ Blank}$

$A_{Total\ Blank} = A_{Sample\ Blank} + A_{Reagent\ Blank}$

TV = Total assay volume = 1.01 ml

F = Dilution factor from sample preparation

180.2 = Molecular weight of glucose

6.22 = Millimolar extinction coefficient for NADH at 340 nm (ml/µmoles)

1 = Light path = 1 cm

1,000 = Conversion factor for µg to mg

SV = Sample volume = 0.01 ml

2.2.1.4 Quantification of total, ferric and ferrous iron levels in PDS and PDF

The level of total, ferrous and ferric iron was determined utilising an iron assay kit from Sigma-Aldrich (catalogue number: MAK025). The concept of this assay is based on the release of iron by the addition of an acidic solution. The PDS and PDF can be tested directly to measure ferrous iron, or reduced to assess the total iron level (ferrous iron +

ferric iron). Following the manufacturer's instructions, the PDS and PDF samples were diluted in the kit's iron assay buffer to be within the detection limits of the assay. One hundred µl of the provided iron standards, PDS or PDF dilutions were pipetted into a 96-well microtiter plate. Next, 5 µl of the kit's iron reducer were added to each standard well. For the PDS and PDF samples, two sets of wells were prepared:

- Five µl of the provided iron assay buffer were added to the first set of wells to measure ferrous iron magnitude.
- Five µl of the kit's iron reducer were pipetted into the second set of wells to measure the total iron level (the level of ferric iron was later obtained by subtracting the ferrous iron value from the total iron value).

Afterward, the microtiter plate was gently mixed using a horizontal shaker, and then incubated for 30 min at RT. Next, 100 µl of the kit's iron probe were added into each well (standards and unknowns), mixed, and the plate was re-incubated for 60 min at RT, protected from light while incubating. The absorbance was read at 595 nm using the ELISA reader. The absorbance values were exported into a Prism file, and the data was used to generate a standard curve (Appendix IV). The amount of iron (nmole) of the PDS and PDF samples was determined by comparing it with the standard curve, and then used in the following equation to determine the final iron concentration in the PDS and PDF samples:

$C = S_a/S_v$

C = The concentration of iron in unknown PDS and PDF samples (nmole/µl).

S_a = The amount of iron in unknown PDS and PDF samples from the standard curve (nmole).

S_v = The sample volume added into the wells (µl).

The concentration of iron in nmole/µl was multiplied by iron's atomic mass (55.85 ng/nmole) to yield the concentration in ng/µl.

2.2.1.5 Measurement of the catecholamine contents of PDF

The level of catecholamine stress hormones (noradrenaline [NE], adrenaline [Adr] and dopamine [Dop]) in PDF samples were determined using a tri cat ELISA kit, purchased from DRG Diagnostic, Germany (reference number: EIA-4080). Apart from the standards, controls and buffers provided, the kit comes with extraction plates coated with boronate affinity gel, and pre-coated plates with corresponding catecholamine hormones (NE, Adr and Dop). The detection limits for the catecholamines were as follows: NE, 50

pg/ml, Adr, 10 pg/ml and Dop, 25 pg/ml. The assay is based on the competitive ELISA concept, and all of the incubation steps, were performed at RT on a horizontal shaker (Digital Microplate Shaker Incubator Thermostat) at 600 rounds per min. The PDF samples were centrifuged at 800×g for 10 min, and the supernatants were acidified to a pH of 2 using concentrated HCl. Three hundred µl of the acidic supernatants and 10 µl of the provided standards and controls were pipetted into the wells of the extraction plates. Then, 250 µl of nanopure water were added only to the wells containing the standards and controls. Next, 50 µl of the assay buffer and 50 µl of the extraction buffer were added into all of the wells. The extraction plates were incubated for 30 min at RT. Next, the wells were washed in duplicate using the provided washing buffer. The extracted catecholamine stress hormones were then acylated by adding 150 µl of the acylation buffer and 25 µl of the acylation reagent. The plates were re-incubated for 15 min. A further washing step was performed, followed by adding 150 µl of HCl (0.025 M) and an extra incubation for 10 min. Next, 25 µl of the kit's enzyme solution were pipetted into all wells of the NE, Adr and Dop microtiter strips. Afterward, specific volumes of the extracted standards, the extracted controls and the extracted PDFs were pipetted into the NE, Adr and Dop microtiter strips as follows:

- Twenty-five µl of the extracted standards, controls and PDFs were added into the wells of the NE plate.
- One hundred µl of the extracted standards, controls and PDFs were added into the Adr plate wells.
- Twenty-five µl of the extracted standards and controls and 50 µl of the extracted PDFs were added into the wells of the Dop plate.

The plates were incubated for 30 min; then, 50 µl of NE antiserum, Adr antiserum, or Dop antiserum were pipetted into the wells of the corresponding plate. The plates were re-incubated for two hours to allow competition between stress hormones in the samples and pre-coated antigens to the stress hormone antiserum. After incubation, the free antigens and free antigen-antiserum complexes were removed by washing. The antiserums bound to the solid phase antigen were detected with an anti-rabbit IgG-peroxidase conjugate using tetramethylbenzidine as a substrate. The reactions were monitored at 450 nm, with a reference wavelength at 620 nm. The absorbance values were exported into a Prism file, and the data was used to generate a standard NE, Adr and Dop curve (Appendix V). The measurement of stress hormones in unknown PDFs was performed by comparing their absorbance readings with the standard curves prepared

from the known concentrations. The NE and Adr concentration readings were divided by 30, whereas the Dop concentration values were divided by 60.

2.2.1.6 Assessment of the protein quantity of PDF

The protein concentration of the PDFs was determined using a Bio-Rad protein assay kit (catalogue number 500-0006). The kit involves an acidic solution known as Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) at a known concentration as a standard protein. The assay applies the principle of the Bradford method (Bradford, 1976), in which the acidic dye is added to the protein solution, and the subsequent absorbance is measured at 595 nm via the Varian Cary® 50 UV-Visible Scanning Spectrophotometer. To generate a standard curve, range concentrations of BSA were made up in 800 µl of distilled water and mixed thoroughly with 200 µl of the acidic dye for a final volume of 1,000 µl. Next, the mixtures were incubated at RT for 15 min before their absorbance was measured. Finally, the absorbance values were exported into a Prism file, and the data was used to produce a standard protein curve (Appendix VI). The protein concentrations of the unknown samples were determined by comparing them with the standard curve and then multiplying them by the dilution factor.

2.2.1.7 Characterisation of proteins lost in PDF

After the protein concentration of the PDFs was measured, the PDFs were concentrated via lyophilisation using a freeze dryer (Thermo Fisher Scientific), then dissolved in 2x SDS-PAGE gel protein sample loading buffer to the concentration required. Afterward, the diluted protein samples were denatured by heating them at 95°C for five min. The denatured proteins were loaded directly into different percentage SDS-PAGE gels. The molecular weights of PDF protein samples were estimated with the co-addition of 5 µl of protein molecular weight marker. Protein separation was performed by using a mini (8 cm x 10 cm) protein separation system from Bio-Rad, UK at a constant 25 milliamperes per gel (25 mA/gel) for around 45 min. The electrophoresis of SDS-PAGE gels was accomplished by using a 1x SDS-PAGE running buffer. When the run was over, the SDS-PAGE gels were either stained with Coomassie Brilliant Blue stain, or Western blotted and antibody probed, as required.

2.2.1.8 Protein sequencing

SDS-PAGE gels containing proteins to be sequenced were sent to the Protein Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester (link is shown below). The PDF proteins were digested with trypsin and the peptides produced sequenced using peptide fingerprinting (matrix assisted laser desorption/ionization-time of flight mass spectrometry; MALDI-TOF MS). The peptide sequences were compared with the Mascot search against the UniProtKB-SwissProt database. Liquid chromatography-mass spectrometry (LC-MS) was also a sequencing technique used in this study to identify the most abundant protein present within one protein band. The LC-MS results were searched against the UniProt database using Scaffold software (version 4.3.3). The protein identity was acceptable if it had more than 95% probability and consisted of a minimum of three identified peptides.
http://www2.le.ac.uk/colleges/medbiopsych/facilities-and-services/cbs/protein-and-dna-facility/pnacl/proteomics

2.2.1.9 Transferrin detection in PDF

The Western blotting technique was performed on the SDS-PAGE gels to detect the presence of transferrin in PDF samples. It was also used on urea polyacrylamide gels to investigate the transferrin iron binding status. First, the prepared protein samples were loaded into SDS-PAGE gels or into urea polyacrylamide gels to separate the proteins. Then, the proteins were transferred into a polyvinyliden fluoride membrane (PVDF, Immobilon-P, Millipore, UK, catalogue number IPVH00010), by using Trans-Blot electrophoresis Western transfer cell (Bio-Rad, UK). The protein transfer was performed using a Western transfer buffer, at a constant current of 250 mA for one hour. The blotted membranes were incubated in Western blocking buffer at 4°C overnight. Next, the membranes were washed with Western washing buffer and incubated in a primary antibody (anti-transferrin antibody developed in goat, which was diluted 1:20,000 in Western blocking buffer) for three hours at RT. After that, the membranes were washed and incubated in a secondary antibody (anti-goat IgG peroxidase antibody produced in rabbit, which was diluted as the primary antibody) for one hour at RT. The membranes were finally washed and developed using an enhanced chemiluminescence (ECL) detection system (Novex Chemiluminescent Substrates Reagent Kit, catalogue number WP20005) and visualised using an X-ray film (Amersham Hyperfilm ECL, GE Healthcare, product number 28906837). By utilising an imaging densitometer, which was

linked to the GS-710 Quantity One Software (Bio-Rad, UK), the gels and the Western X-ray films were scanned, and proteins of interest were quantitatively analysed.

2.2.1.10 Assessment of transferrin levels in PDF

The concentration of transferrin in PDFs was determined using an assaymax human transferrin ELISA kit (catalogue number: ET3105-1). The assay is based on the principle of a quantitative sandwich enzyme immunoassay approach, which measures human transferrin in less than four hours at RT. According to the producer's instructions, the PDFs were centrifuged at 800×g for 10 min, and the supernatants were diluted in the kit's mix diluent to be within the detection limits of the assay. Fifty µl of the provided human transferrin standards or PDF dilutions were pipetted into the assay's 96-well polystyrene microplate, which was coated in advance with polyclonal transferrin antibodies. Transferrin in the standards and in the PDF dilutions was sandwiched by adding 50 µl of the biotinylated polyclonal transferrin antibodies. The plate was incubated for one hour to allow specific binding between the transferrin (in the standards and in the PDFs) and the biotinylated antibodies. Afterward, a manual washing of the plate was performed five times by adding 200 µl per well of the kit's washing buffer and inverting the plate each time on an absorbent paper towel to decant the contents and remove the liquid completely. Next, 50 µl of streptavidin-peroxidase conjugate was pipetted into each well, and the plate was re-incubated for 30 min. Further washing steps were performed five times by adding 200 µl per well of the kit's washing buffer and inverting the plate each time on an absorbent paper towel to decant the contents and remove the liquid completely, followed by the addition of chromogen substrate (50 µl per well) for about 12 min until the ideal blue colour developed. In order to halt the colour development reaction, 50 µl of stop solution was added to each well, and the absorbance was read at 450 nm using the ELISA reader. The absorbance values were exported into a Prism file, and the data was used to generate a standard transferrin curve (Appendix VII). The transferrin concentration of the PDFs was determined by comparing it with the standard curve and then multiplying it by the dilution factor.

2.2.2 Investigation of mechanism by how transferrin induced staphylococcal growth in PDF

2.2.2.1 Urea-PAGE

To investigate the transferrin iron binding status of PDF, the PDF samples were mixed with 2x urea gel protein sample loading buffer, then loaded directly onto a urea polyacrylamide gel. Next, electrophoresis was performed at 70 volts for around five hours at 4°C in 1x Tris-borate/EDTA buffer. After electrophoresis, the urea polyacrylamide gels were Western blotted as described in Section 2.2.1.9.

2.2.2.2 Effect of glucose PD solution on iron-binding capacity of apo-transferrin

To investigate the ability of human apo-transferrin to acquire iron from PDS, a method described by Freestone *et al.* (2009) was adapted. A 1-cm diameter dialysis membrane (4-kDa cut-off, Scientific Industries International Inc., Loughborough, UK) was placed vertically in PDS. Next, 100 µl of 5 mg/ml human apo-transferrin was injected inside the dialysis membrane. Following overnight incubation at 4°C, transferrin from within the dialysis membrane was extracted and analysed by urea-PAGE.

2.2.2.3 PDF iron uptake investigations

2.2.2.3.1 ^{55}Fe-labelling of transferrin

^{55}Fe- labelled transferrin was formulated using the method described by Freestone *et al.* (2000). Briefly, the human apo-transferrin was incubated for 5 hours at 37°C in a CO_2 incubator with 25 µCi of $^{55}FeCl_3$ per mg of transferrin in a reaction mixture consisting of 1.5 µg of iron per mg of protein, using 2 mM sodium citrate as the iron donor. Unincorporated [^{55}Fe] to [^{55}Fe]-transferrin was eliminated by performing round of spin column chromatography (Micro Bio-Spin P-6 columns; Bio-Rad, U.K., catalogue number 7326221) (Freestone *et al.*, 2000).

2.2.2.3.2 ^{55}Fe-Transferrin uptake in staphylococci in PDS and PDF

Inoculums of staphylococci were grown at 37°C for 24 hours in a TSB medium (Table 2.3). The next day, the bacterial cultures were normalised (final volume 1 ml) with PDS or PDF to 2 at OD_{600}. The normalised bacterial cultures were washed by centrifugation at 10,000 rpm for 10 min. Next, the normalised washed bacterial cultures were mixed with 4 ml of the PDS or PDF sample. One µg/ml of ^{55}Fe-transferrin (2×10^5 cpm) was pipetted

into the mixture and a subsequent incubation step was performed for 4 hours at 37°C in a 5% CO_2 incubator. After incubation, the bacterial cells were centrifuged at 4,000 rpm for 15 min and washed two times in phosphate-buffered saline (PBS) to eliminate any remaining ^{55}Fe-transferrin. After the final washing step, the bacterial cell pellets were re-suspended in 100 µl of PBS and 2 ml of Optiphase Safe scintillation fluid (Canberra-Packard, UK). Radioactivity was measured utilising the tritium channel of a Minaxi Tris-Carb 400 series scintillation counter as described by Freestone *et al.* (2000, 2003).

2.2.2.4 Analysis of the ability of deferoxamine or human apo-transferrin to inhibit staphylococcal growth in PDF

To examine the capability of the chemical chelator deferoxamine mesylate salt or human apo-transferrin in the binding of free iron in the PDF, inoculums of staphylococci were grown overnight in a TSB medium (Table 2.3) at 37°C in a shaking incubator for ~18 hours. Next, a ~10^7 CFU/ml inoculum of the staphylococcal overnight culture was inoculated into the PDFs (as controls), or with deferoxamine mesylate salt or with human apo-transferrin. Afterwards, 150 µl from each PDF inoculated with staphylococci and with or without deferoxamine/apo-transferrin was transferred into a 96-well plate. The plate was placed in the Multiskan Go Spectrophotometer, which was programmed as stated in Table 2.6.

2.2.3 Staphylococcal virulence assays

2.2.3.1 Biofilm formation

An attachment assay was performed using flat-bottomed 96-well microtiter plates, as described by Tendolkar *et al.* (2004), with slight adaptations. Bacteria were grown aerobically at 37°C for 24 hours in a TSB medium (Table 2.3). The bacterial cells were then pelleted at 3,270×g for 10 min, and the bacterial cell pellets were re-suspended in 5 ml of PDS or PDF sample. The OD of the bacterial suspensions were measured at 600 nm using a spectrophotometer (Varian Cary 50 UV-Vis Spectrophotometer) and normalised with PDS or with PDF to a 1:00 dilution. The bacterial cultures were then diluted at 1:40 in fresh PDS or PDF and from the diluted bacterial cultures, 200 µl was dispensed into three wells of a flat-bottomed polystyrene 96-well microtiter plate (Nunc™ Cell Culture Treated, Thermo Fisher Scientific). The dispensed cultures were incubated statically at 37°C in a 5% CO_2 incubator for 24 hours. Afterwards, the culture

supernatants were removed and the wells washed three times with sterile PBS. The wells were allowed to dry for 30 min in a heat cabinet. Next, 200 µl of an 0.2% aqueous crystal violet solution was pipetted into each well, and the plates were incubated at RT for 15 min. Then, the wells were re-washed three times with sterile PBS and air dried at RT for 24 hours. The aqueous crystal violet bound to the bacterial biofilms was extracted by adding 95% ethanol and the plates rotated for 1 min using a plate rotator. Finally, the absorbance at 595 nm was measured using an ELISA reader for the extracted crystal violet. As a negative control, the crystal violet binding to the wells alone was measured for wells containing only PDS or PDF.

2.2.3.1.1 Visual appearance of bacterial cultures in PDS or PDF

As described in Section 2.2.3.1, 1:40 dilutions of bacterial cultures in PDS or PDF samples were prepared. Next, 1 ml of the diluted bacterial cultures was pipetted into the wells of a 24-well plate (Nunc^{TM} Cell Culture Treated, Thermo Fisher Scientific). The plates were incubated statically at 37°C in a 5% CO_2 incubator for 24 hours. Then, the plates were examined under the microscope using a 40x objective lens for biofilm formation by bacterial cultures in different PDS or PDF. Photographs were taken of the appearance of the bacteria using an inverted microscope, which was connected to DeltaPix InSight software.

2.2.3.2 Haemolytic activity

A haemolysis assay was performed to examine the activity of bacterial haemolysins in PDF samples using the method described by Molnar *et al.* (1994), with slight adjustments. First, bacteria were grown aerobically overnight at 37°C in a TSB medium (Table 2.3). Then, the bacterial pellets at ~10^8 CFU/ml were washed three times with the PDF sample by centrifugation at 1,734×g for 10 min each time. After the last wash, the bacterial pellets were re-suspended in 5 ml of the PDF sample and re-incubated statically at 37°C overnight. Then, a 50 µl serial two-fold dilution of the bacterial suspension in sterile PBS were performed across 12 wells of a round-bottomed 96-well microtiter plate (Nunc^{TM} Cell Culture Treated, Thermo Fisher Scientific). Sterile PBS was used as a negative control and *S.aureus* grown in a TSB medium was used as a positive control. Then, 50 µl of 4% (v/v) sheep red blood cells (RBCs; erythrocytes) suspension was added to each well. To prepare the 4% (v/v) erythrocytes suspension, 10 ml of defibrinated sheep blood (Oxoid, UK) was centrifuged at 3,000 rpm (Refrigerated Centrifuge 3-16KL, Sigma) for

15 min at 4°C. Then, the supernatant was removed and 400 µl of the pelleted erythrocytes was mixed gently with 9.6 ml of sterile PBS to yield the 4% (v/v) erythrocytes suspension. After the addition of the 4% (v/v) RBC suspension, the plates were incubated at 37°C in a 5% CO_2 incubator for 24 hours, with readings taken after 30 min, 90 min, 4 hours and 24 hours. Haemolytic activity was assessed macroscopically by determining the dilution at which 50% haemolysis of the RBCs occurred (Figure 2.1). The number of haemolytic units (HUs) was calculated in all wells up to when 50% haemolysis occurred. Haemolytic activity was expressed in HU/ml.

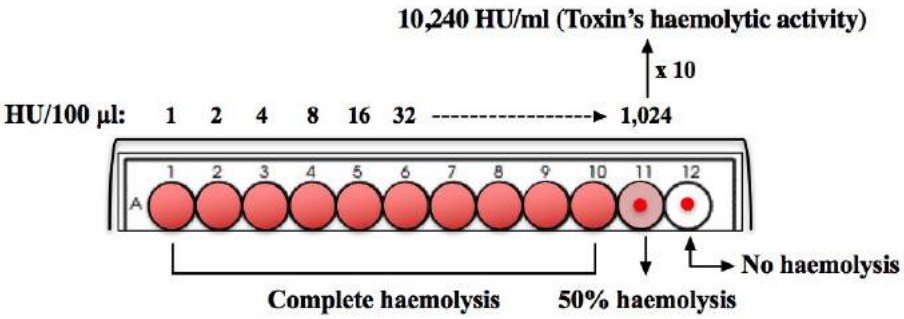


Figure 2.1Determination of haemolytic activity.
The first well contained the PDS or PDF sample, which was previously incubated with the bacterial culture. Then a serial, double-fold dilution was carried out in PBS until the last well, followed by the addition of 50 µl of 4% (v/v) sheep RBCs suspension. The number of haemolytic units (HUs) in 100 µl was calculated according to the toxin dilution up to the haemolytic units required to generate 50% haemolysis. The values were multiplied by 10 to express the haemolytic activity in 1 ml (HU/ml).

2.2.3.3 Proteolytic activity

A proteolysis assay was performed to examine the biological action of bacterial protease enzymes production in PDF samples utilising casein as a substrate present in skimmed milk agar (SMA), as described by Kanekar *et al.* (2002) and Morris *et al.* (2012). The SMA plates were prepared by mixing 200 ml of milk (British 0.1% Fat Skimmed Milk, UK) and 250 ml of 1.5% (w/v) sterile agar (Oxoid, U.K). Bacteria were grown overnight at 37°C in a TSB medium (Table 2.3). Then, the bacterial pellets at ~10^8 CFU/ml were washed three times with the PDF sample by centrifugation at 1,734×g for 10 min each time. After the final wash, bacterial pellets were re-suspended in the PDF sample and incubated at 37°C overnight. The overnight bacterial cultures in the PDF samples were centrifuged at 1,734×g for 10 min to separate the supernatant from the bacterial pellets. The supernatant, which contained exogenous protease, was filter sterilised through a 0.2-µm pore size syringe unit filter, whereas bacterial pellets, which had endogenous proteases, were washed three times with 10 mM Tris-HCl pH 7.5. Finally, 10 µl of the

filter sterilised supernatant, washed pellet and PDF sample alone were inoculated onto the casein milk agar. The plates were incubated at 37°C in a 5% CO_2 incubator for 48 hours. The proteolytic activity of bacteria was evaluated by measuring the diameter (cm) of the clear zones indicating proteolytic activity around the bacterial colonies.

2.2.4 Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 6.02 for Macs (Graph Pad Software, San Diego, California, US). The data were recorded in the text as mean ± standard error of the mean (SEM) of technical replicates. Due to the low numbers of patients in this study, the association between each PDF biochemical factor level and *S.aureus* or *S.epidermidis* growth had to be determined using univariable linear regression analysis with 95% confidence intervals. Statistical significance was represented by a P value of less than 0.05.

CHAPTER 3 Characterisation studies of peritoneal dialysis fluid (PDF)

3.1 Introduction

PD, as compared to haemodialysis and kidney transplantation, is the simplest and most economical therapeutic technique for ESKD patients (Chaudhary *et al.*, 2011; Ellam & Wilkie, 2011; Han *et al.*, 2008; Zorzanello, 2004). The removal of waste products and excess water from the body takes place in the peritoneal cavity via the aseptic infusion of peritoneal dialysis solution (PDS) (Levy *et al.*, 2015). Afterwards, the transport of solutes and fluids occurs across the peritoneal membrane between the PDS and the distributed peritoneal capillaries (Levy *et al.*, 2015). However, because PD involves repeated and daily administration of large volumes of the PDS into the sterile peritoneal cavity, this form of dialysis has two major clinical complications: loss of proteins into the peritoneal cavity and infectious peritonitis. The latter is a life-threatening complication, which also reduces the time PD can be performed (Chaudhary *et al.*, 2011; Ellam & Wilkie, 2011; Zarrinkalam *et al.*, 2001; Blumenkrantz *et al.*, 1981; Levy *et al.*, 2015). As a result of protein loss into the peritoneal cavity during the PD exchange, the PDS (which contains glucose, electrolytes and buffer) becomes a fluid rich in waste products and a mixture of proteins; thus, it is called peritoneal dialysis fluid (PDF). Normal kidney functions serve to retain proteins and allow their reabsorption into the circulation (Guyton & Hall, 2006). Consequently, the protein loss experienced as part of routine therapy is a unique category of the PD modality.

Limited information is available about the association between protein loss and risk of developing infectious peritonitis (Dong *et al.*, 2013). Hence, obtaining an understanding of the relationship between the protein loss and the risk of developing infectious peritonitis becomes a key issue in PD patients. In other words, identifying the proteins that might be associated with infection risk could lead to possible improvements in PD patient care. In the case of infectious peritonitis, bacteria gain access to the peritoneal cavity via the PD implemented catheter or via a perforation of the gastrointestinal tract (Williams *et al.*, 1995; Ahmetagic *et al.*, 2013). As bacteria translocate from one place (e.g. the skin in the case of *S.aureus* and *S.epidermidis*) to another (the peritoneal cavity), they have to adapt to the new environment in terms of colonisation and virulence (Williams *et al.*, 1995). Hence, on bacterial entry and through the development of infectious peritonitis, bacteria will come into contact with environmental signals present within the peritoneal cavity that accommodate the PDF. These include pH, body temperature and nutrients (in particular glucose and iron), which collectively are needed

for bacterial survival and growth (Williams *et al.*, 1995). Therefore, the influence of these different PDF biochemical parameters on the growth of the most predominant bacteria that cause infectious peritonitis, *S.aureus* and *S.epidermidis*, was investigated in the present chapter. The first question investigated in this chapter was to what extent the PDF (which contains protein molecules, hormones and other factors) and the PDS (which contains glucose, electrolyte and buffer) were supportive for *S.aureus* and *S.epidermidis* growth. The next question considered was, what were the biochemical changes that occurred in the PDS during PD exchange, which accordingly made it more supportive of bacterial growth?

3.2 Results

3.2.1 PD patients' clinical information

The present study involved PDF sampling from patients with kidney failure who attended the John Walls Renal Unit at Leicester General Hospital and were placed on PD as a first choice RRT modality. Patients were selected randomly in terms of age and other clinical parameters. Nine follow-up PD patients kindly participated in this study by donating their peritoneal dialysis fluids (PDFs). In this group of patients, 77.8% (n=7) were females, the mean age was 55.7 ± 18.3 years and 22.2% (n=2) were diabetic. The reasons behind kidney failure were unknown in 55.5% of the patients, while others (44.5%) had kidney failure due to renal cortical necrosis, lithium toxicity, hypertensive nephrosclerosis and systemic lupus erythematosus. None of them regularly used oral iron. In terms of hypertension management drugs, 44.4% (n=4) of them were hypertensive, taking either alpha- or beta-adrenergic blocker or a combination of both alpha- and beta-adrenergic blockers. The mean time spent on the PD programme was 11.78 ± 7.96 months, with a minimum time of two months and a maximum of 25 months. The main causes of dropout from PD treatment were infectious peritonitis, kidney transplantation, patient's personal preference and death with 33.3%, 33.3%, 22.2% and 11.1%, respectively.

The rate of peritonitis was expressed as risk of peritonitis episode per patient months and calculated in accordance with the International Society for Peritoneal Dialysis recommendations (Li *et al.*, 2010). Thus, the total number of months of PD patients for the entire PD programme was calculated and then divided by the total number of peritonitis episodes. As a result, the rate of peritonitis for all the PD patients in this study was one episode every 9.6 patient months. The clinical data from the participating PD

patients is summarised in Table 3.1; additional medical information for these PD patients, including the drug therapy they received, is located in Table 3.1B.

Table 3.1A Clinical characteristics for the nine PD patients enrolled in the current study.

Patient ID	Gender	Age	PD modality	Had used α/β-adrenergic blockers?	Had experienced peritonitis?	Number of peritonitis episodes	Time spent on PD (months)	Cause of PD termination
1	M	78	CAPD and APD	α-blockers	Yes	5 (3 within first year of PD)	15	Infectious peritonitis
2	F	51	CAPD	No	Yes	2 within first year of PD	25	Kidney transplant
3	F	40	CAPD	No	No	0	17	Patient's preference
4	F	63	CAPD	No	No	0	5	Kidney transplant
5	F	63	CAPD	No	No	0	2	Death
6	F	54	APD	β-blockers	Yes	3 within first year of PD	14	Infectious peritonitis
7	M	82	CAPD	α- and β-blockers	Yes	1 within first two months of PD	2	Infectious peritonitis
8	F	46	CAPD	α- and β-blockers	No	0	18	Kidney transplant
9	F	24	CAPD	No	No	0	8	Patient's preference

Key: M: Male; F: Female. α- and β- refer to alpha- and beta-adrenergic blockers, respectively. CAPD and APD refer to continuous ambulatory peritoneal dialysis and automated peritoneal dialysis, respectively.

Table 3.1B The types of drug therapy used by the PD patients involved in this study.

Patient ID	Medications
1	Alfacalcidol, Aspirin, Atorvastatin, Calcium, Acetate (Phosex), Doxazosin, Tamsulosin, Epoetin alpha – Eprex, Lactulose, Lanthanum carbonate, Docusate sodium and Senna.
2	Alfacalcidol, Amlodipine, Lactulose, Senna, Simvastatin, Darbepoetin – Aranesp, Furosemide, Lisinopril and Sevelamer.
3	Alfacalcidol, Cetirizine, Esomeprazole, Folic acid, Sevelamer, Warfarin, Erythrocyte stimulating agent, Thyroxine, Phosex, Quinine sulphate, Zopiclone and Darbepoetin – Aranesp.
4	Alfacalcidol, Calcium, Acetate (Phosex), Furosemide, Lithium carbonate, Sodium bicarbonate and Epoetin alpha – Eprex.
5	Alfacalcidol, Lithium carbonate, Epoetin alpha – Eprex, Clonazepam, Lactulose, Procyclidine, Quinine, Senna and Tetrabenazine.
6	Alfacalcidol, Atorvastatin, Bisoprolol, Digoxin, Furosemide, Losartan, Paracetamol, Phosex, Spironolactone, Darbepoetin – Aranesp, Erythromycin, Gliclazide, Lansoprazole, Sitagliptin and Tramadol.
7	Alfacalcidol, Allopurinol, Bisoprolol, Doxazosin, Furosemide, Lactulose, Quinine, Ranitidine, Senna, Simvastatin, Sodium bicarbonate, Warfarin and Darbepoetin – Aranesp.
8	Alendronic acid, Alfacalcidol, Amlodipine, Beclomethasone, Bisoprolol, Calcichew, Doxazosin, Gliclazide, Lactulose, Mycophenolate, Paracetamol, Prednisolone, Salbutamol, Simvastatin, Sodium bicarbonate, Darbepoetin – Aranesp, Epaderm, Furosemide and Sevelamer.
9	Alfacalcidol, Amlodipine, Chlorpheniramine, Docusate sodium, Furosemide, Lanthanum carbonate, Oramorph, Quinine sulphate, Tramadol and Epoetin Alfa – Eprex.

3.2.2 Follow-up PDF

Approximately 2 L of the PDF were collected from each patient at each visit, which were then used for *in vitro* studies. In total, there were 20 PDFs obtained from a range of nine PD patients at the beginning of their dialysis and at different points in time, as described in Table 3.2. After the PDFs were collected, they were transported to the laboratory within three hours, and then under aseptic conditions were aliquoted into sterile tubes and kept at -20°C to avoid protein deterioration and microbial contamination. The PD solutions utilised by PD patients enrolled in this study were composed of glucose as an osmotic agent (Table 2.2). The PDS used contained 13.6 g/L or 22.7 g/L or 15 g/L of glucose. The PD solutions were sterile and did not contain any specific bacteriostatic agent as they were produced for intraperitoneal administration only. The PD solutions were stored at RT until used.

Table 3.2 Follow-up PDF collection from the nine PD patients at different points in time and the percentage of glucose used in PDS.

Patient ID	Follow-up PDF collection time and the percentage of glucose used in PDS					
1	3 days; 2.27% glucose	7 months; 2.27% glucose	11.5 months; 2.27% glucose			
2	1 day; 2.27% glucose	6 months; 1.36% glucose	11 months; 2.27% glucose	17 months; 2.27% glucose	24 months; 1.36% glucose	
3	1 day; 1.36% glucose	6 months; 2.27% glucose	11 months; 2.27% glucose			
4	3 days; 1.36% glucose					
5	7 days; 1.5% glucose					
6	22 days; 2.27% glucose	7 months; 2.27% glucose				
7	7 days; 1.5% glucose					
8	7 days; 1.36% glucose	6 months; 2.27% glucose	15 months; 2.27% glucose			
9	7 days; 1.36% glucose					

Key: Red: PD terminated due to infectious peritonitis; Green: Patient had kidney transplant; Orange: PD terminated because of patient's preference; Grey: PD terminated because patient died.

3.2.3 Investigation of the growth profiles of staphylococci in PDS and PDF

Before beginning the characterisation of the biochemical features of the PDS and PDF, it was worthwhile to first study the growth behaviour of *S.aureus* and *S.epidermidis* in PDS (which is the solution that is infused into the peritoneal cavity and contains glucose, electrolytes and buffer) and in PDF (which is the fluid collected after the PD exchange and has been modified with host-protein molecules, hormones and other factors). This aim was addressed following the method described in Section 2.2.1.1. Briefly, *S.aureus* or *S.epidermidis* was inoculated at ~10^7 CFU/ml into PDS or PDF; then, their growth was monitored by measuring OD at 600 nm over 24 hours.

3.2.3.1 Did variations in the glucose concentration of PDS have a marked influence on the growth pattern of staphylococci?

Because PD patients used variable glucose percentages in their PD solutions, it was examined whether different glucose formulations of the PDS had an impact on staphylococcal growth. Four different glucose concentrations in the PDS were analysed: 1.36%, 2.27%, 3.86% (Baxter Healthcare Ltd.) and 1.5% (Fresenius Medical Care Ltd.). Figure 3.1 demonstrated that 1.5% glucose PDS (the red line on the graph) was the most supportive medium for *S.aureus* and *S.epidermidis* growth. Notably, the 1.5% glucose PDS was produced by a different company than that which made the 1.36%, 2.27% and 3.86% glucose PDS; thus, different PDS formulations had a dissimilar effect on *S.aureus* and *S.epidermidis* growth. This could be due to differences in the composition of the buffers between different PD solutions (Table 2.2). The growth profile of *S.aureus* in 1.36% glucose PDS was similar to that in 2.27% or 3.86% glucose PDS. On the other hand, 3.86% glucose PDS (the purple line on the graph) was the most bacteriostatic habitat for *S.epidermidis* multiplication, as compared to a 1.36% and 2.27% glucose PDS. For *S.epidermidis*, it seemed that the higher the glucose concentration the more inhibitory to growth it was.

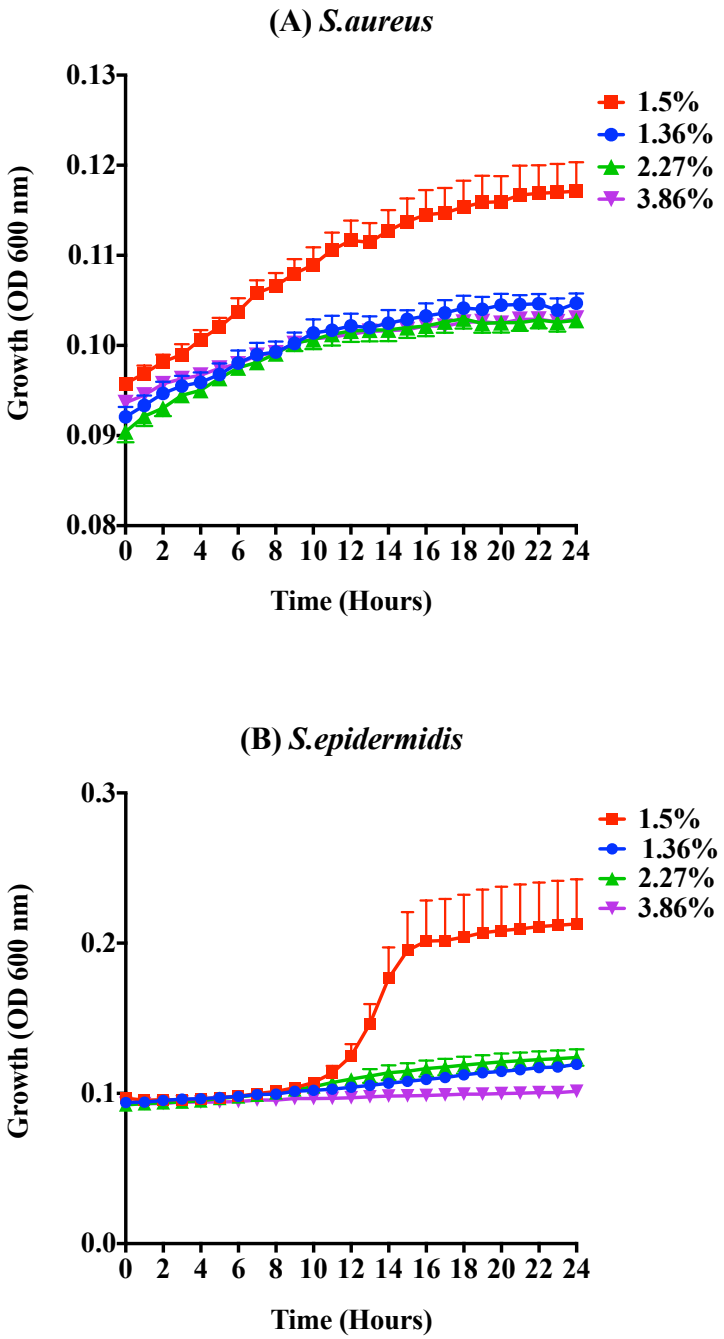


Figure 3.1 Time course of *S.aureus* and *S.epidermidis* growth in different concentrations of glucose PDS over 24 hours.
The 1.36%, 2.27% and 3.86% glucose PD solutions were manufactured by Baxter Healthcare Ltd., whereas the 1.5% glucose PDS was produced by Fresenius Medical Care Ltd. Approximately 10^7 CFU/ml inoculum of *S.aureus* or *S.epidermidis* culture was inoculated into different PD solutions and incubated at 37°C for 24 hours. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.1.1); n=3.

3.2.3.2 Did PDF differ from PDS in stimulating staphylococcal growth?

In contrast to the PDS, the PDF, as expected, was more supportive for *S.aureus* and *S.epidermidis* growth (Figure 3.2). Furthermore, the growth assessment experiments were performed on the PDF obtained from nine different patients at the start of PD therapy to determine if variations between patients did exist. Interestingly, there were clear differences in the growth profiles of *S.aureus* and *S.epidermidis* in the various PDFs tested. The PDF of Patient 4 (the purple line on the graph) was by far the most favorable fluid for *S.aureus* and *S.epidermidis* growth. The OD readings of *S.aureus* growth in the PDF of Patient 4 rose sharply during the logarithmic phase from 0.1 (± 0.009) at 2 hours, to a high of 0.4 (± 0.008) at 10 hours, while *S.epidermidis* growth increased slowly but to a lower level over the same period. This finding suggests that this particular PDF contained a considerable quantity of growth stimulating factors, whose identity will be investigated later in this chapter. To conclude, there were marked differences in the growth response of *S.aureus* and *S.epidermidis* between PD patients at the start of PD therapy, which could clarify why certain patients were more susceptible to infection than others.

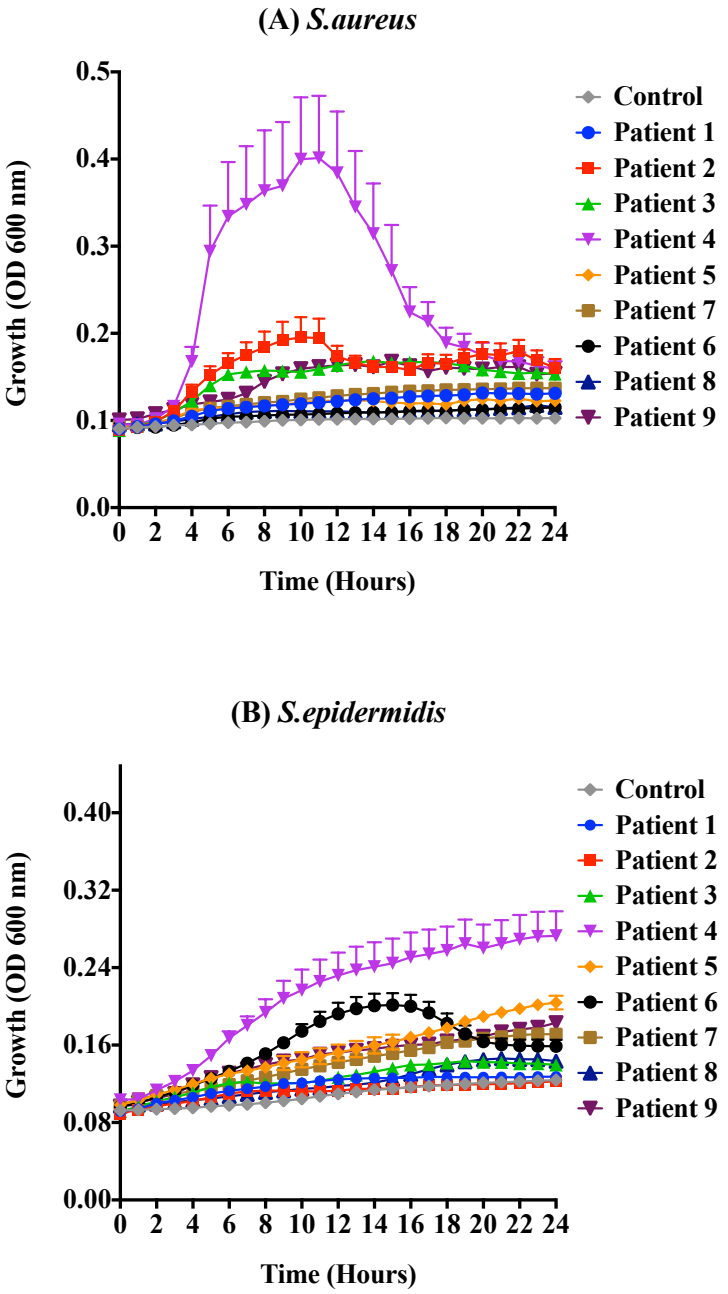


Figure 3.2 Time course of *S.aureus* and *S.epidermidis* growth in PDF derived from nine PD patients. PDFs were collected at the beginning of PD therapy. Approximately 10^7 CFU/ml inoculum of *S.aureus* or *S.epidermidis* culture was inoculated into different PDFs and incubated at 37°C for 24 hours. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.1.1); n=3. The 'control' refers to the PD solution.

3.2.3.3 Did the time spent on peritoneal dialysis influence the growth of staphylococci in PDF?

Comparison studies of the growth responsiveness of *S.aureus* and *S.epidermidis* to the PDF of follow-up PD patients (Patients 1, 2, 3, 6 and 8) were performed. In general, the growth trends of *S.aureus* and *S.epidermidis* in the PDF within each patient varied over the period from start to follow-up. For Patient 1, the first PDF (collected after three days on PD) was by far the most optimal medium for *S.aureus* growth, as compared to the second and the third PDFs (collected after seven months and 11.5 months on PD, respectively), Figure 3.3 A. There was also a clear increase in the growth of *S.epidermidis* when the PDF (collected after seven months on PD was used as a growth medium), as opposed to the first and last PDFs (obtained after three days and after 11.5 months on PD, respectively), Figure 3.3 B. The staphylococcal growth response to the PDFs from Patient 2 is shown in Figure 3.4. The PDF (collected after the patient had spent six months on PD) was by far the most stimulatory fluid for both *S.aureus* and *S.epidermidis* growth. The growth results of the PDFs of Patient 3 were quite similar to that of Patient 2, as the second PDF (collected after six months on PD) was the maximum supportive fluid for *S.aureus* and *S.epidermidis* growth, as compared to the PDFs that were obtained after one day and after 11 months on PD (Figure 3.5). According to the time courses graphs shown in Figure 3.6, the first PDF collected from Patient 6 (after 22 days on PD) was more supportive of *S.epidermidis* than *S.aureus* growth, when compared to the PDF collected seven months later. There was a clear variation in *S.aureus* and *S.epidermidis* growth in the follow-up PDFs of Patient 8 as shown in Figure 3.7.

In conclusion, the pattern of staphylococcal growth in the follow-up PDFs of each patient fluctuated over the course of PD therapy. Such differences in the growth profile of *S.aureus* and *S.epidermidis* may result from changes in the biochemical characteristics of the patient PDF over time. An investigation of what changes might cause the PDF to become more growth permissive were then investigated.

Patient 1

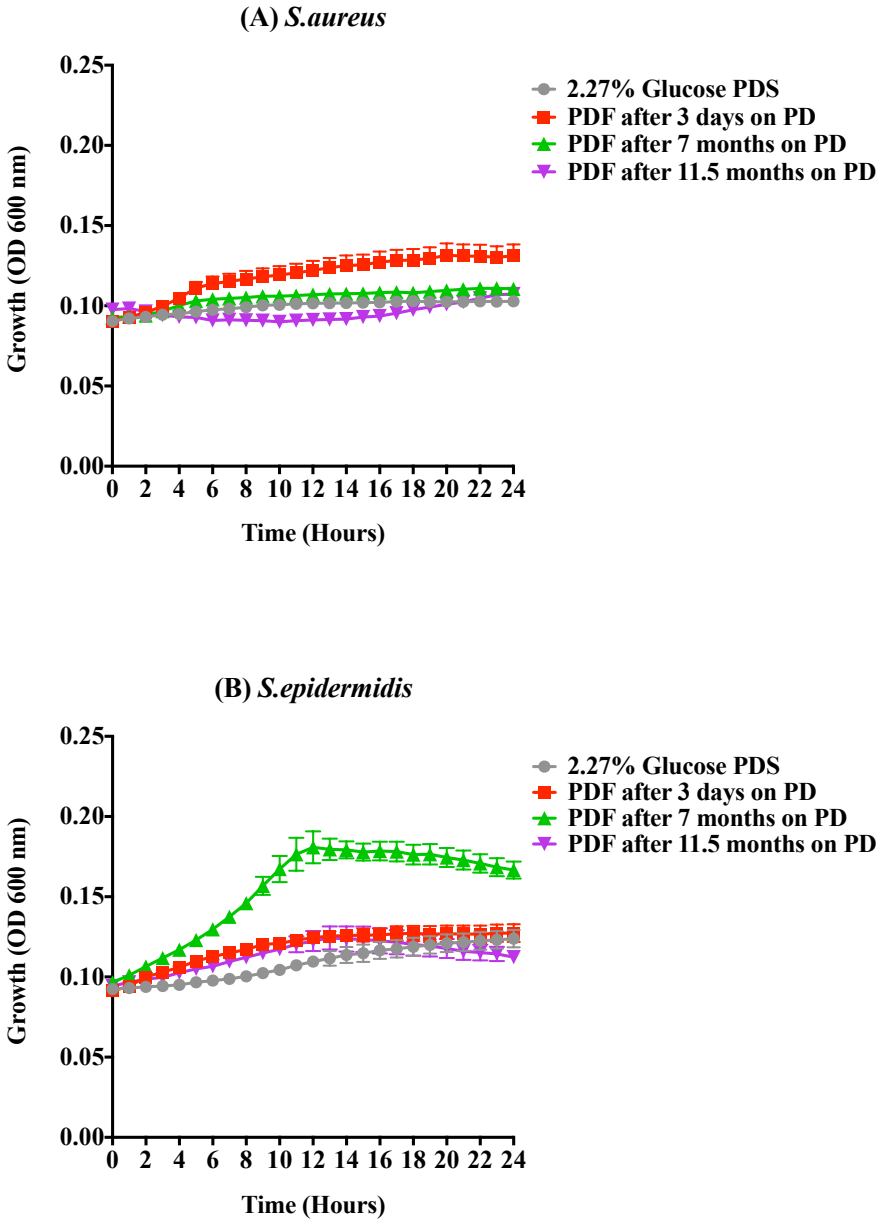


Figure 3.3 Time course of *S.aureus* and *S.epidermidis* growth in the follow-up PDFs collected from Patient 1.

Approximately 10^7 CFU/ml inoculum of *S.aureus* or *S.epidermidis* culture was inoculated into the PDF and incubated at 37°C for 24 hours. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.1.1); n=3.

Patient 2

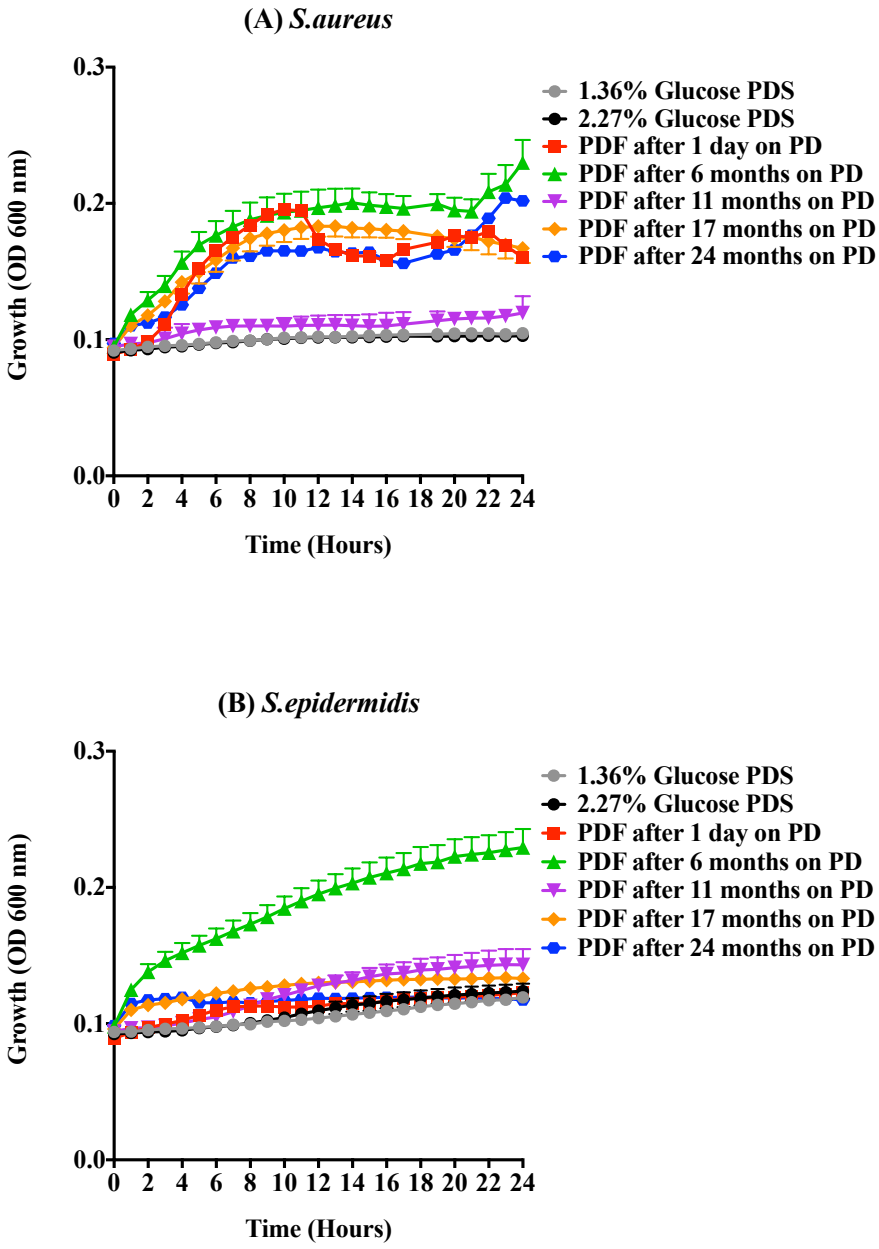


Figure 3.4 Time course of *S.aureus* and *S.epidermidis* growth in the follow-up PDFs collected from Patient 2.

Approximately 10^7 CFU/ml inoculum of *S.aureus* or *S.epidermidis* culture was inoculated into the PDF and incubated at 37°C for 24 hours. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.1.1); n=3.

Patient 3

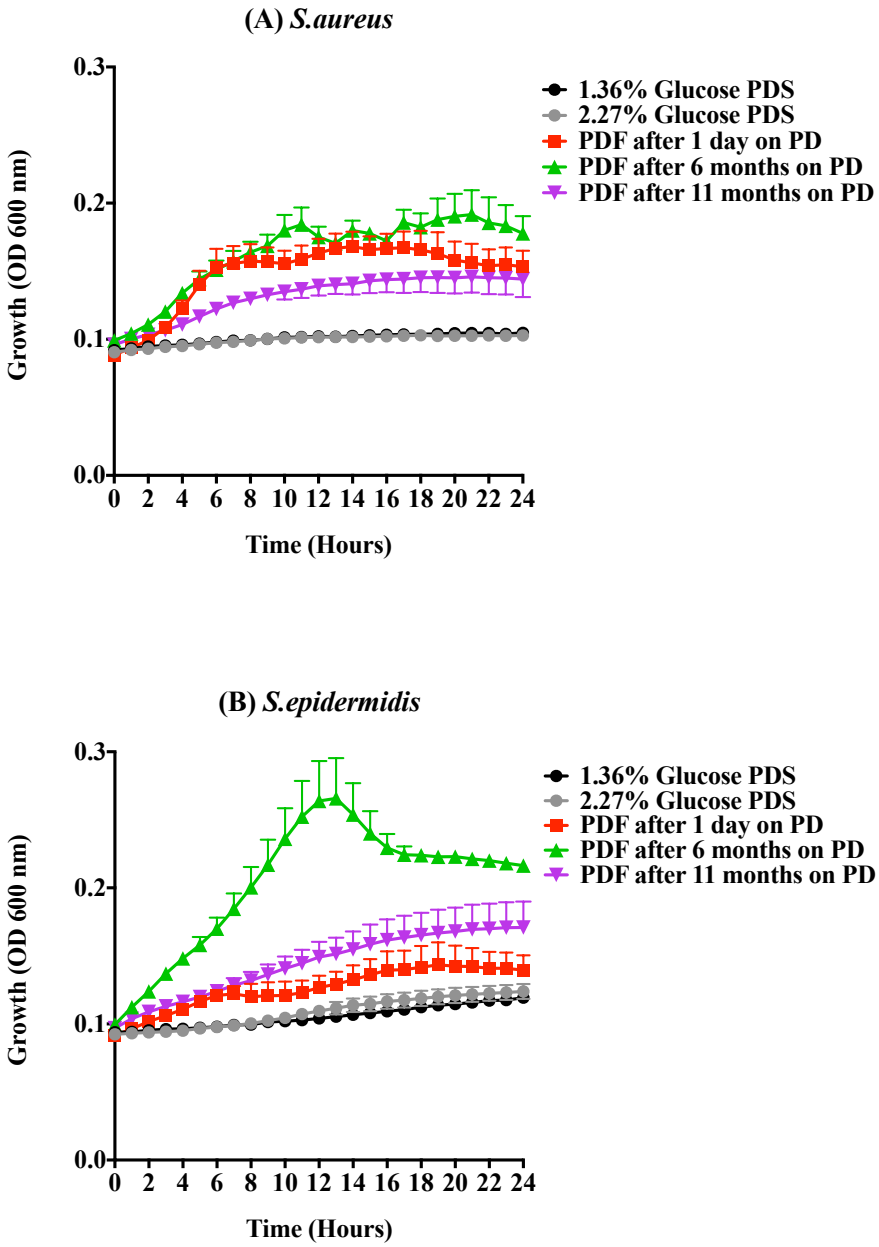


Figure 3.5 Time course of *S.aureus* and *S.epidermidis* growth in the follow-up PDFs collected from Patient 3.

Approximately 10^7 CFU/ml inoculum of *S.aureus* or *S.epidermidis* culture was inoculated into the PDF and incubated at 37°C for 24 hours. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.1.1); n=3.

Patient 6

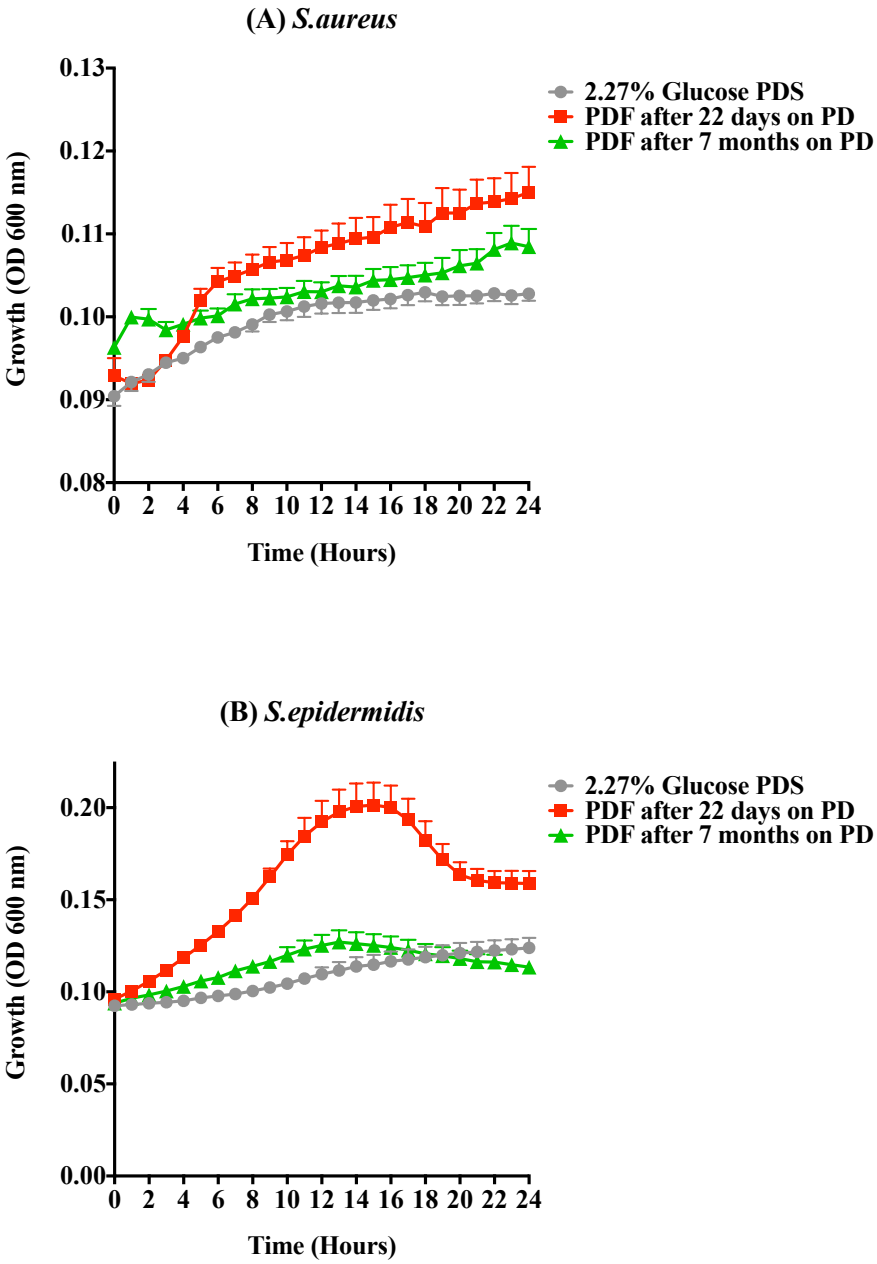


Figure 3.6 Time course of *S.aureus* and *S.epidermidis* growth in the follow-up PDFs collected from Patient 6.

Approximately 10^7 CFU/ml inoculum of *S.aureus* or *S.epidermidis* culture was inoculated into the PDF and incubated at 37°C for 24 hours. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.1.1); n=3.

Patient 8

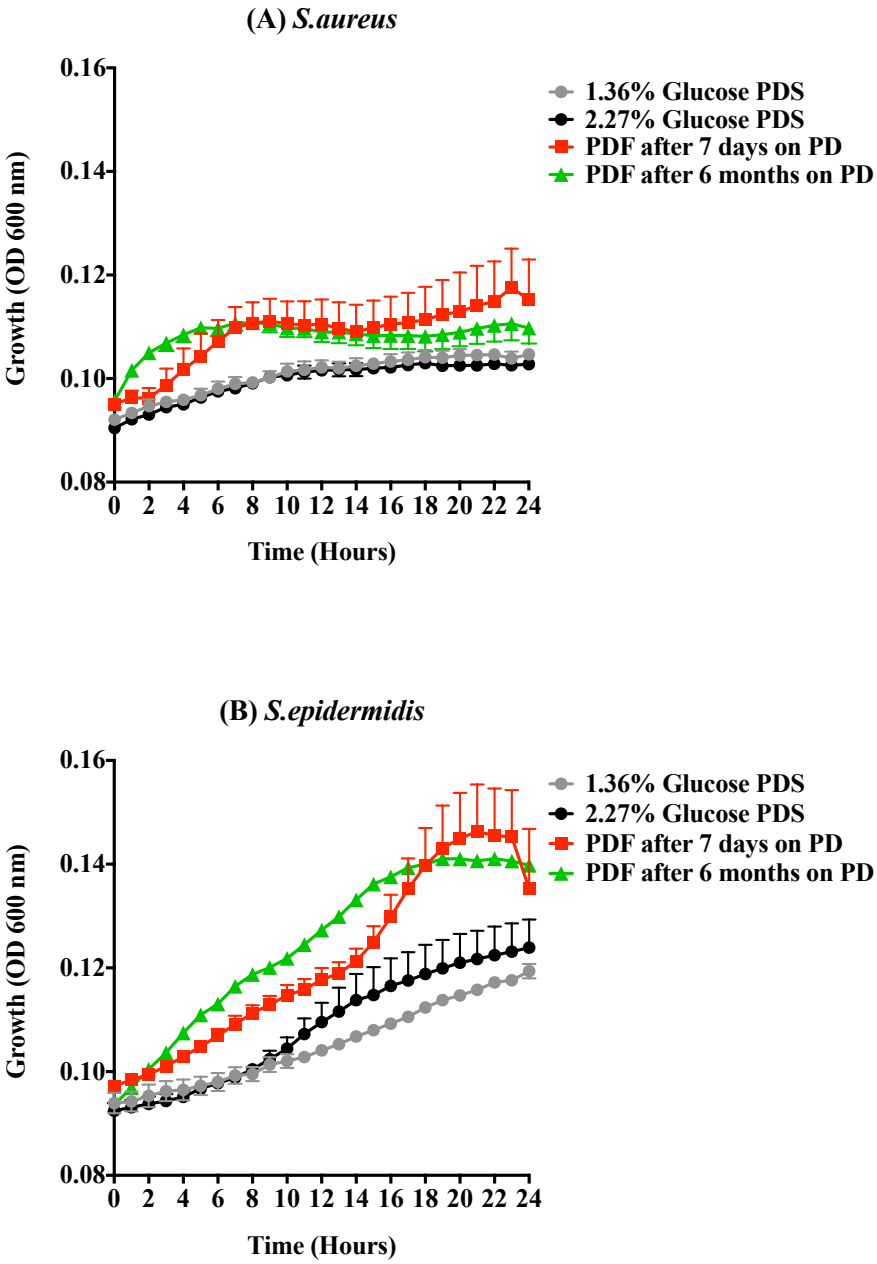


Figure 3.7 Time course of *S.aureus* and *S.epidermidis* growth in the follow-up PDFs collected from Patient 8.

Approximately 10^7 CFU/ml inoculum of *S.aureus* or *S.epidermidis* culture was inoculated into the PDF and incubated at 37°C for 24 hours. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.1.1); n=3.

3.2.4 Investigation of pH changes in PDS during peritoneal dialysis

pH measurements of PDFs were made to determine if there were changes in the acidity of the PDS after the dialysis process. An additional objective of this experiment was to assess the change in pH when comparing the initial PDFs with the follow-up ones. The pH level was determined using a pH meter (3510 pH Meter Jenway) (Section 2.2.1.2). Both the initial PDFs, which were obtained from nine PD patients, and follow-up PDFs, which were collected from five PD patients (Patients 1, 2, 3, 6 and 8) (Table 3.2), were analysed. As shown in Figure 3.8, the pH values of the PDS changed markedly after being in the peritoneal cavity. The pH value of the original PDS was acidic at 5.5; however, the pH readings of the PDFs increased to 8.1 ± 0.27. No major pH differences were found between the initial PDFs and the follow-up PDFs. The acidic environment of the PDS is not optimal for staphylococcal growth; however, after a PD exchange, the pH shifted from acidic to basic, which is more preferable for staphylococcal growth (Todar, 2006).

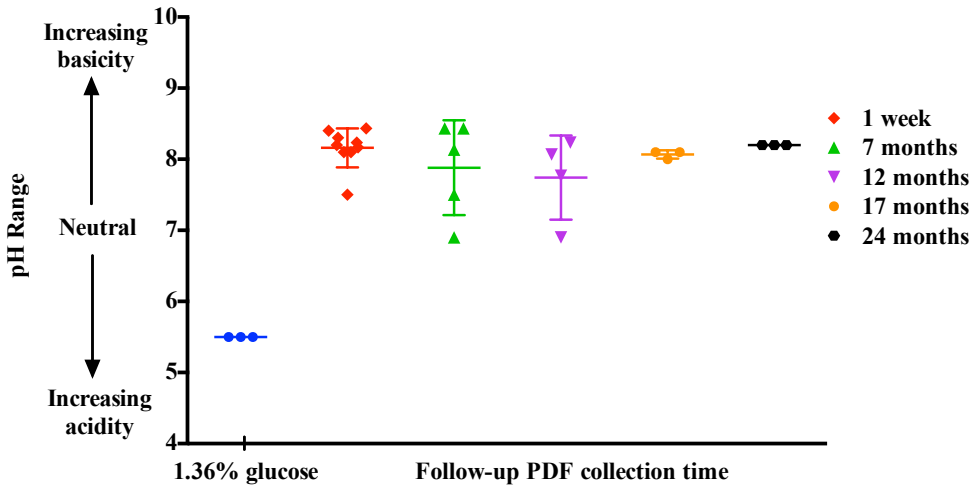


Figure 3.8 pH measurement of PDS and PDF.
The pH level was determined using the pH meter (3510 pH Meter Jenway) (Section 2.2.1.2). Follow-up PDF collection time from different PD patients is indicated beside the graph.

3.2.5 Glucose concentration of PDF

The group of PD patients enrolled in this study used different concentrations of glucose-based PDS during their PD therapy (Table 3.2); hence, it was decided to evaluate changes that occurred in the glucose level after a PD exchange. Bacterial cells can use glucose as an energy source; therefore glucose availability in PDF could contribute to microbial growth induction (Dey & Rosen, 1995). Determination of glucose concentration in the PDF was performed by using a glucose assay kit (Section 2.2.1.3). The measurements were first carried out on the initial PDFs collected from nine PD patients at the beginning of their PD therapy. Although PD patients used different PDS glucose concentrations, a consistently clear reduction in the glucose level of the PDFs was found (Figure 3.9). This suggests that there was peritoneal absorption of glucose from the PDS (Hain & Kessel, 1987; Levy *et al.*, 2015).

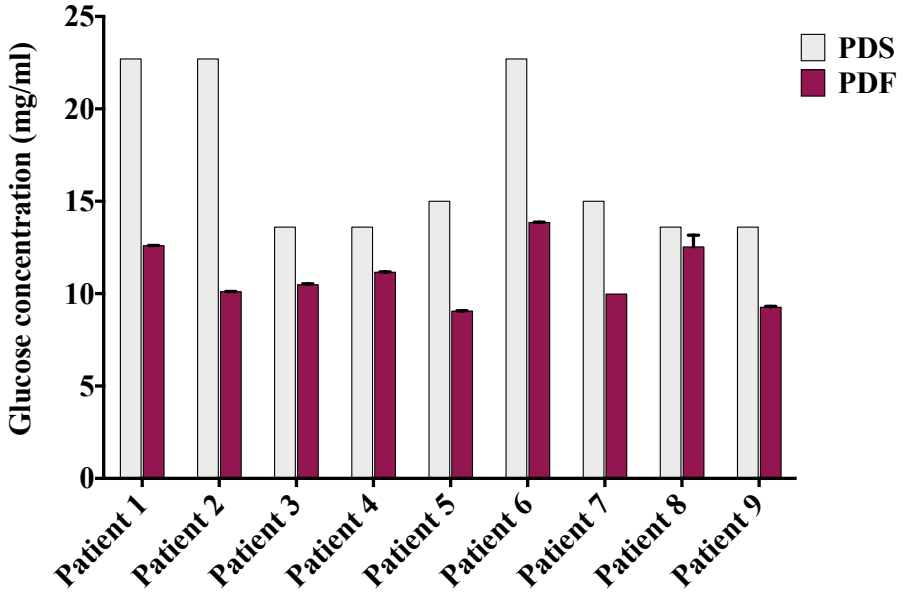


Figure 3.9 Glucose concentration in PDS and PDF.
The glucose concentration was determined using the glucose assay kit (Section 2.2.1.3). Initial PDFs, which were obtained from nine PD patients at the beginning of their therapy, were analysed. Patients 1, 2 and 6 used 2.27% glucose PDS; Patients 3, 4, 8 and 9 used 1.36% glucose PDS; and Patients 5 and 7 utilised 1.5% glucose PDS.

Analysis of the glucose availability in the PDF over a PD therapy period involved the study of the follow-up PDFs, which were collected from Patients 1, 2, 3, 6 and 8 at different points in time (Table 3.2). Generally speaking, the glucose content of the PDF declined over the PD period. The bar charts illustrated in Figure 3.10 A provide information about the glucose concentration of the follow-up PDFs of Patient 1, who used 2.27% glucose PDS over the PD therapy duration. In comparison with the PDS, there was a decline in the glucose level of the PDF during the PD course. The mean glucose concentration of the follow-up PDFs was approximately half the concentration of the PDS used. Similarly, the PDFs of Patient 2 demonstrated lower glucose levels than the PDS (Figure 3.10 B). Patient 2 used two different formulas of glucose based PDS—that is, 1.36% and 2.27% glucose PDS. Nevertheless, the glucose availability in the PDFs steadily decreased over the PD therapy period and reached the lowest level after two years of PD duration. Patient 3 used 1.36% glucose PDS at the beginning of PD therapy, and six months later switched to 2.27% glucose PDS. As shown in Figure 3.10 C, the level of glucose in the PDS declined markedly after being in the peritoneal cavity. Furthermore, despite the fact that Patients 6 and 8 were diabetic, the trend of glucose concentration in the PDF during a PD therapy course was quite similar to that in the previous patients analysed (Patients 1, 2 and 3), who were non-diabetic. The concentration of glucose in the follow-up PDFs of Patient 6 had lower levels than the PDS used, which was a 2.27% glucose PDS (Figure 3.10 D). Moreover, the PDFs of Patient 8 demonstrated a marked decrease in glucose levels during the PD course and reached the lowest glucose level after 15 months on PD (Figure 3.10 E). Overall, the glucose concentrations were lower in PDF than in PDS, and PDF glucose levels continued to decrease as patient on dialysis time increased.

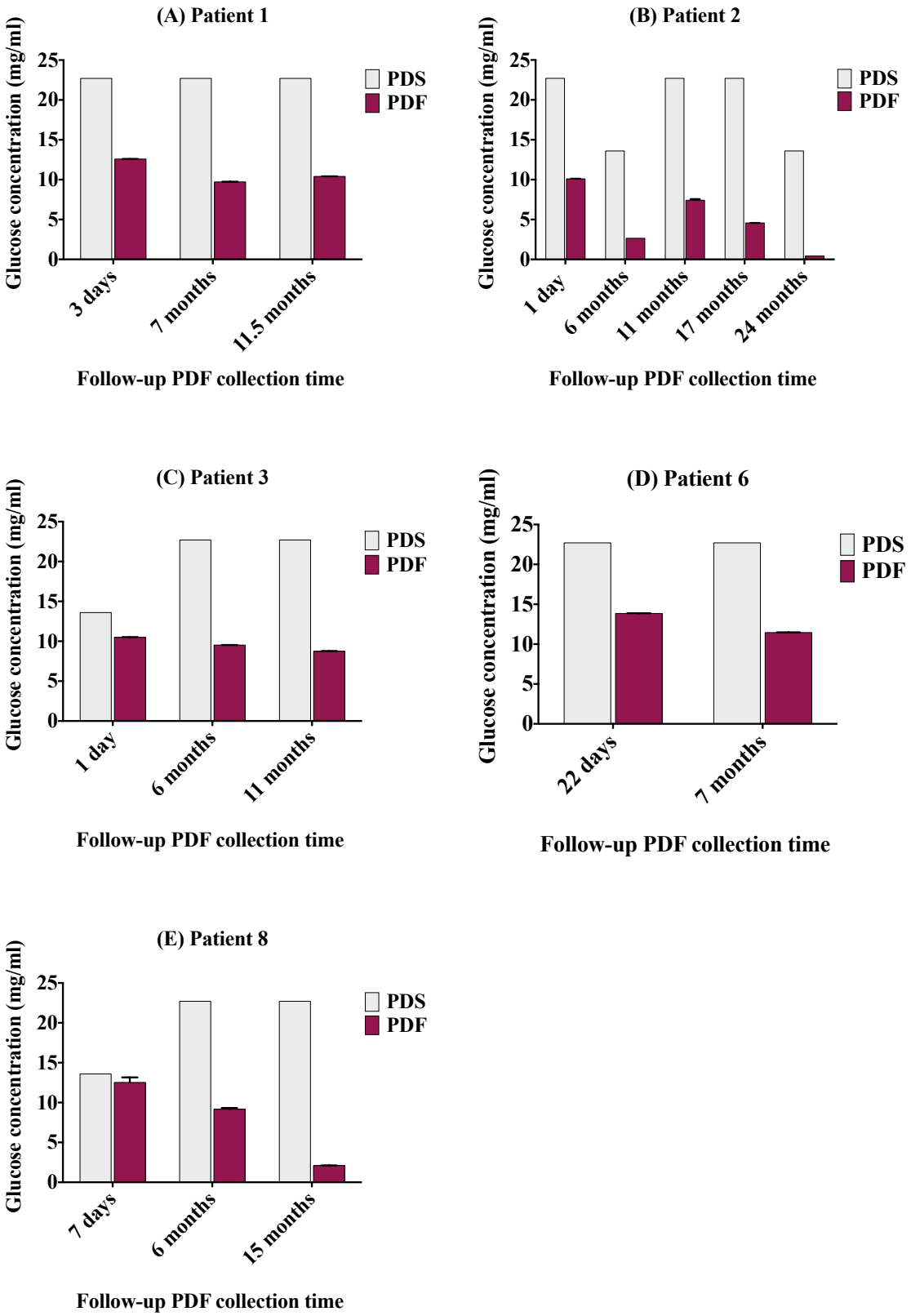


Figure 3.10 Glucose levels in PDS and PDF of five follow-up PD patients.
The time of the follow-up PDF collection is indicated for each patient. The glucose concentration was determined using the glucose assay kit (Section 2.2.1.3).

3.2.6 Investigation of iron availability in PDS and PDF

Iron is a key nutrient for bacterial growth and multiplication (Skaar & Schneewind, 2004). Measurements of iron levels in the PDS and PDF were important to this study, because if iron was available in the PDS and PDF, this could stimulate bacterial growth, which consequently could increase the risk of a peritoneal infection. The iron concentration was determined using an iron assay kit (Section 2.2.1.4).

3.2.6.1 Total PDS and PDF iron

The PD solutions which had variable glucose concentrations were examined for their total iron (ferrous iron [Fe^{+2}] + ferric iron [Fe^{+3}]) levels. The solutions which were examined were 1.36%, 1.5%, 2.27% and 3.86% glucose PDS. It can be seen in Figure 3.11 that the iron was available in all the PD solutions analysed. The mean iron concentration in the PD solutions was 45.52 ± 11.96 µM, with a minimum value of 35.24 ± 1.27 µM and a maximum value of 60.74 ± 4.24 µM for the 1.5% and 3.86% glucose PD solutions, respectively. With the exception of the 1.5% glucose PDS, increasing the glucose concentration clearly increased the iron availability, as the 3.86% glucose PDS had the highest iron concentration.

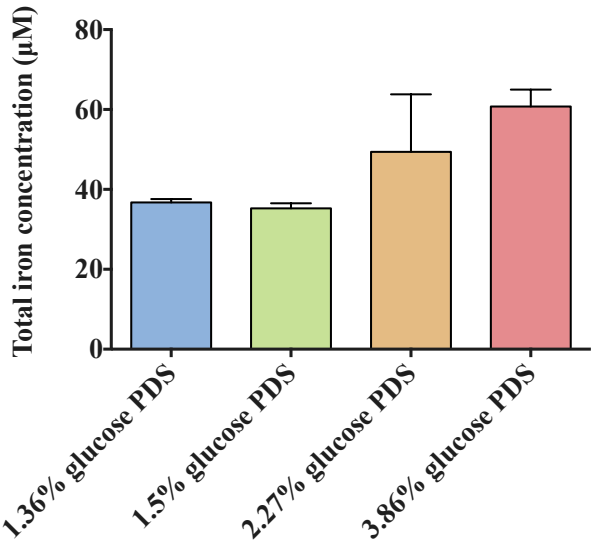


Figure 3.11 Total iron concentration in different PD solutions.
The total iron concentration was determined using the iron assay kit (Section 2.2.1.4).

The total iron level was also measured in the PDFs, which were obtained from nine PD patients at the beginning of their dialysis. As expected, iron was detectable in all the PDFs analysed. Variations in the total iron levels between the patient PDFs can be seen in Figure 3.12. The PDF of the nine patients had a higher total iron level than the corresponding PDS. The highest level of the total iron was detected in the PDF of Patient 3, followed by the PDF of Patient 4, whereas the PDF of Patient 7 had the lowest total iron concentration.

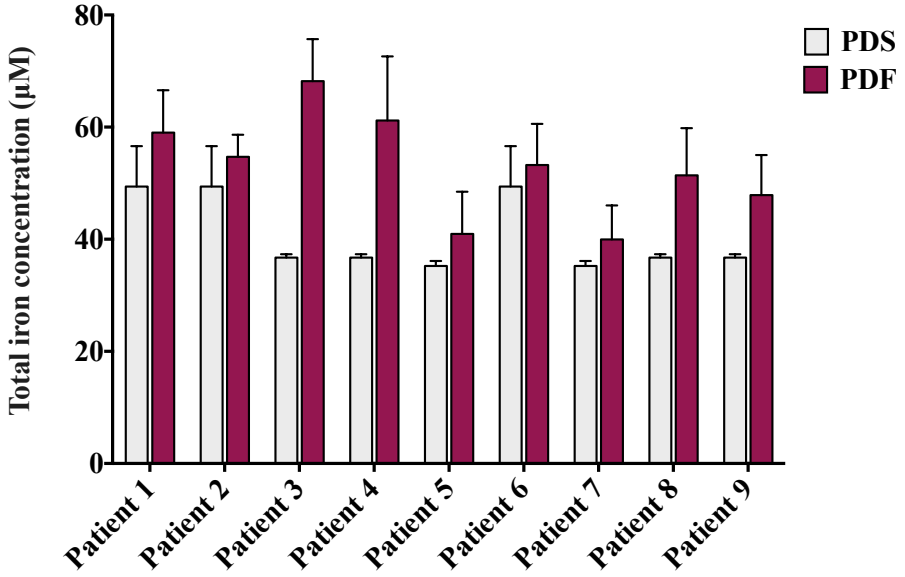


Figure 3.12 Total iron concentration of the PDFs, which were obtained from nine PD patients at the starting of their dialysis and the corresponding PD solutions.
Patients 1, 2 and 6 used 2.27% glucose PDS; Patients 3, 4, 8 and 9 used 1.36% glucose PDS; and Patients 5 and 7 utilised 1.5% glucose PDS. The total iron concentration was determined using the iron assay kit (Section 2.2.1.4).

The PDFs of the follow-up patients (Patients 1, 2, 3, 6 and 8) were analysed in terms of the total iron concentration in their PD fluids over the course of the PD. The majority of the follow-up PDFs studied, in comparison to the PDS, showed higher levels of total iron (Figure 3.13). The PDF of Patient 2 (collected after 11 months on PD) and Patient 3 (collected after 1 day on PD) demonstrated a marked increase in their total iron levels as compared to the PDS.

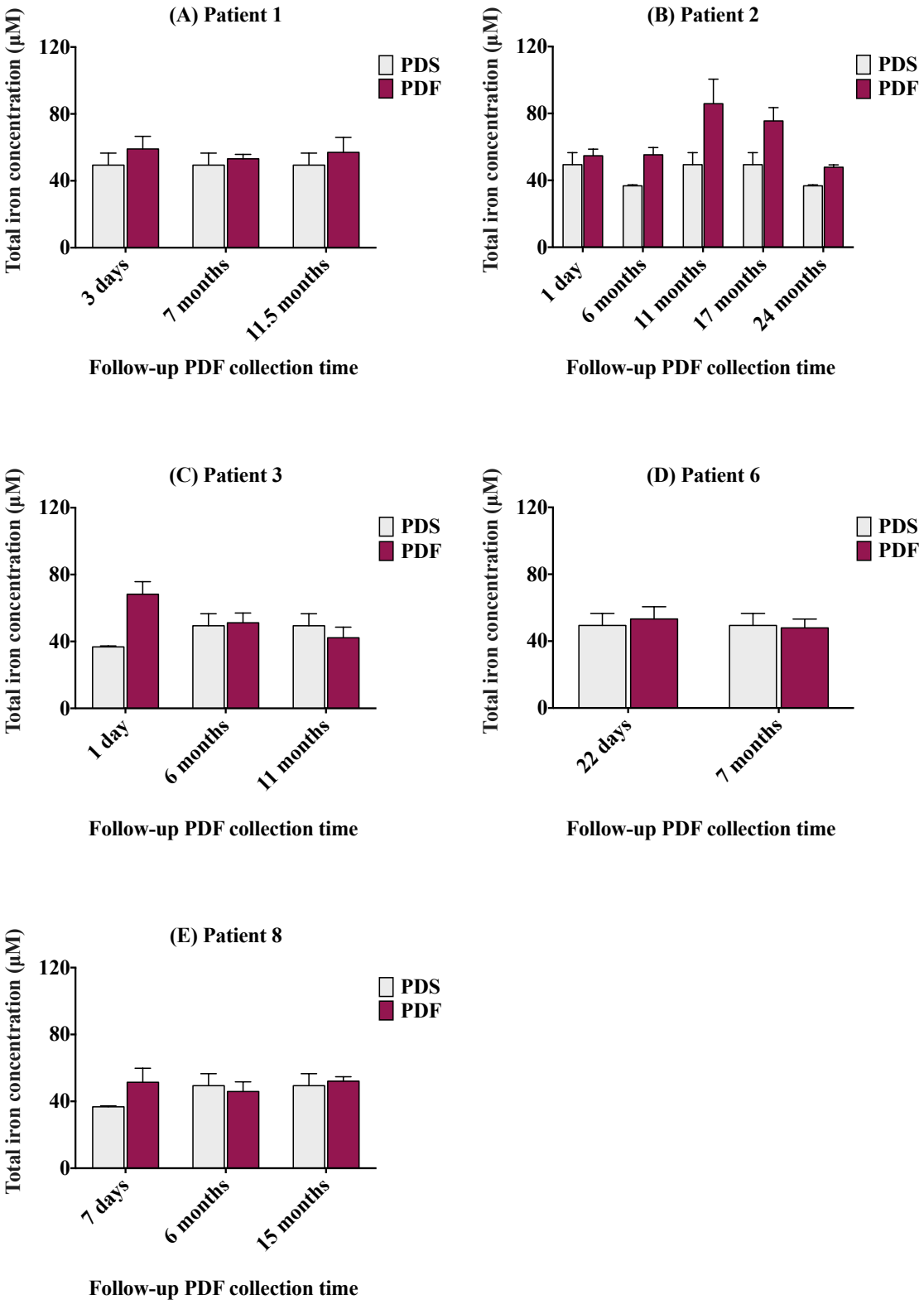


Figure 3.13 Total iron concentration in PDS and PDF of five follow-up PD patients.
The time of the follow-up PDF collection is indicated for each patient. The total iron concentration was determined using the iron assay kit (Section 2.2.1.4).

3.2.6.2 Comparison studies on the level of Fe^{+2} and Fe^{+3} within PDF

The iron assay kit can also quantify the concentration of Fe^{+2} (ferrous iron; also termed free iron), as well as Fe^{+3} (ferric iron), which exists in combination with molecules such as transferrin (Gomme *et al.*, 2005; Modun *et al.*, 1994; Parkkinen *et al.*, 2002). Less is known about transferrin-iron binding in PDF and so it was interesting to measure the levels of Fe^{+2} and Fe^{+3} in the PDF samples. The 2.27% glucose PDS was examined because it was used most commonly by PD patients as a dialysis solution. As shown in Figure 3.14, the Fe^{+2} concentration in 2.27% glucose PDS was very low compared to the Fe^{+3} level. The Fe^{+3} concentration in the PDFs of Patients 5, 6 and 9 was higher than the PDF Fe^{+2} concentration. On the other hand, the concentration of Fe^{+2} was greater than the level of Fe^{+3} in the PDFs of Patients 3 and 4. Thus, Patients 3 and 4, who had the highest levels of total iron in their PDFs, also had higher levels of Fe^{+2} than Fe^{+3}. Interestingly, the PDF of Patient 4 was the most supportive medium for staphylococcal growth (Figure 3.2).

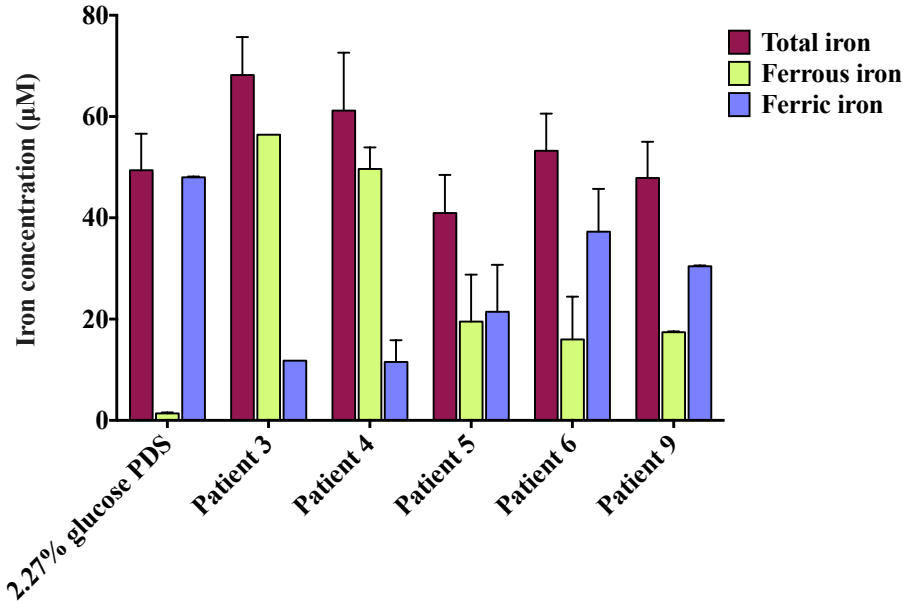


Figure 3.14 Total, ferrous and ferric iron concentrations in PDS and PDF of Patients 3, 4, 5, 6 and 9 at the starting of their dialysis.
The total, ferrous and ferric iron concentrations were determined using the iron assay kit (Section 2.2.1.4).

The Fe^{+2} and Fe^{+3} levels were also measured using a range of follow-up PDFs obtained from Patients 1, 2, 6, and 8. Overall, both Fe^{+2} and Fe^{+3} were detected in the PDFs. The Fe^{+3} concentration in the follow-up PDF of Patients 1, 6 and 8 was higher than the PDF Fe^{+2} concentration, whereas the level of Fe^{+2} was higher in the follow-up PDF of Patient 2 (Figure 3.15).

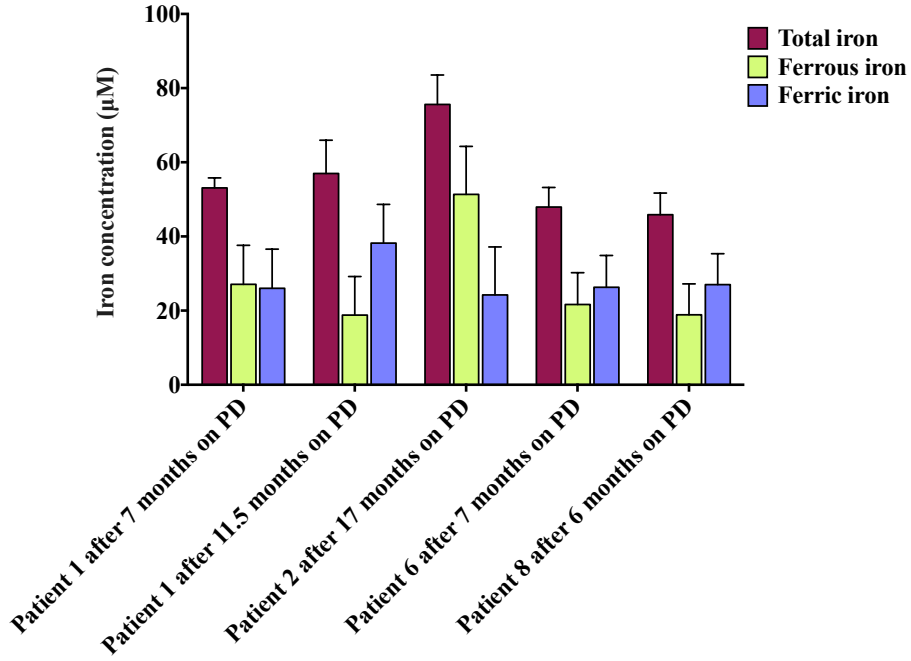


Figure 3.15 Total, ferrous and ferric iron concentrations in follow-up PDF from Patients 1, 2, 6, and 8.
PDFs were obtained at different points in time, as indicated in the bar chart. The total, ferrous and ferric iron concentrations were determined using the iron assay kit (Section 2.2.1.4).

3.2.7 Investigation of catecholamine stress hormones levels in PDF

In those with normal kidney function, or for renal patients on haemodialysis, metabolites such as the catecholamine stress hormones are largely eliminated in the urine or haemodialysis dialysate (Guyton & Hall, 2006). For PD patients, it is not fully clear where catecholamine elimination takes place. Measurements of noradrenaline (NE), adrenaline (Adr) and dopamine (Dop) levels in PDF were therefore performed using a tri cat ELISA kit (Section 2.2.1.5). First, the PDFs, which were obtained from the nine PD patients at the beginning of their dialysis, were analysed. It can be seen in Figure 3.16 that catecholamine stress hormones, in particular NE, were present in all of the PDFs. The NE concentration was variable among the nine PD patients, with the mean NE concentration varying from 0.1716 ± 0.1515 ng/ml to a maximum value of 0.5182 ± 0.153 ng/ml. With

the exception of the PDFs of Patients 7, 8 and 9, the level of Adr was detectable in the PDFs of PD patients and there were differences in the Adr concentration between them. The mean concentration of Adr was lower than the mean concentration of NE in all PDFs. The PDF of Patient 4 had the highest Adr concentration, whereas the lowest Adr level was detected in the PDF of Patient 5. The highest level of NE as well as Adr was found in the PDF of Patient 4. This finding could explain why the PDF of Patient 4 was by far the most supportive medium for staphylococcal growth (Figure 3.2). It will be interesting to examine if the transferrin level in that patient's PDF is similarly high (which will be investigated later in this chapter). In contrast to the mean NE and Adr levels, the mean Dop concentration had the lowest catecholamine presence in the PDF of all the PD patients. To summarise, the catecholamine stress hormones (NE, Adr and Dop) were ultimately present at different levels in the PDFs of the PD patients.

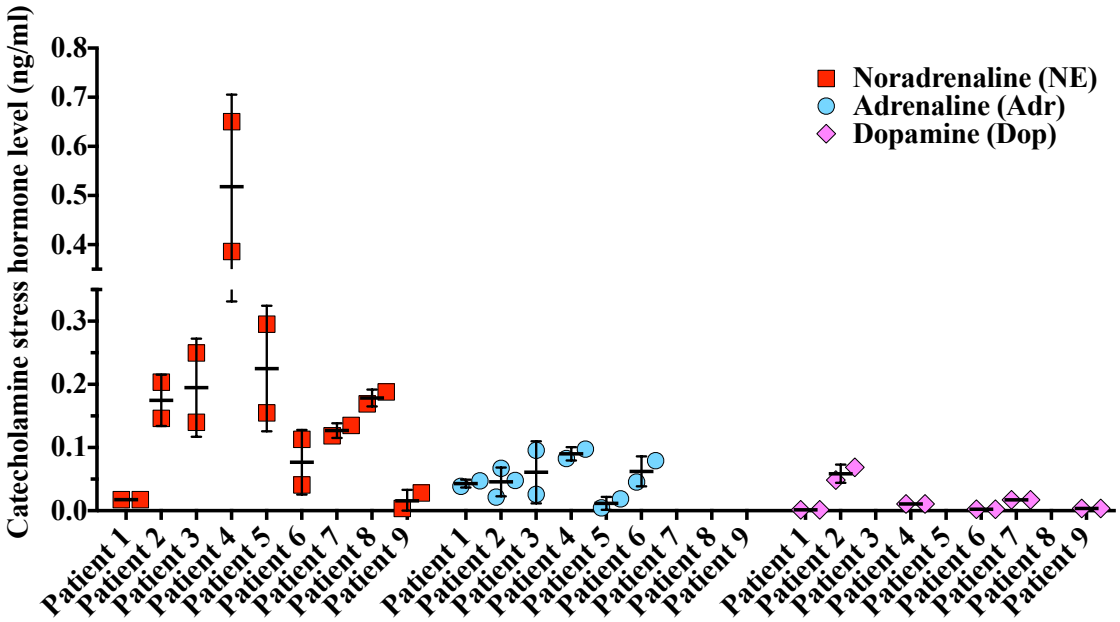


Figure 3.16 Measurement of catecholamine stress hormones levels in PDF from nine PD patients at the start of their dialysis.
The NE, Adr and Dop levels were determined using the tri cat ELISA kit (Section 2.2.1.5).

In addition to the analysis of NE, Adr and Dop in the PDFs at the beginning of PD therapy, it was also worthwhile to monitor changes in catecholamine concentrations over the duration of the patients PD therapy. For this purpose, the PDFs of the follow-up PD patients (Patients 1, 2, 3, 6 and 8) were examined. On the whole, NE, and not Adr or Dop, was present in all the follow-up PDFs analysed. Moreover, its concentration, in comparison with Adr and Dop, was the highest of the catecholamines. The concentration

of NE in the PDF of Patient 1 changed most clearly over the course of the PD period and reached a peak after the patient had spent seven months on PD (Figure 3.17 A). Interestingly, that particular PDF was the most favourable fluid for *S.epidermidis* growth (Figure 3.3 B). This PDF also had the maximum level of Adr and Dop. An analysis of the level of stress hormones over a longer PD period showed that the concentration of NE also changed over time. For example, the follow-up PDFs of Patient 2, who spent 25 months on PD therapy, showed the most changes in NE levels (Figure 3.17 B). The highest level of NE was detected in the PDF that was obtained after 17 months, while the lowest concentration of NE was found in the PDF collected after 11 months on PD. Patient 3 showed changes in NE concentration over the duration of PD therapy (Figure 3.18 A). As shown in Figure 3.18 B, the concentration of NE as well as Adr increased in the PDF of Patient 6 that was received after seven months on PD, as compared to the fluid collected at the start of PD therapy. Similar to the results of Patients 1, 2 and 3, Dop in the PDF of Patient 6 was only detectable at extremely low levels, as opposed to NE and Adr concentrations. Last but not least, there was a reduction in the concentration of NE in the dialysates of Patient 8 over the course of PD therapy (Figure 3.19). The maximum NE concentration was found in the PDF that was collected after seven days on PD. Then, the NE level decreased gradually, but steadily, and reached the minimum level after Patient 8 had spent 15 months on PD. Adr and Dop were only detected in the PDF after Patient 8 had spent 15 months on PD. To conclude, NE was present in all of the PDFs analysed, and its concentration changed over the PD therapy course. It was also the most abundant stress hormone detected in the PDF samples analysed.

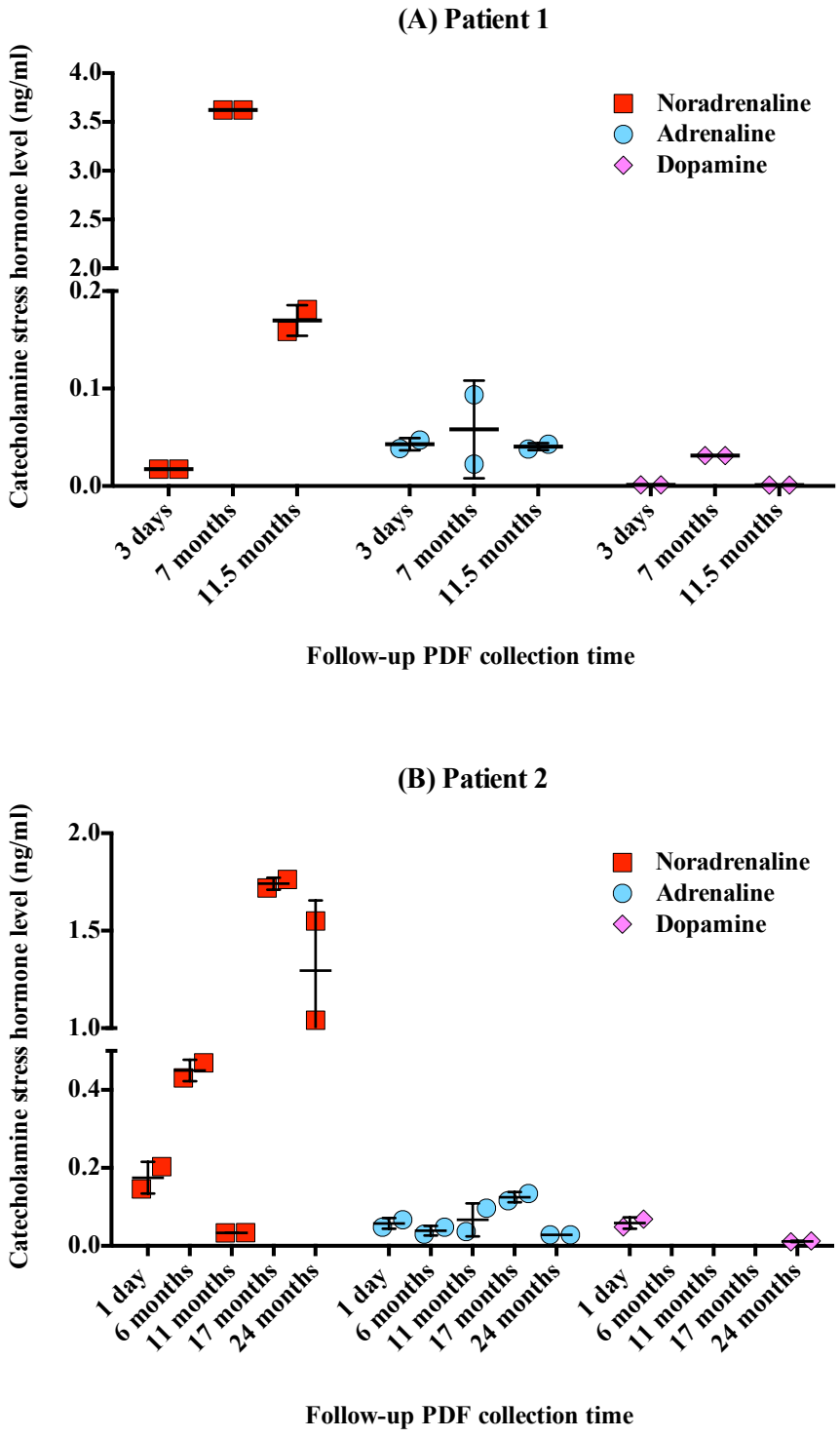


Figure 3.17 Catecholamine stress hormones levels in PDFs of Patients 1 and 2.
The noradrenaline, adrenaline and dopamine levels were determined using the tri cat ELISA kit (Section 2.2.1.5).

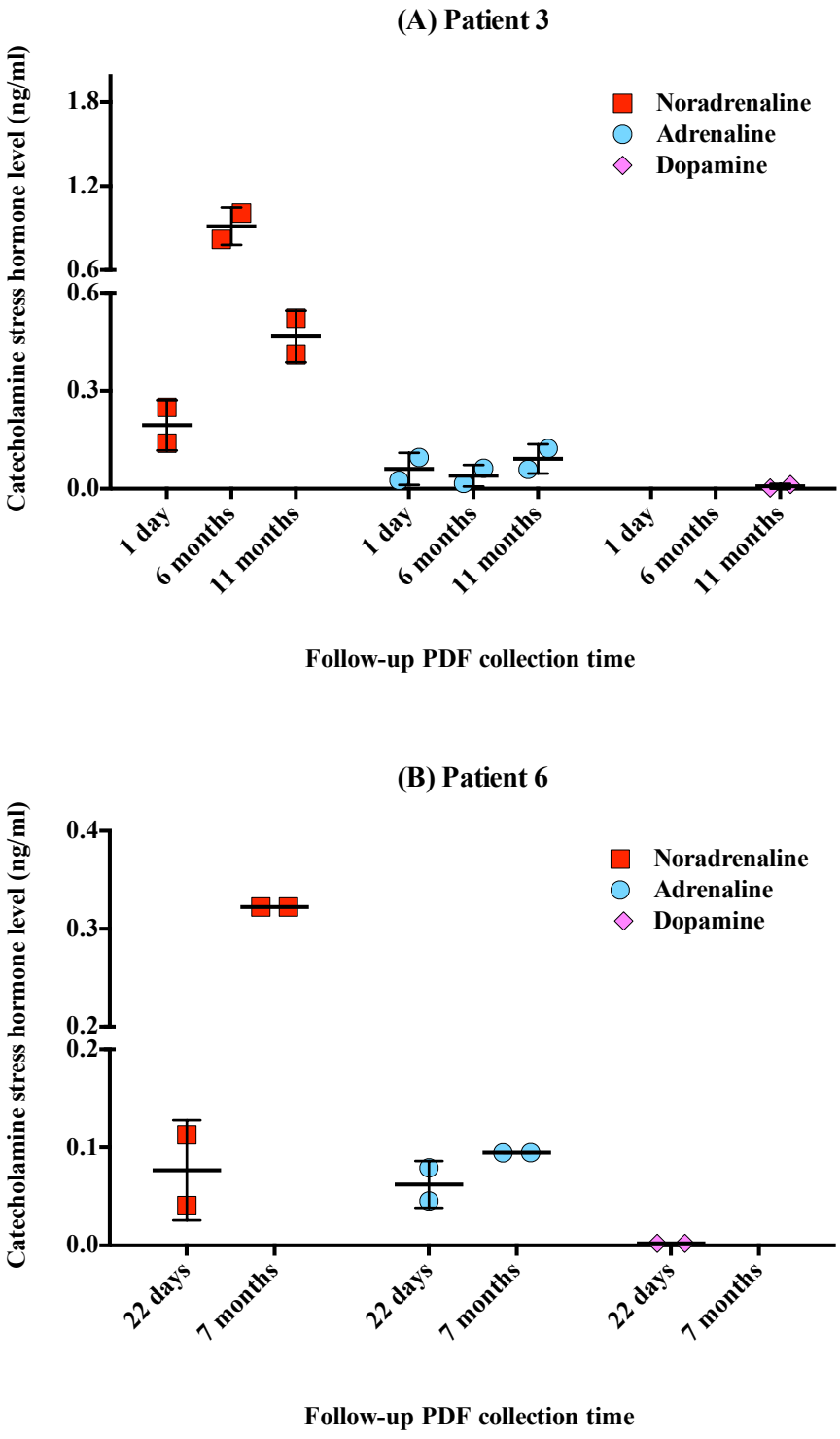


Figure 3.18 Catecholamine stress hormones levels in PDFs of Patients 3 and 6.
The noradrenaline, adrenaline and dopamine levels were determined using the tri cat ELISA kit (Section 2.2.1.5).

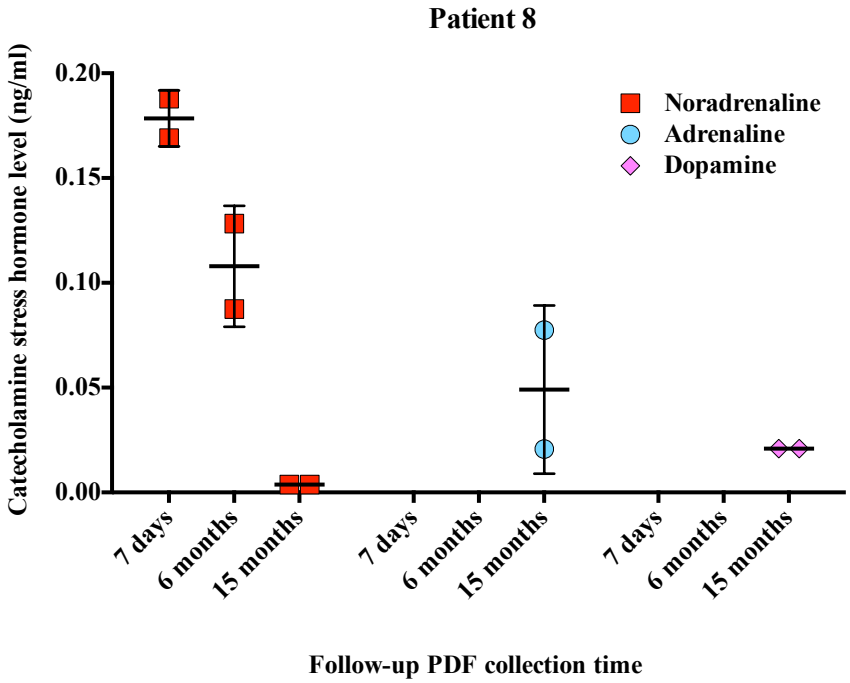


Figure 3.19 Catecholamine stress hormones levels in PDFs of Patient 8.
The noradrenaline, adrenaline and dopamine levels were determined using the tri cat ELISA kit (Section 2.2.1.5).

3.2.8 Comparative analysis of protein concentration in patient PDF

As mentioned in Chapter 1 (Section 1.5.1), protein loss into the PDF is one of the major obstacles of PD therapy (Blumenkrantz *et al.*, 1981) and, as hypothesised, may play an important role in the development of infectious peritonitis. Therefore, the concentration of protein within PDF was examined. Determination of the protein concentration in the PDF was performed using a Bio-Rad protein assay kit (Section 2.2.1.6). Measurements of protein concentration in the PDFs obtained from nine PD patients at the beginning of their dialysis and from later collections were performed. It can be seen in Figure 3.20, there were marked variations between PD patients in the concentration of protein in their PDFs. The mean protein concentration among all the patients was 1.844 ± 1.29 g/L. The highest protein concentration was detected in the PDF of Patient 9, while the lowest protein concentration was found in the PDF of Patient 6. Notably, Patient 6 was the only patient who used APD as a PD modality, which suggests that the PD style could influence the level of protein loss into the PDF. Patients 1, 2, 3, 4, 5, 7, 8 and 9 used CAPD. Each CAPD patient used 2 L of PDS per exchange, whereas the APD patient used 12 L of PDS per exchange, which allowed the amount of protein lost by the PD patients during a dialysis exchange to be determined (Table 3.3).

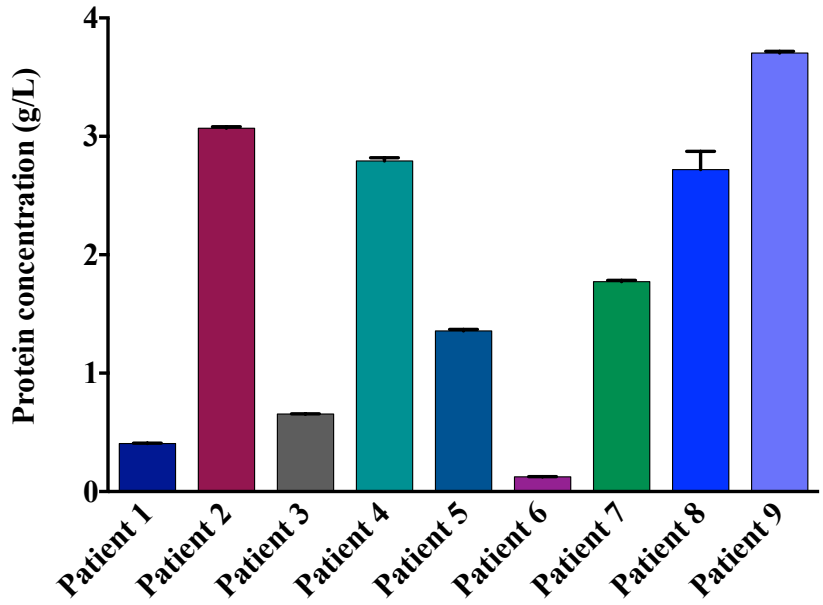


Figure 3.20 Protein concentration in PDF of nine PD patients.
The protein concentration was measured using the Bio-Rad protein assay kit (Section 2.2.1.6).

Table 3.3 Mean protein concentration and the estimated protein loss in PDF of the nine PD patients at the beginning of their dialysis.

Patient ID	Protein concentration (g/L)	Protein loss (g)/PD exchange
Patient 1	0.4050 ± 0.001	0.810 ± 0.003
Patient 2	3.069 ± 0.004	6.139 ± 0.009
Patient 3	0.6546 ± 0.006	1.309 ± 0.001
Patient 4	2.792 ± 0.011	5.584 ± 0.022
Patient 5	1.358 ± 0.004	2.715 ± 0.010
Patient 6	0.1246 ± 0.006	1.495 ± 0.008
Patient 7	1.773 ± 0.004	3.546 ± 0.009
Patient 8	2.720 ± 0.062	5.440 ± 0.124
Patient 9	3.703 ± 0.006	7.406 ± 0.012

In terms of investigation the protein concentration during the PD course, the PDF of the follow-up patients (Patients 1, 2, 3, 6 and 8) were analysed. For each follow-up patient, the trend of the protein concentration in the PDF during the PD therapy period varied markedly. The PDF of Patients 1, 2 and 8 at the beginning of dialysis represented a higher protein concentration than was seen in the follow-up PD fluids (Figure 3.21 A, B and E). However, the PDFs that were obtained from Patients 3 and 6 after about six months on PD demonstrated more protein loss than the other PD fluids (Figure 3.21 C and D). The protein concentrations in the PDFs eventually varied between the PD patients. The estimated amount of protein lost by the PD patients during a dialysis exchange is shown in Table 3.4.

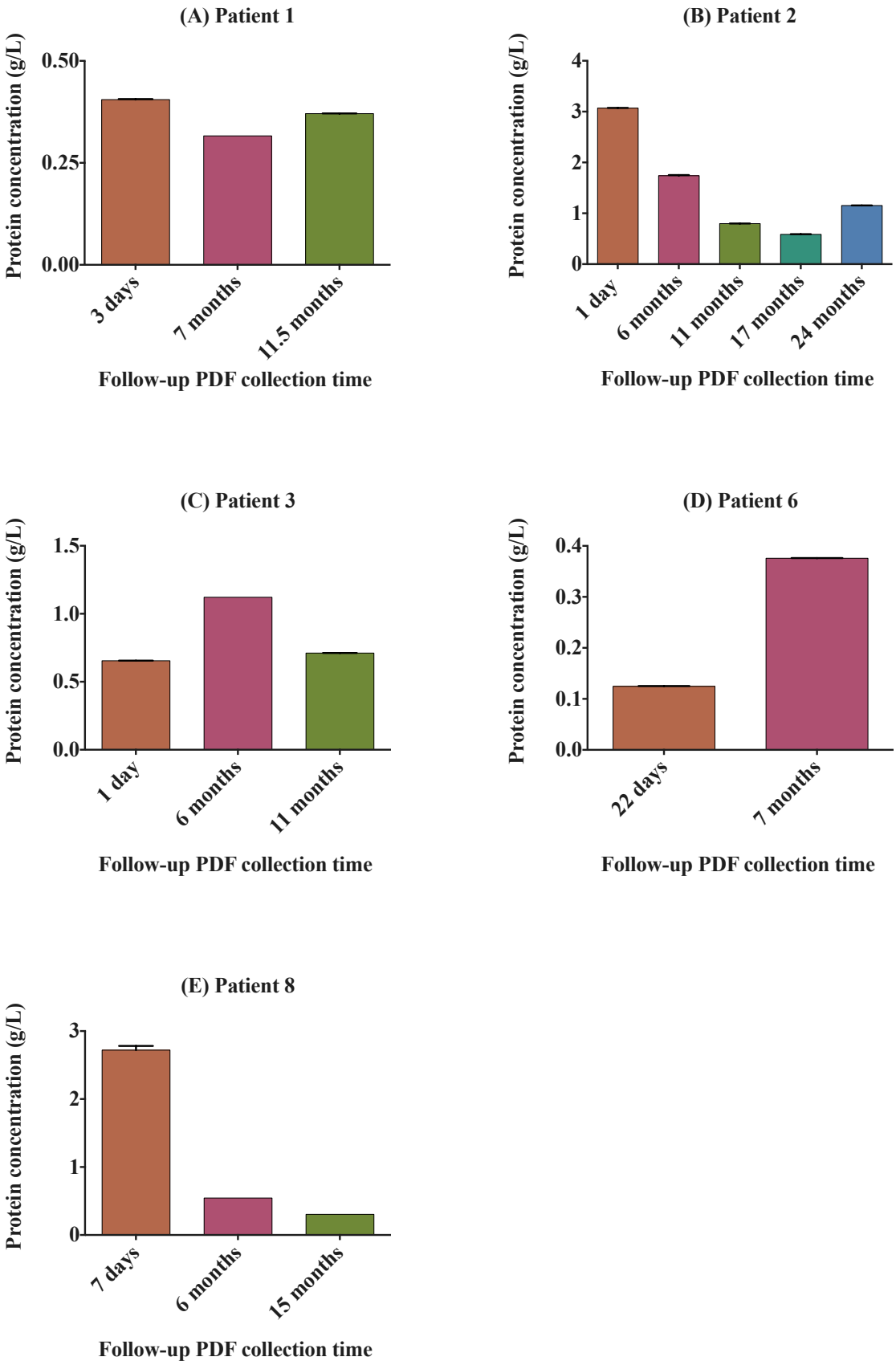


Figure 3.21 Protein concentration in PDF of follow-up PD patients.
The protein concentration was measured using the Bio-Rad protein assay kit (Section 2.2.1.6).

Table 3.4 Mean protein concentration and the estimated protein loss in follow-up PDF of five PD patients.

Patient ID	Follow-up PDF collection time	Protein level (g/L)	Protein loss (g)/PD exchange
Patient 1	3 days	0.4050 ± 0.0012	0.810 ± 0.003
	7 months	0.3163 ± 0.0001	0.633 ± 0.0001
	11.5 months	0.3707 ± 0.0004	4.449 ± 0.006
Patient 2	1 day	3.069 ± 0.004	6.139 ± 0.009
	6 months	1.740 ± 0.011	3.479 ± 0.023
	11 months	0.7974 ± 0.002	1.595 ± 0.004
	17 months	0.5857 ± 0.003	1.171 ± 0.006
	24 months	1.154 ± 0.001	2.307 ± 0.002
Patient 3	1 day	0.6546 ± 0.0006	1.309 ± 0.001
	6 months	1.122 ± 0.0001	2.244 ± 0.0001
	11 months	0.7108 ± 0.001	1.422 ± 0.002
Patient 6	22 days	0.125 ± 0.0007	1.495 ± 0.008
	7 months	0.376 ± 0.0005	4.508 ± 0.006
Patient 8	7 days	2.720 ± 0.062	5.440 ± 0.124
	6 months	0.5439 ± 0.0005	1.088 ± 0.001
	15 months	0.3043 ± 0.0004	0.609 ± 0.001

3.2.9 Investigations of PDF protein profiles

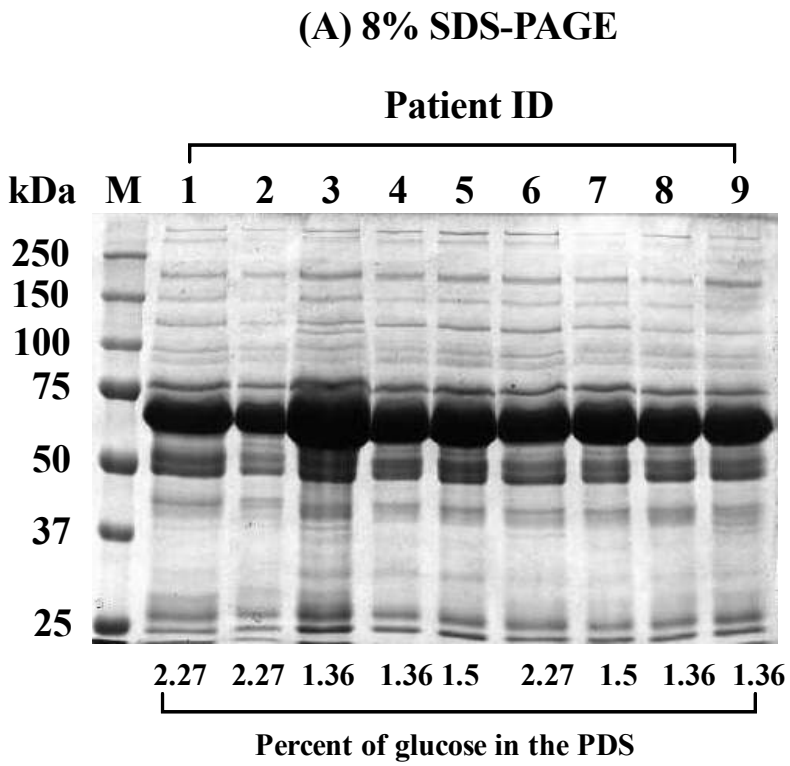
In Table 3.4, it was recognised that there was a marked variability in the protein concentration of PDF between different PD patients. It was also noticed that within the same patient the total protein level varied over PD therapy time. Based on these findings, it was decided to investigate further whether PD patients lost different types of proteins during PD therapy, with the hope of finding protein(s) which might be potential infection-risk biomarker(s). In terms of the results obtained, various classes of proteins were identified whose origin was locally (from an intraperitoneal source), from close to the peritoneal cavity, or from the blood (Dulaney & Hatch, 1984).

3.2.9.1 Did protein profile of PDF of variable peritoneal dialysis patients have similar patterns?

PDFs were obtained from the nine PD patients at the beginning of PD therapy and subjected to SDS-PAGE analysis (Section 2.2.1.7). Figure 3.22 shows the 8% and 12% SDS-PAGE gels of the PDFs. The protein profiles showed a striking similarity between the different PD patients. The 8% SDS-PAGE gel which was used to optimise separation of proteins above 50 kDa, showed very similar protein profiles. In terms of the lower molecular weight proteins, the 12% SDS-PAGE gel showed the PD patients released largely the same proteins into their PDFs. However, release of some proteins showed variability in levels, such as a 25 kDa protein and proteins in the 50-75 kDa range.

3.2.9.2 Did different glucose concentration in PDS influence the types of proteins that are appearing in PDF?

As mentioned earlier in this chapter, the PD patients used different glucose formulations in their dialysis solutions (Table 3.2). Patients 1, 2 and 6 used a 2.27% glucose PDS, whereas Patients 3, 4, 8 and 9 used a 1.36% glucose PDS. On the contrary, a 1.5% glucose PDS was utilised by Patients 5 and 7. As shown in Figure 3.22, there were no marked differences in the protein profiles of the patients who used 1.36% glucose PDS, as opposed to the patients who used 2.27% or 1.5% glucose PDS. Consequently, a different glucose concentration in the PDS had no effect on the protein profile of the PDF.



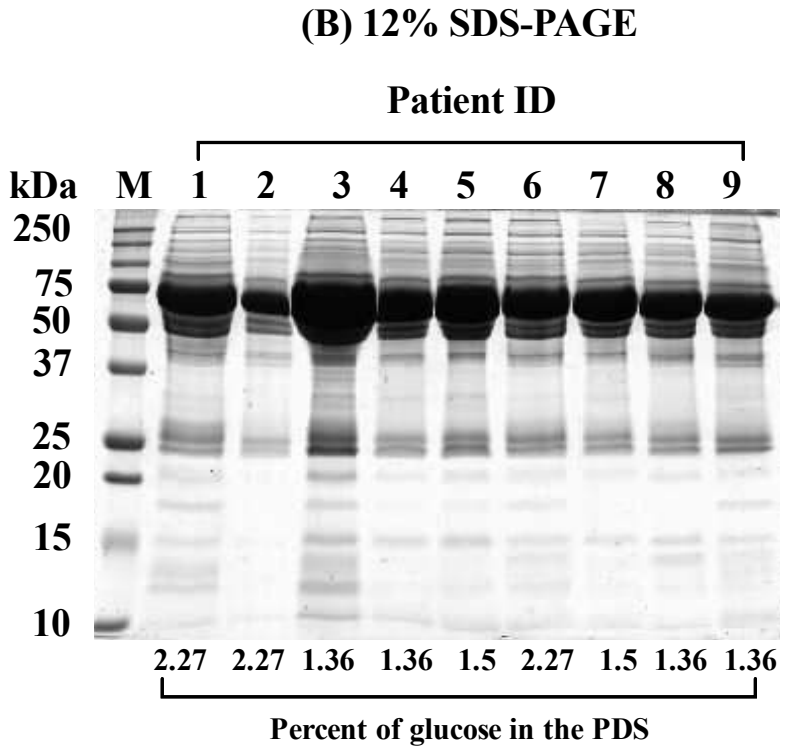


Figure 3.22 Comparative protein profiles of PDF from nine PD patients using SDS-PAGE gels. PDFs were collected from the patients at the beginning of their dialysis. A constant quantity of 20 µg of the protein of each PDF were loaded per lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7). The M.W of the PDF proteins was estimated using protein molecular weight markers (M). 'kDa' (kilodalton).

3.2.9.3 Were novel proteins starting to appear the longer a patient was on peritoneal dialysis?

The PDFs of follow-up patients were analysed using 8% and 12% SDS-PAGE gels, to determine if novel proteins appeared over PD therapy. The follow-up PDFs were collected at different points in time (Table 3.2). A fixed, protein quantity of 20 μg per lane was resolved on the SDS-PAGE gels. The gels shown in Figures 3.23 to 3.27 show the protein profiles in the PDF of the follow-up PD dialysates.

In general, the numbers of proteins were similar and no major differences were noted in levels of proteins released into the PDF. Also, the percentage of the glucose in the PDS appeared to have no influence on the protein profile of the PDF during the PD period. However, some of the follow-up PDFs showed changes in protein amounts when compared to the protein profile of the first PDF collected. For instance, for Patient 1 (Figure 3.23), levels of two proteins on the 8% SDS-PAGE gel (> 250 kDa) were lower in the follow-up PDFs than in the PDF taken from Patient 1 after three days on PD. In comparison with the PDF collected at the beginning of dialysis, there was higher level for some proteins (between 50 kDa and 10 kDa) in the PDFs derived from Patient 1 after seven months on PD and then after 11.5 months on PD. The PDF taken from Patient 3 after one day on PD showed greater protein level compared to the follow PDFs (Figure 3.25). After seven months on APD, the PDF of Patient 6 showed higher levels of the low M.W proteins (< 20 kDa) than the PDF collected after 22 days on PD (Figure 3.26). The first PDF collected from Patient 8 (after seven days on PD) had lower levels of proteins between 100 kDa and 50 kDa and in low M.W proteins between 15 kDa and 10 kDa than the follow-up PDFs (Figure 3.27). In conclusion, SDS-PAGE gels provided no obvious evidence of new proteins appearing during dialysis time; however, the levels of proteins released changed over time.

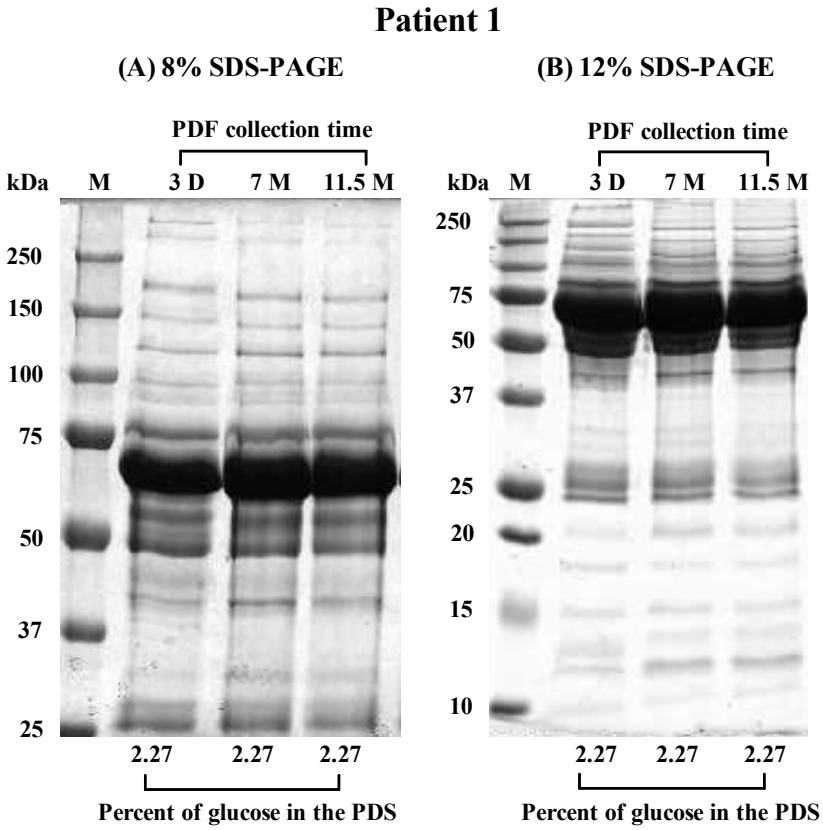


Figure 3.23 Protein profiles of PDF of Patient 1 resolved by SDS-PAGE gels.
PDFs were collected from Patient 1 at different points in time, as shown above the gels (D: refers to days; M: refers to months). The percentage of glucose of the corresponding PDS is indicated at the bottom of each gel. A constant quantity of 20 µg of the protein of each PDF was loaded in each lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7). The M.W of the PDF proteins was estimated using protein molecular weight markers (M). 'kDa' (kilodalton).

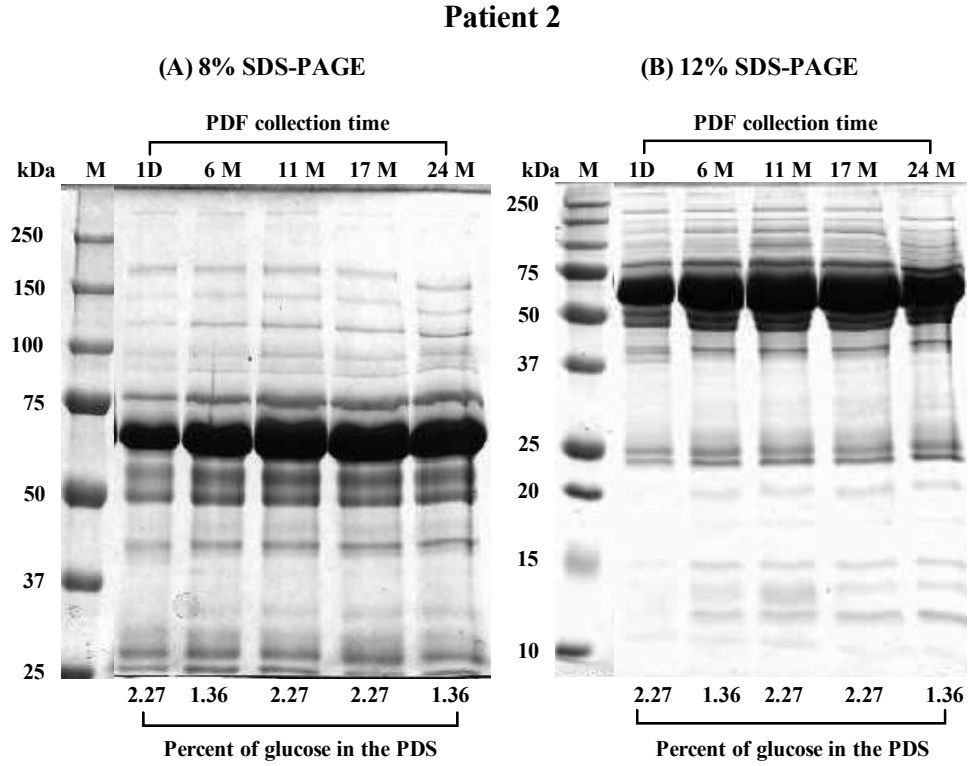


Figure 3.24 Protein profiles of PDF of Patient 2 resolved by SDS-PAGE gels.
PDFs were collected from Patient 2 at different points in time, as shown above the gels (D: refers to days; M: refers to months). The percentage of glucose of the corresponding PDS is indicated at the bottom of each gel. A constant quantity of 20 µg of the protein of each PDF was loaded in each lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7). The M.W of the PDF proteins was estimated using protein molecular weight markers (M). 'kDa' (kilodalton).

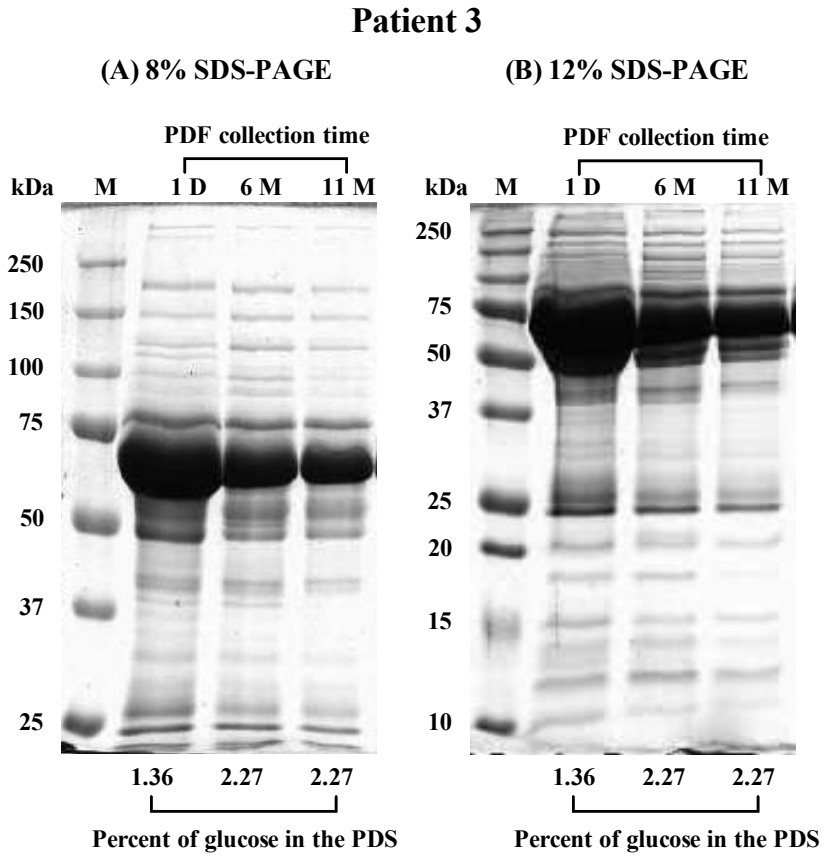


Figure 3.25 Protein profiles of PDF of Patient 3 resolved by SDS-PAGE gels.
PDFs were collected from Patient 3 at different points in time, as shown above the gels (D: refers to days; M: refers to months). The percentage of glucose of the corresponding PDS is indicated at the bottom of each gel. A constant quantity of 20 µg of the protein of each PDF was loaded in each lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7). The M.W of the PDF proteins was estimated using protein molecular weight markers (M). 'kDa' (kilodalton).

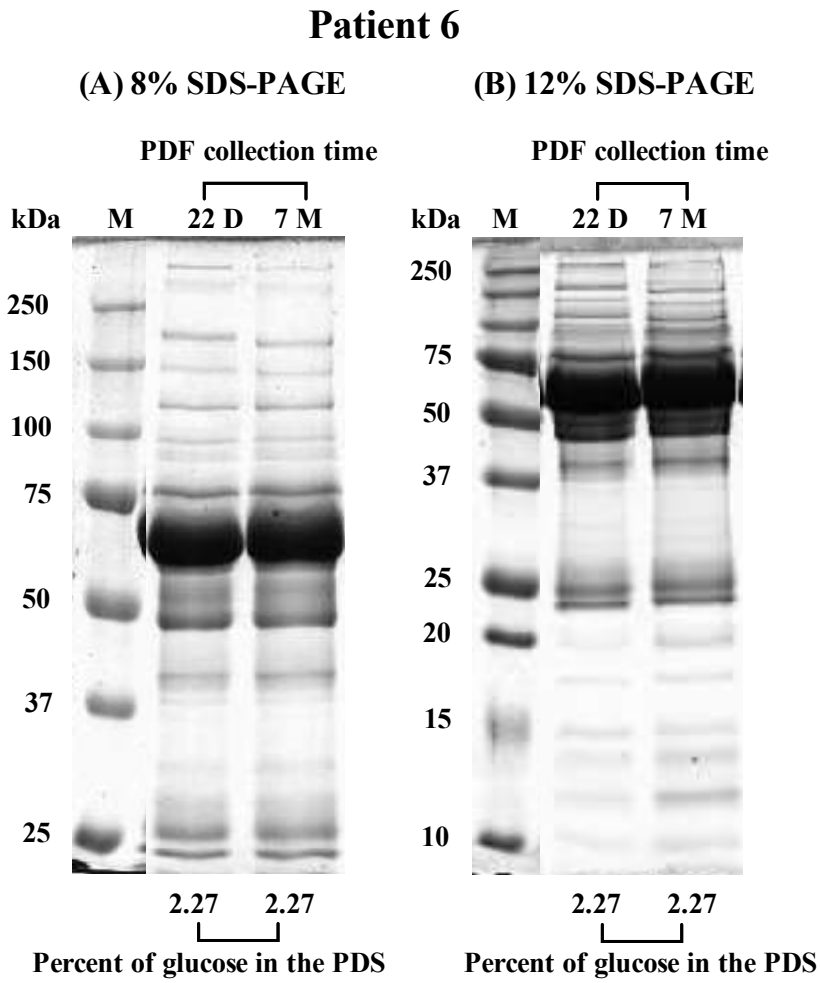


Figure 3.26 Protein profiles of PDF of Patient 6 resolved by SDS-PAGE gels.
PDFs were collected from Patient 6 at different points in time, as shown above the gels (D: refers to days; M: refers to months). The percentage of glucose of the corresponding PDS is indicated at the bottom of each gel. A constant quantity of 20 µg of the protein of each PDF was loaded in each lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7). The M.W of the PDF proteins was estimated using protein molecular weight markers (M). 'kDa' (kilodalton).

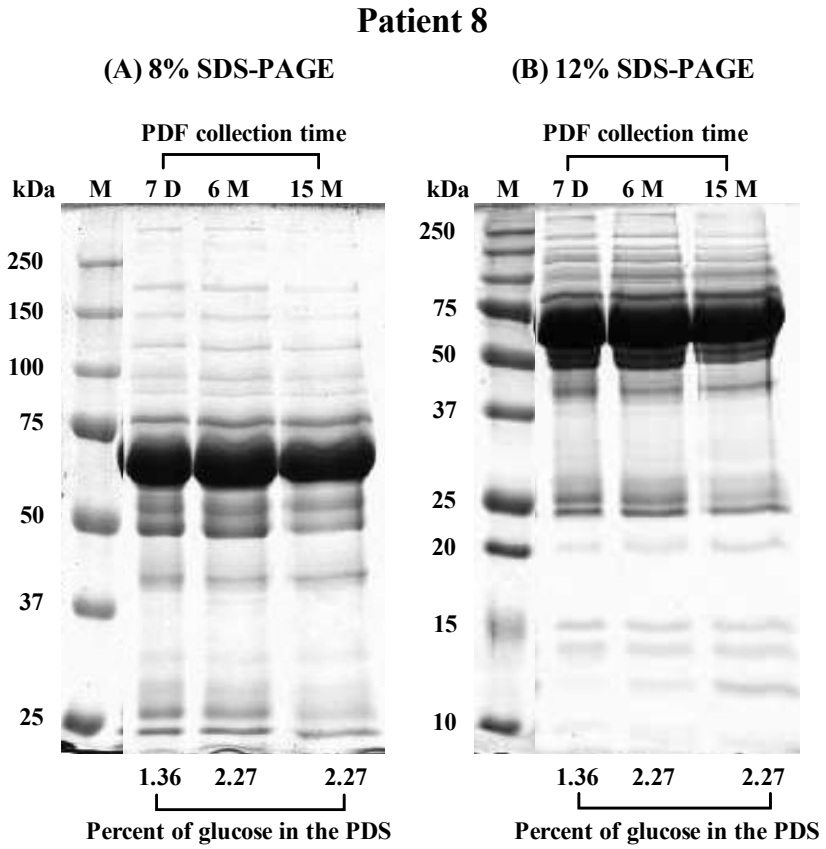
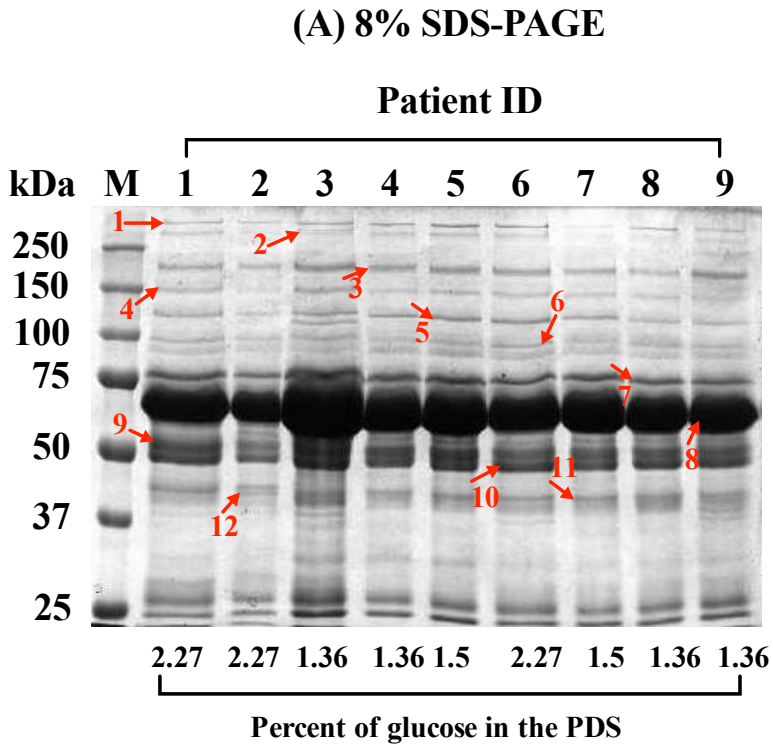


Figure 3.27 Protein profiles of PDF of Patient 8 resolved by SDS-PAGE gels.
PDFs were collected from Patient 8 at different points in time, as shown above the gels (D: refers to days; M: refers to months). The percentage of glucose of the corresponding PDS is indicated at the bottom of each gel. A constant quantity of 20 µg of the protein of each PDF was loaded in each lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7). The M.W of the PDF proteins was estimated using protein molecular weight markers (M). 'kDa' (kilodalton).

3.2.9.4 Did peritoneal dialysis patients release protein(s) into their PDFs that might be associated with infection risk?

PD patients generally had the same protein profile as viewed by 8% and 12% SDS-PAGE gels (Figure 3.22). However, it was constructive to know the identity of the PDF proteins. Sequencing studies of the proteins within the PDFs were performed as described in Section 2.2.1.8. SDS-PAGE gels that contained the PDF proteins were sequenced at the PNACL laboratory using MALDI-TOF MS or LC-MS. The numbered proteins shown in Figure 3.28 refer to the proteins that were identified by sequencing and Table 3.5 gives the protein identity and biological functions. More details of the protein sequences are attached in Appendix VIII.



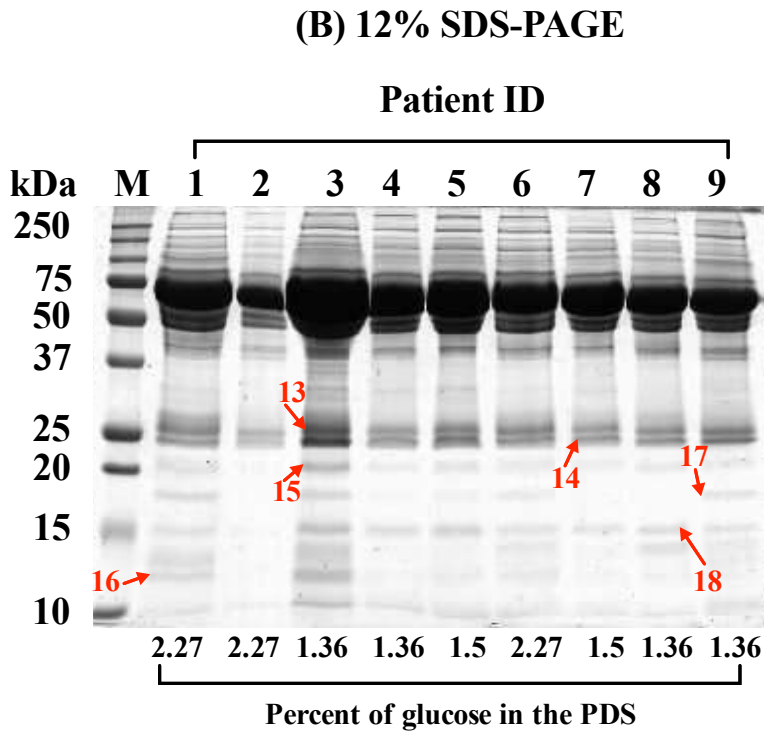


Figure 3.28 Identification of proteins in PDF of peritoneal dialysis patients.
The figure shows 8% and 12% SDS-PAGE gels of the proteins from PDF of nine PD patients at the beginning of their dialysis. The percentage of glucose of the corresponding PDS is indicated at the bottom of each gel. The arrow and number beside each protein refer to protein sent for sequencing. Protein numbers 1, 2, 3, 4, 5, 6, 15, 16 and 18 were sequenced by LC-MS, whereas protein numbers 7, 8, 9, 10, 11, 12, 13, 14 and 17 were sequenced by MALDI-TOF MS (Section 2.2.1.8).

Table 3.5 The identity and protein functions of the PDF proteins identified in Figure 3.28.

Protein ID	Protein identity	M.W (kDa)	Biological roles
1	Apolipoprotein B-100	515	A serum protein and a primary structure of very low-density lipoprotein and low-density lipoprotein. It is part of an innate immune response and could work against invasive *S.aureus* infection (Peterson *et al.*, 2008).
2	Fibronectin	262	An extracellular matrix protein involved in the innate immunity. It is used by the fibronectin-binding proteins of *S.aureus* to mediate attachment to host cells and to fibronectin-coated biomaterials (Menzies, 2003).
3	Alpha-2-macroglobulin	163	A plasma protein and an innate immunity component. It works as a proteinase inhibitor. Its concentration is elevated in nephrotic syndrome patients (Rabelink *et al.*, 1998).
4	Ceruloplasmin	122	A serum ferroxidase enzyme and copper-binding protein. It has a role in iron metabolism (Hellman & Gitlin, 2002).
5	Complement component 3	187	The most available complement protein in the serum and a key protein in complement system activation. It promotes a local inflammatory response against infectious agents (Sahu & Lambris, 2001).
6	Fibrinogen gamma chain	52	A plasma protein involved in blood coagulation process and in innate immune response (Mosesson, 2005).
7	Transferrin	77	An iron-binding protein present in plasma. It is a key part of the innate immunity as it makes extracellular body fluids bacteriostatic due to its ability to bind free iron and thus inhibit the growth of bacteria (Afzali & Goldsmith, 2004).
8	Albumin	71	A plasma protein, which has a role in the preservation of colloid osmotic pressure (Don & Kaysen, 2004).
9	Alpha-1-antitrypsin	46	A serum protein, which has a primary role in inhibiting human serine protease, including neutrophil esterase (Brantly *et al.*, 1988).
10	tRNA-dihydrouridine synthase	55	Catalyses the synthesis of dihydrouridine in tRNA. The level of dihydrouridine is increased in tumour cells (Kasprzak *et al.*, 2012).
11	Apolipoprotein A-IV	45	A plasma protein that participates in lipid metabolism and could work as a positive acute phase protein (Khovidhunkit *et al.*, 2004).
12	Fibrinogen beta chain	56	A plasma protein involved in blood coagulation process and in the innate immune response (Mosesson, 2005).
13	Immunoglobulin kappa chain C region	11	An important component of humoral immunity. Immunoglobulins protect the human body from microbial agents (Vidarsson *et al.*, 2014).
14	Apolipoprotein A-I	30	A major component of high-density lipoprotein and a negative acute phase protein (Burger & Dayer, 2002).
15	Retinol-binding protein 4	23	A plasma protein responsible for retinol transportation. It interacts with transthyretin to form a macromolecular complex that inhibits its filtration through the kidney glomeruli. It also has a role in glucose homeostasis (Cho *et al.*, 2006).
16	Beta-2-microglobulin	13	A plasma protein and a major component in dialysis-related amyloidosis (Liabeuf *et al.*, 2012).
17	Transthyretin	15	A plasma protein and a major carrier protein for the thyroid hormone thyroxin (Episkopou *et al.*, 1993).
18	Haptoglobin	22	A plasma protein that captures and links with free haemoglobin in the plasma to prevent the toxicity of haem iron in the kidneys (Alayash, 2011).

Considered collectively, 18 proteins were identified in the patient PDF samples that were of plasma origin, as defined in Table 3.5. The majority of proteins identified were basically involved in the immune system: fibronectin, alpha-2-macroglobulin, beta-2 microglobulin, complement component 3, albumin, alpha-1-antitrypsin and fibrinogen (gamma chain and beta chain) (Menzies, 2003; Rabelink *et al.*, 1998; Liabeuf *et al.*, 2012; Sahu & Lambris, 2001; Don & Kaysen, 2004; Brantly *et al.*, 1988; Mosesson, 2005). Immunoglobulin also belongs to the immune system, which was detected as kappa light chain (Vidarsson *et al.*, 2014). Apolipoproteins that were involved in the innate immunity response and in the lipid metabolism were detected: apolipoprotein B-100, apolipoprotein A-IV and apolipoprotein A-I (Peterson *et al.*, 2008; Khovidhunkit *et al.*, 2004; Burger & Dayer, 2002). In addition, iron, copper, thyroxin and retinol binding proteins were also identified: transferrin, ceruloplasmin, transthyretin and retinol-binding protein, respectively (Afzali & Goldsmith, 2004; Hellman & Gitlin, 2002; Episkopou *et al.*, 1993; Cho *et al.*, 2006). The proteins involved in dihydrouridine synthesis in tRNA and in inhibiting the toxicity effect of haem iron in the kidneys were tRNA-dihydrouridine synthase and haptoglobin, respectively (Kasprzak *et al.*, 2012; Alayash, 2011).

Transferrin in particular is a key component of the innate immunity. It is an iron-holding protein that makes extracellular body fluids bacteriostatic due to its ability to bind free iron and thus inhibit the growth of bacteria (Afzali & Goldsmith, 2004; Gomme *et al.*, 2005). However, it has been shown by Freestone *et al.* (2000) and Sandrini *et al.* (2010) that in the presence of catecholamine stress hormones (NE, Adr and Dop), the transferrin becomes a bacterial iron source and can promote bacterial infection induction. Interestingly, as examined in this chapter (Figures 3.16 to 3.19), the catecholamine stress hormones—NE in particular—were detected at variable concentrations in all the PDFs analysed. As a result, it was necessary to investigate the levels of transferrin in the PDFs, as they could be associated with infection risk.

3.2.10 Investigations of the transferrin levels in PDF

As hypothesised, the levels of catecholamine stress hormones and iron-binding proteins (such as transferrin) in the PDF may increase PD patient susceptibility to peritoneal infection by stimulating bacterial growth via iron-providing. Catecholamine stress hormones were present in the PDFs, as investigated earlier in this chapter (Figures 3.16 to 3.19). Therefore, testing for the levels and iron-binding status of the transferrin in the PDFs were of major interest in this study.

In addition to the sequencing results of the PDFs shown in the previous analysis (Section 3.2.9.4), which represented the presence of transferrin in the PDFs (protein number 7 in Figure 3.28 and Table 3.5), the Western blotting technique was also performed using all the PDFs to confirm the presence of transferrin. The PDFs were loaded on 12% SDS-PAGE gels, Western blotted onto PVDF membranes and the blots probed with anti-transferrin antibodies developed in goat (Section 2.2.1.9). The initial PDFs from the nine PD patients at the beginning of their dialysis, were tested for the presence of transferrin. Figure 3.29 confirms the presence of transferrin in the PDFs of the PD patients. In terms of transferrin presence in the PDFs during the later PD period, the PDFs of the follow-up patients were examined. The Western blots shown in Figure 3.30 also confirm the presence of transferrin in all the PDFs of the follow-up patients.

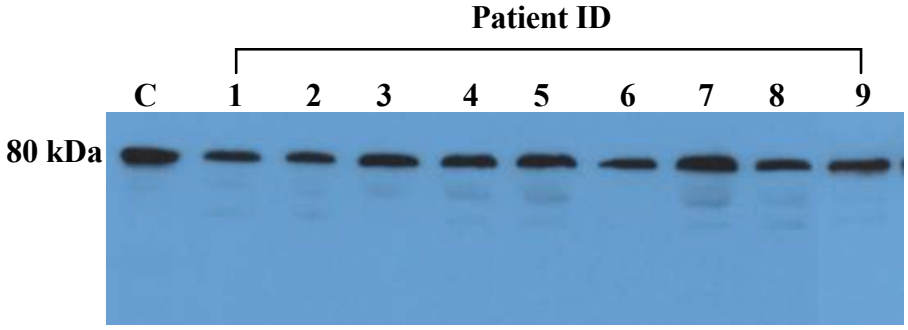


Figure 3.29 Transferrin presence in PDF of nine PD patients at the beginning of their dialysis.
The proteins in PDFs were separated first on a 12% SDS-PAGE gel, then Western blotted onto a PVDF membrane and probed with an anti-transferrin antibody developed in goat (Section 2.2.1.9). Key: C: positive control, which was pure human transferrin (0.5µg); 80 kilodaltons (kDa) refers to the molecular weight of transferrin. For PDF, 15 µl of each dialysate sample was loaded per well.

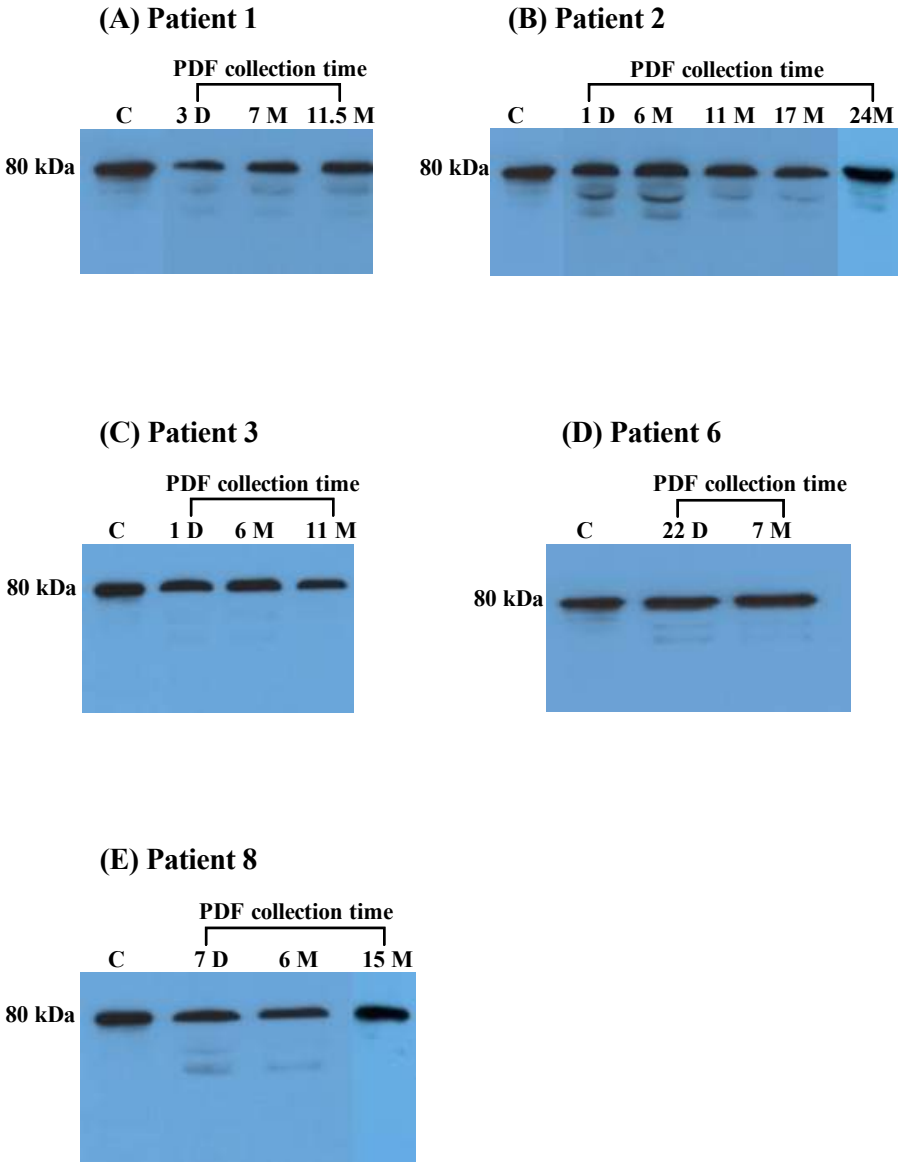


Figure 3.30 Transferrin detection in PDF of follow-up PD patients.
PDFs were collected from Patients 1, 2, 3, 6 and 8 at different points in time, as shown above the blots. The proteins in PDFs were separated first on 12% SDS-PAGE gels, then Western blotted onto PVDF membranes and probed with anti- transferrin antibodies developed in goat (Section 2.2.1.9). Key: C: positive control, which was pure human transferrin (0.5µg); 80 kilodaltons (kDa) refers to the molecular weight of the transferrin; D: refers to days; M: refers to months. For PDF, 15 µl of each dialysate sample was loaded per well.

Accordingly, a clear transferrin presence was shown using both sequencing and immunoblotting analysis. Following that, it was decided to investigate whether PD patients released different levels of transferrin in their dialysis fluids. The transferrin concentration was measured using a commercial human transferrin ELISA kit (Section 2.2.1.10). In the first place, the PDFs, which were picked up from the PD patients at the beginning of their dialysis, were analysed. Figure 3.31 shows variable transferrin levels among the nine PD patients, with the greatest level of transferrin detected in the PDF of Patient 4 and the lowest level of transferrin in the PDF of Patient 1. Hence, the maximum level of transferrin and the highest level of NE and Adr (Figure 3.16) were detected in the PDF of Patient 4. These findings could explain why the PDF of Patient 4 was by far the most growth stimulating medium for *S.aureus* and *S.epidermidis* (Figure 3.2).

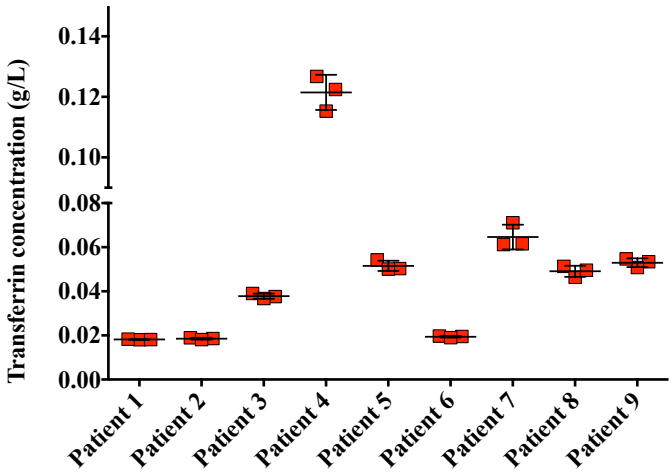


Figure 3.31 Transferrin concentration in PDF of nine PD patients at the beginning of their dialysis. The concentration of transferrin was determined using the human Tf ELISA kit (Section 2.2.1.10).

The transferrin concentrations were also measured in the PDF of follow-up PD patients to determine if their transferrin levels changed during their dialysis time. On the whole, relative to transferrin levels in initial PDF samples the transferrin levels in the follow-up PDFs of each patient changed over the course of PD therapy time (Figure 3.32). The data for Patients 1, 6 and 8 clearly showed the greatest release of transferrin into their PDFs at the beginning of dialysis (Figure 3.32 A, D and E). Interestingly, the PDFs of Patients 1 and 8 were the most supportive media for *S.aureus* growth, whereas the PDF of Patient 6, which showed the highest level of transferrin, was the most optimal growth medium for *S.aureus* and *S.epidermidis* (Figures 3.3, 3.6 and 3.7). Figure 3.32 B demonstrates that there was a fluctuation in transferrin levels in the PDF of Patient 2 during the PD therapy time. The level of transferrin reached its highest at nearly the end of the PD therapy (after 24 months). Regarding Patient 3, it can be seen in Figure 3.32 C that the PDF obtained at six months had the highest level of transferrin, as well as the maximum supportiveness for *S.aureus* and *S.epidermidis* growth, when compared with the first and last PDFs collected (Figure 3.5). Overall, the concentrations of transferrin varied in the PDF of all the follow-up PD patients. Apart from the PDFs of Patient 2, there was a direct association between the level of transferrin and the growth stimulation of bacteria.

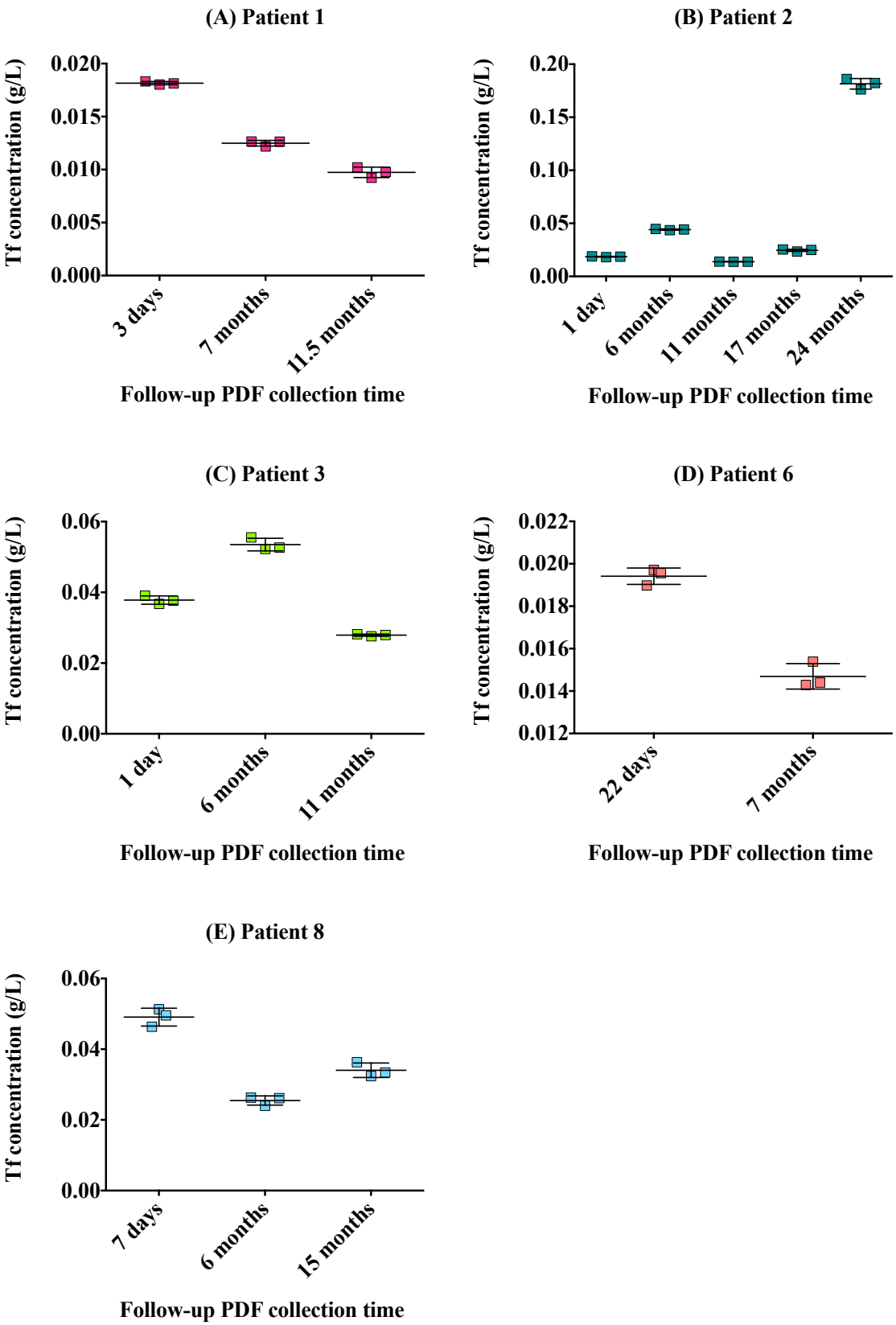


Figure 3.32 Transferrin concentration in PDF at various times during peritoneal dialysis.
The concentration of transferrin (Tf) was determined using the human transferrin ELISA kit (Section 2.2.1.10).

3.2.11 Investigation of mechanism by how transferrin induced staphylococcal growth in PDF

So far, the present study had investigated the hormonal and protein factors within the PDF that might participate in *S.aureus* and *S.epidermidis* growth induction. Collectively, the PDFs were alkaline, which is optimal for bacterial growth (Todar, 2006) (Figure 3.8). Glucose and iron were available in the PDFs; these are essential nutritional factors for staphylococcal survival (Austin *et al.*, 2012; Hammer & Skaar, 2012) (Figures 3.9 and 3.12). The catecholamine stress hormones, particularly NE, were detected in the PDFs (Figure 3.16), as was also transferrin (Figure 3.31), which can act as a bacterial iron source. The conclusion in the previous text (Section 3.2.10) stated that in most PDFs, a linear association was established between high level of transferrin and growth stimulation of staphylococci. Furthermore, the PDF of Patients 3, 4 and 8 suggested a direct association between the level of transferrin, the concentration of NE and the stimulation of staphylococcal growth in the PDF. However, this raised the following question: what was the iron-binding status of transferrin within PDF?

3.2.11.1 Was transferrin in PDF protective or non-protective?

Transferrin is a key innate immune component because it makes extracellular body fluids bacteriostatic due to its ability to bind free iron (Fe^{+2}); because the iron is a key nutritional element for bacterial growth, this activity of transferrin inhibits the growth of bacteria (Afzali & Goldsmith, 2004; Gomme *et al.*, 2005). Normally, serum transferrin is partially saturated with ferric iron (Fe^{+3}) to maintain the level of Fe^{+2} at ~10^{-18} M, which is too low to enable microbial growth; thus, the transferrin is protective against development of infection (Gomme *et al.*, 2005; Modun *et al.*, 1994; Parkkinen *et al.*, 2002). Therefore, it was necessary to investigate whether the transferrin present within the PDF was partially iron saturated (protective) or iron saturated (non-protective). Transferrin can exist in four isoforms depending on the number of Fe^{+3} atoms bound to it (Von Bonsdorff *et al.*, 2002). These isoforms are differic transferrin isoform (transferrin molecule with two Fe^{+3} atoms; known as saturated transferrin or holo-transferrin), *C*-terminal monoferric isoform (transferrin with one Fe^{+3} atom in the *C*-lobe), *N*-terminal monoferric isoform (transferrin with a single Fe^{+3} atom in the *N*-lobe) and the apo-transferrin isoform (transferrin molecule without Fe^{+3} atoms; also known as iron-free transferrin) (Von Bonsdorff *et al.*, 2002). The iron-binding status of the transferrin in PDFs was examined by using urea

polyacrylamide gels. Urea is a denaturant substance that allows unfolding of the transferrin molecule into four isoforms depending on the transferrin electrophoretic mobility (Williams & Moreton, 1980). These are the apo-transferrin, which is the least mobile isoform; the *C*-terminal monoferric isoform; the *N*-terminal monoferric isoform and the differic transferrin, which is the most mobile isoform (Williams & Moreton, 1980). The PDFs of the nine PD patients enrolled to the current study were examined for their transferrin iron binding status by electrophoresis in 6% urea polyacrylamide gels (Section 2.2.2.1). In comparison to the investigation of the transferrin iron binding status in the PDFs, a serum sample from one of the PD patients (Patient 6) was also analysed. However, the transferrin bands of the PDFs and of the serum sample could not be clearly visualised after urea gel electrophoresis; the results obtained were like smear proteins (data not shown). Nevertheless, use of a novel combination of urea gel electrophoresis and Western immunoblotting was able to clarify the results. Surprisingly, a significant presence of differic transferrin (iron-saturated transferrin) was observed in all of the PDFs of the nine PD patients (Figure 3.33). In contrast to the detection of the iron-saturated transferrin in the PDFs, the serum sample, as expected, demonstrated the presence of the apo-transferrin and monoferric transferrin isoforms and no iron-saturated transferrin was found. These findings mean that the transferrin, which normally works as a major innate immune defence, inhibiting microbial growth by limiting iron accessibility in blood, is actually iron-saturated in PDF. As a result, a microbe that may be present in the PDF, is present within a non-bacteriostatic medium. Indeed, the transferrin in the PDFs is no longer protective.

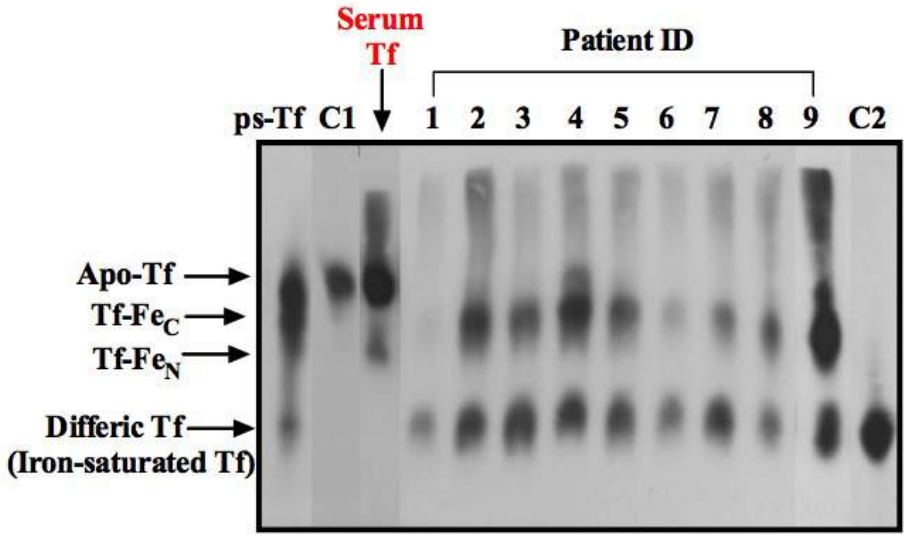


Figure 3.33 Transferrin (Tf) iron-status in PDF of nine PD patients.
Key: ps-Tf: partially iron-saturated human Tf consisting of apo-Tf (iron-free Tf), Tf-Fe_c (Tf with single Fe^{+3} atom in the *C*-lobe), Tf-Fe_N (Tf with one Fe^{+3} atom in the *N*-lobe) and differic Tf (iron-saturated Tf); C1: control 1 (pure human apo-Tf); C2: control 2 (pure human differic Tf). The ps-Tf, apo-Tf and differic Tf were used at 1 µg/µl; they were purchased from Sigma. 10 µl of each PDF was mixed with 10 µl of 2x urea gel protein sample loading buffer, and then a total 20 µl was loaded. On the other hand, 2 µl of serum sample was mixed 8 µl of 2x urea gel protein sample loading buffer, and then the net 10 µl of the mixture was loaded. The serum sample was kindly provided by Patient 6. The controls, PDFs and serum sample were initially loaded onto a urea gel. Next, the urea gel was Western blotted onto a PVDF membrane and then probed with an anti-Tf antibody developed in goat (Section 2.2.2.1).

3.2.11.2 Where did the transferrin in PDF acquire its iron?

As shown in Figure 3.11, glucose-based PDS contained iron (Gyurcsik & Nagy, 2000). With the exception of the 1.5% glucose PDS, increasing the glucose level noticeably increased the iron availability; the 3.86% glucose PDS had the highest iron concentration, at 60.74 ± 4.24 µM. To examine the ability of the human apo-transferrin to acquire iron from the PDS, a method described by Sandrini *et al.* (2010) was utilised (Section 2.2.2.2). Basically, 0.5 µg/µl of the human apo-transferrin was inserted into a dialysis membrane placed in 25 ml of 1.36% glucose PDS. After overnight incubation at 4°C, the transferrin from within the dialysis membrane was extracted and loaded onto a urea polyacrylamide gel. Figure 3.34 shows that some of the human apo-transferrin, which is iron-free, picked up iron from the PDS and became a *C*-terminal monoferric isoform (transferrin with a single Fe^{+3} atom in the *C*-lobe). This shows that the transferrin released into the PDF could pick up iron from the PDS and so become more iron-saturated.

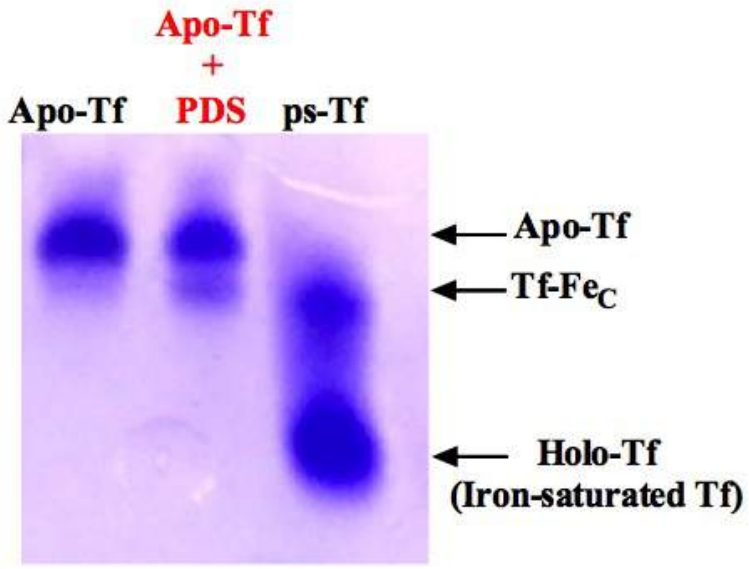


Figure 3.34 Urea polyacrylamide gel shows the effect of PDS on iron-binding capacity of human apo-transferrin (Tf).
Key: Apo-Tf: pure human apo-Tf (iron-free Tf), used at 1 µg/µl; PDS: 1.36% glucose PDS; ps-Tf: partially iron-saturated human Tf consisting of Tf-Fe$_c$ (Tf with a single Fe^{+3} atom in the *C*-lobe) and differic Tf (iron-saturated Tf), used at 1 µg/µl. The apo-Tf (0.5 µg/µl) was inserted into a dialysis membrane placed in 25 ml of 1.36% glucose PDS. Following incubation overnight at 4°C, the apo-Tf was extracted and loaded onto the urea gel. After urea gel electrophoresis, the gel was stained using Coomassie Brilliant Blue urea gel staining solution (Section 2.2.2.2).

3.2.11.3 Could PD pathogens access PDF transferrin iron?

Bacteria transferrin iron uptake analyses in PDS and PDF were performed following a methodology described by Freestone *et al.* (2000). Normalised cultures of *S.aureus* and *S.epidermidis* (OD 2 at 600 nm) were prepared as described in Section 2.2.2.3 and mixed with 4 ml of 2.27% glucose PDS or patient PDF chosen because of their differences in catecholamine levels. These include PDFs containing high NE levels, intermediate in terms of concentrations and low NE levels. This range was chosen because it has been shown (Freestone *et al.*, 2000; Freestone *et al.*, 2003) that NE and other catecholamine hormones can provide iron for bacteria in a medium containing transferrin. Table 3.6 gives information about the PDFs that were utilised, along with their NE levels. For the transferrin iron uptake experiments, 1 µg/ml of ^{55}Fe-transferrin (1 x 10^5 cpm) was added to PDS or PDF containing *S.aureus* or *S.epidermidis*. The levels of iron uptake by staphylococci were measured over the course of 4 hours of incubation using scintillation counting (Section 2.2.2.3).

Table 3.6 NE concentration in PDFs that were used for ^{55}Fe-transferrin iron uptake analysis.

Identity of PDF used	NE level (ng/ml) ± SD
PDF of Patient 4 after 3 days on PD	0.518 ± 0.153
PDF of Patient 6 after 7 months on PD	0.332 ± 0.001
PDF of Patient 1 after 11.5 months on PD	0.170 ± 0.013

The results for uptake of transferrin iron showed a different pattern for the different staphylococci (Figure 3.35). For *S.aureus*, which is a more aggressive pathogen than *S.epidermidis* and contains a greater array of host iron uptake systems (Skaar & Schneewind, 2004) the bacteria were able to acquire substantially more iron (in the form of ^{55}Fe) from the ^{55}Fe-transferrin. In only the PDS, which is the solution that goes into the peritoneal cavity, for both species, uptake of iron was much greater than in any of the PDFs. This suggests that there is/are factor(s) present within the PDF that can interfere with bacterial uptake of iron, and it would be interesting in future studies to characterise this/these factor(s). Interestingly, for *S.aureus*, the catecholamine content of the patient PDF influenced iron acquisition from transferrin, and there was a clear direct association between NE concentration and the level of transferrin iron the bacteria were able to assimilate. Patient 4 had the highest catecholamine levels (Figure 3.16), and Figure 3.2 shows this patient's PDF supported the highest staphylococcal growth compared to the other PDFs. Hence, the catecholamine level could indeed influence bacterial acquisition of transferrin iron in the PDFs and so increase bacterial growth.

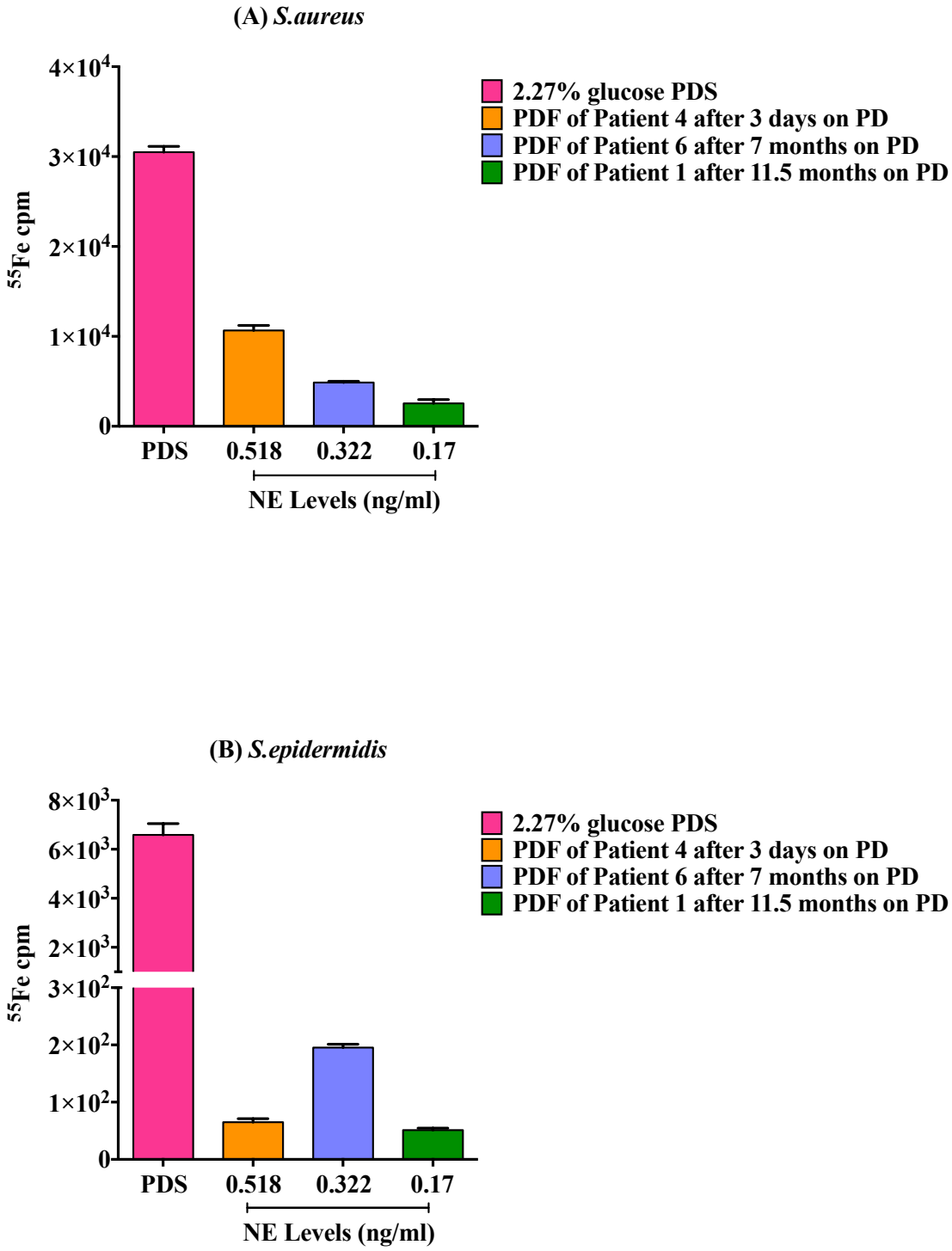


Figure 3.35 Uptake of ^{55}Fe from ^{55}Fe-labelled transferrin in PDS or PDF.
The histograms show uptake of iron (in the form of ^{55}Fe from ^{55}Fe-labelled transferrin) by *S.aureus* and *S.epidermidis* incubated in patient's PDF or PDS (Section 2.2.2.3); n=3. The identity of PDFs analysed is indicated in the bar charts along with NE levels.

3.2.12 Investigations into the abilities of iron chelators apo-transferrin and deferoxamine to inhibit staphylococcal growth in PDF

In this study, iron accessibility was shown to be a key factor in explaining the ability of staphylococci to grow in PDF. Apart from the iron-saturated transferrin (Figure 3.33), there was also non-transferrin-bound iron, commonly known as free iron or Fe^{+2}, detected in the PDFs (Figures 3.14 and 3.15). In other words, the simultaneous presence of iron-saturated transferrin and free iron in the PDFs could influence the degree of bacteriostatic nature of the PDFs. Furthermore, an association was established between the NE level of the PDF and transferrin-iron uptake of *S.aureus* (Figure 3.35). Therefore, it was appropriate to consider the use of an iron chelator, which could bind free iron in the PDF and restore bacteriostatic conditions there.

Two iron chelator agents were utilised in this study to examine their ability to inhibit staphylococcal growth in the PDF. First, human apo-transferrin (iron-free transferrin) was used at 0.1 and 0.3 mg/ml, as described by Von Bonsdorff *et al.* (2003). An additional intermediate human apo-transferrin concentration was used at 0.2 mg/ml. Secondly, the chemical chelator deferoxamine mesylate was also examined using a range of clinical infusion doses at 0.05, 0.1, 0.15 and 0.2 mg/ml recommended by Cohen *et al.*, (1990). Clinically, deferoxamine is used to eliminate excess iron from the body in patients with chronic iron overload due to blood transfusion associated anaemia (Miller & Morgan, 2014). Examinations of the effects of deferoxamine and apo-transferrin on bacterial growth were performed using a range of PDFs, which had differences in total iron concentration and NE levels (Table 3.7). *S.aureus* and *S.epidermidis* growth in the PDFs was assessed following the addition of deferoxamine or apo-transferrin, or without any such additions (as controls). The growth assays were performed as described in Section 2.2.2.4.

Table 3.7 The PDFs used in apo-transferrin and deferoxamine studies, along with their total iron and NE levels.

The PDF used	Total Fe level (µM)	NE level (ng/ml)
PDF of Patient 1 after 7 months on PD	53	3.62
PDF of Patient 1 after 11.5 months on PD	56	0.17
PDF of Patient 2 after 17 months on PD	75	1.7
PDF of Patient 3 after 1 day on PD	68	0.21
PDF of Patient 6 after 7 months on PD	47	0.33

Generally, *S.epidermidis* growth in the PDFs responded to apo-transferrin more than *S.aureus* growth. First, the addition of apo-transferrin at concentrations of 0.1, 0.2 and 0.3 mg/ml, respectively, to the PDFs with total iron levels of 56 μM and 75 μM increased *S.aureus* growth (Figure 3.36 B and C). However, the use of 0.1 and 0.3 mg/ml of apo-transferrin into the PDF containing 68 μM total iron (Figure 3.36 D), decreased *S.aureus* growth. Additionally, apo-transferrin had no impact on *S.aureus* growth in the PDFs with the lowest total iron levels, at 53 μM and 47 μM (Figure 3.36 A and E). *S.epidermidis* growth generally declined when apo-transferrin was added (Figure 3.37). The PDF with an iron level of 68 μM, for example, was responsive to apo-transferrin in terms of inhibiting *S.epidermidis* growth (Figure 3.37 D). Interestingly, multiplication of *S.epidermidis* in the PDF with the highest total iron level (75 μM) decreased when apo-transferrin was used at 0.2 mg/ml, but not at 0.1 mg/ml or 0.3 mg/ml (Figure 3.37 C). Moreover, the PDFs with the lowest total iron levels produced inhibition effects of apo-transferrin on *S.epidermidis* growth added at any concentration (Figure 3.37 A and E). Lastly, the administration of apo-transferrin at 0.1 and 0.3 mg/ml caused a decline in *S.epidermidis* growth in the PDF with 56 μM total iron level (Figure 3.37 B). The PDFs shown in Table 3.7 contained variable concentrations of NE. Thus, it was interesting to investigate whether NE concentrations had an impact on the ability of apo-transferrin to inhibit staphylococcal growth in the PDFs. Generally speaking, it was found that apo-transferrin additions to PDFs inhibited *S.epidermidis* growth and that there was no direct association between the NE level of the PDF and the effect of apo-transferrin on *S.epidermidis*.

In conclusion, despite differences in the total iron and NE levels in the PDFs, the incorporation of apo-transferrin did inhibit the growth of *S.epidermidis*, and on rare occasions that of *S.aureus*. Furthermore, the concentration of apo-transferrin injected influenced the behaviour of staphylococcal growth in the PDFs and total iron and NE levels could indeed influence apo-transferrin's ability to restore the bacteriostatic characteristics of PDFs. Potentially, this iron availability concept might be used to reduce the growth of bacteria, or perhaps even in conjunction with the injection with antibiotics to treat infectious peritonitis.

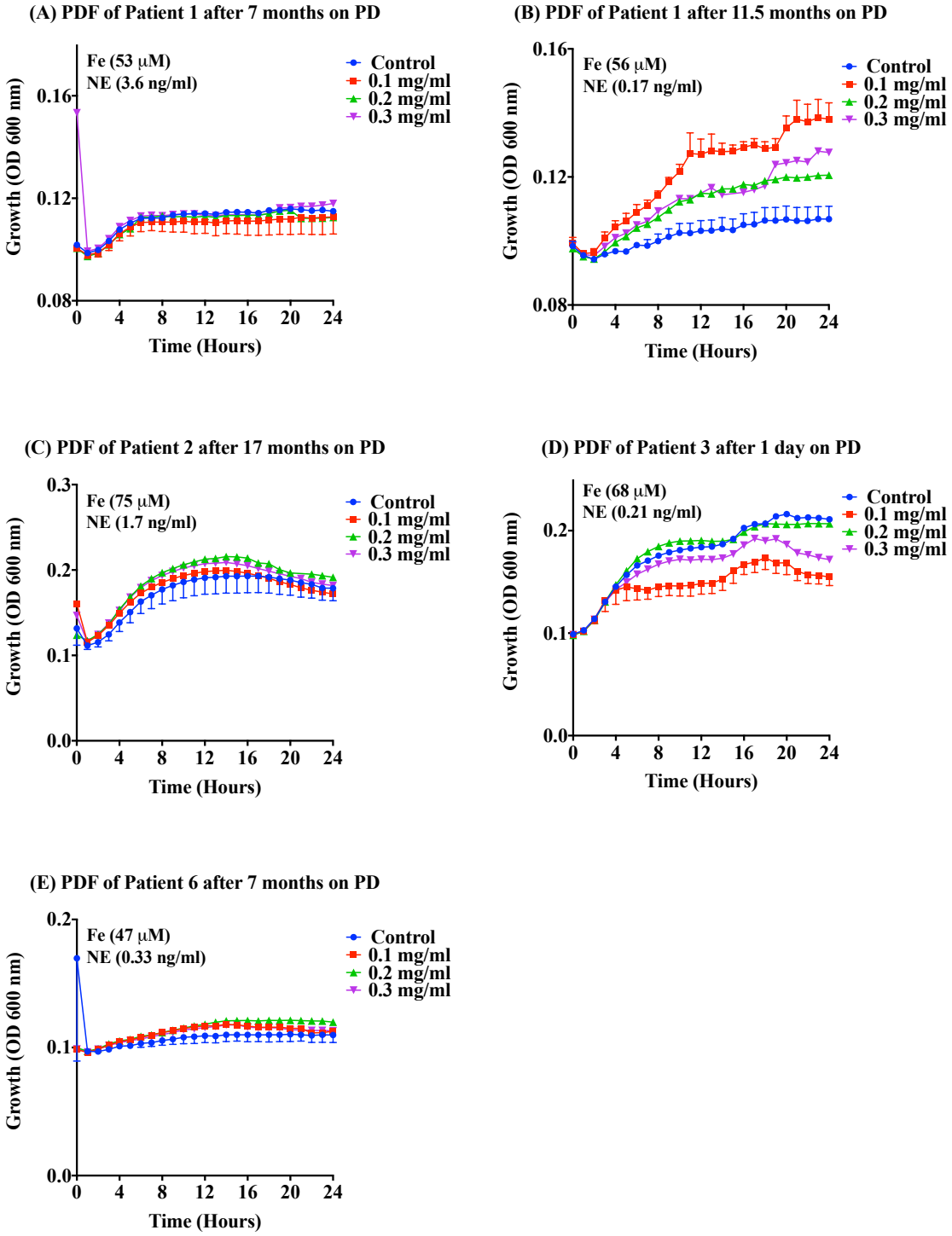


Figure 3.36 Effect of apo-transferrin on *S.aureus* growth in PDF.
Human apo-transferrin was added to each PDF at 0.1, 0.2 and 0.3 mg/ml. 'Control' refers to *S.aureus* growth in PDF without apo-transferrin addition. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.2.4); n=3.

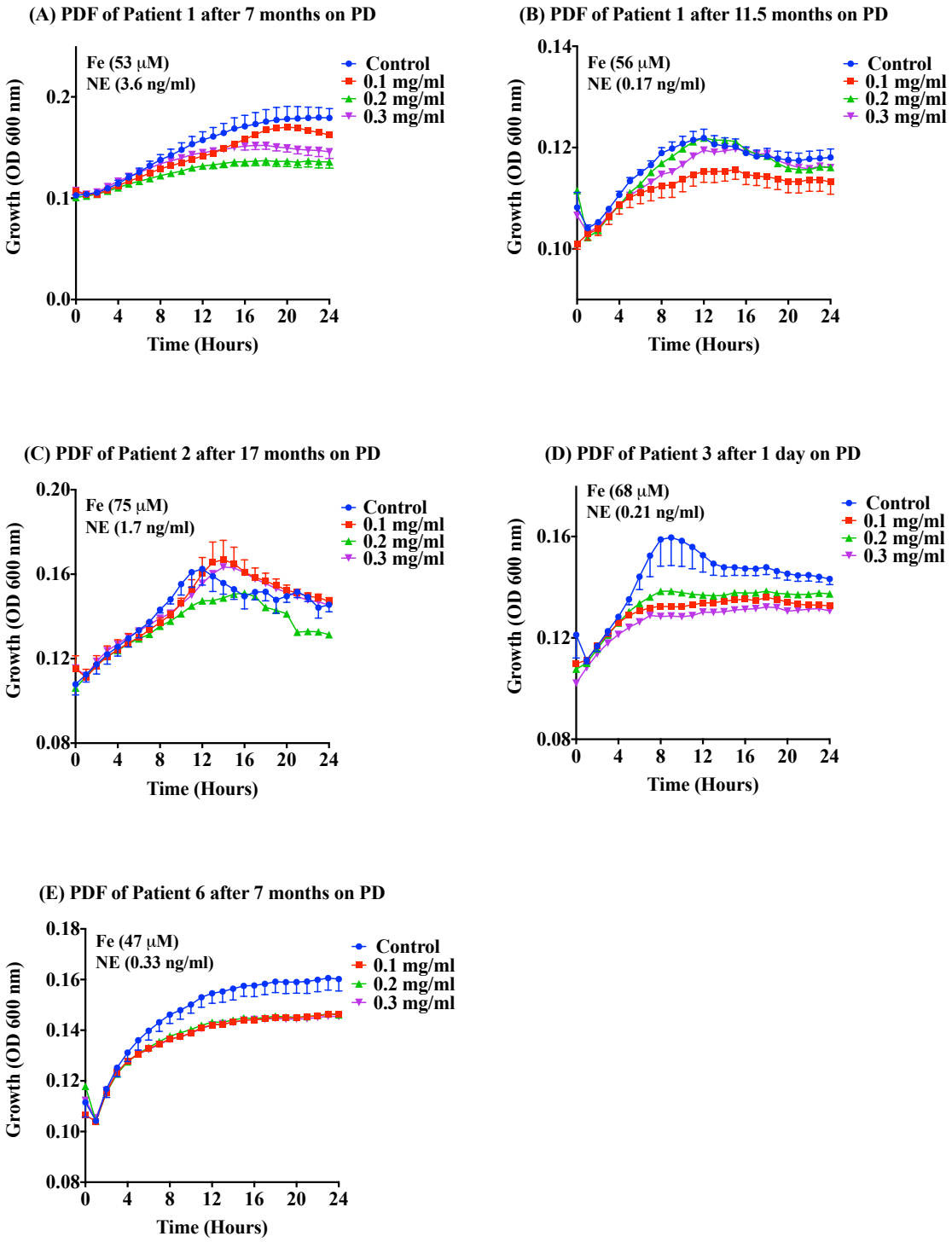


Figure 3.37 Effect of apo-transferrin on *S.epidermidis* growth in PDF.
Human apo- transferrin was added to each PDF at 0.1, 0.2 and 0.3 mg/ml. 'Control' refers to *S.epidermidis* growth in PDF without apo-transferrin addition. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.2.4); n=3.

The same strategy used to investigate the effect of the apo-transferrin on staphylococcal growth in the PDF was used to observe the influence of the chemical chelator deferoxamine. A range of PDFs with a variety of total iron and NE levels were analysed in this portion of the study (Table 3.7). It was shown in Figures 3.36 and 3.37 that apo-transferrin had more of an impact on *S.epidermidis* growth in the PDFs than it did on *S.aureus* growth. However, in the case of deferoxamine, different results were obtained. Deferoxamine was seen to occasionally inhibit the growth of *S.aureus*, but not that of *S.epidermidis*. In particular, additions of deferoxamine at concentrations of 0.05, 0.1, 0.15 and 0.2 mg/ml, respectively, impaired *S.aureus* growth, but not that as *S.epidermidis*, in PDFs that contained the lowest total iron levels (53 μM and 47 μM) (Figure 3.38 A & E and Figure 3.39 A & E). The growth of both *S.aureus* and *S.epidermidis* in the PDF with an iron level of 56 μM (Figure 3.38 B and Figure 3.39 B) showed no response to the deferoxamine, whereas at a higher total iron level (68 μM), the deferoxamine did stimulate *S.aureus* and *S.epidermidis* growth in PDF (Figure 3.38 D and Figure 3.39 D). Furthermore, the administration of deferoxamine into the PDF with the greatest total iron level (75 μM) showed no clear impact on *S.aureus* growth, although it did stimulate *S.epidermidis* growth (Figure 3.38 C and Figure 3.39 C).

The inhibitory effect of deferoxamine appeared to relate more to the growth of *S.aureus*, but not to that of *S.epidermidis*. Also, there was no association between deferoxamine's influence on *S.aureus* growth and catecholamine levels in the PDFs. Thus, it is evident that deferoxamine is able to mediate the iron uptake of staphylococci in PDF and is not suitable as a PDF bacterial growth inhibitor.

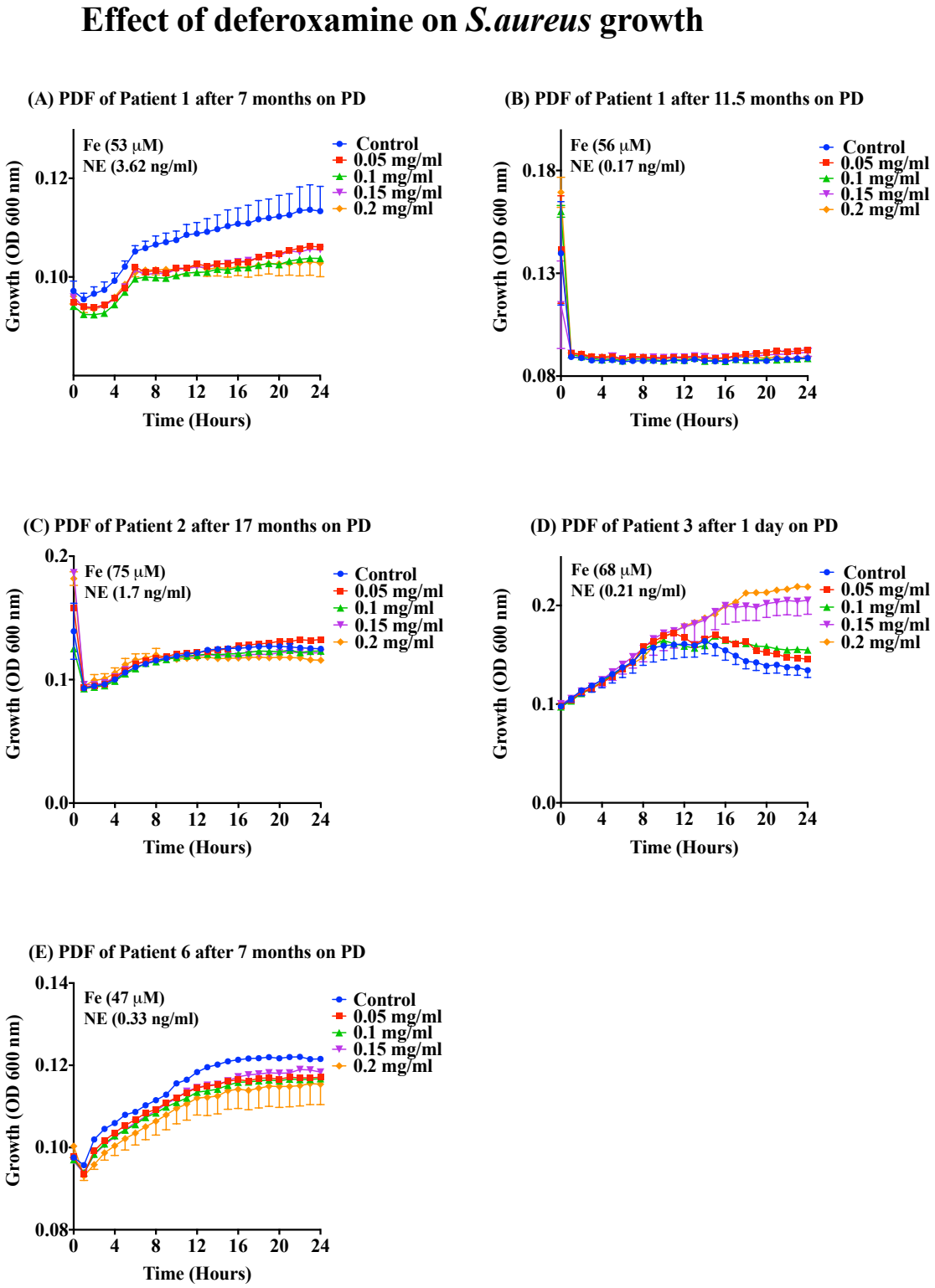


Figure 3.38 Effect of deferoxamine on *S.aureus* growth in PDF.
Deferoxamine was added to each PDF at 0.05, 0.1, 0.15 and 0.2 mg/ml. 'Control' refers to *S.aureus* growth in PDF without deferoxamine addition. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.2.4); n=3.

Effect of deferoxamine on *S.epidermidis* growth

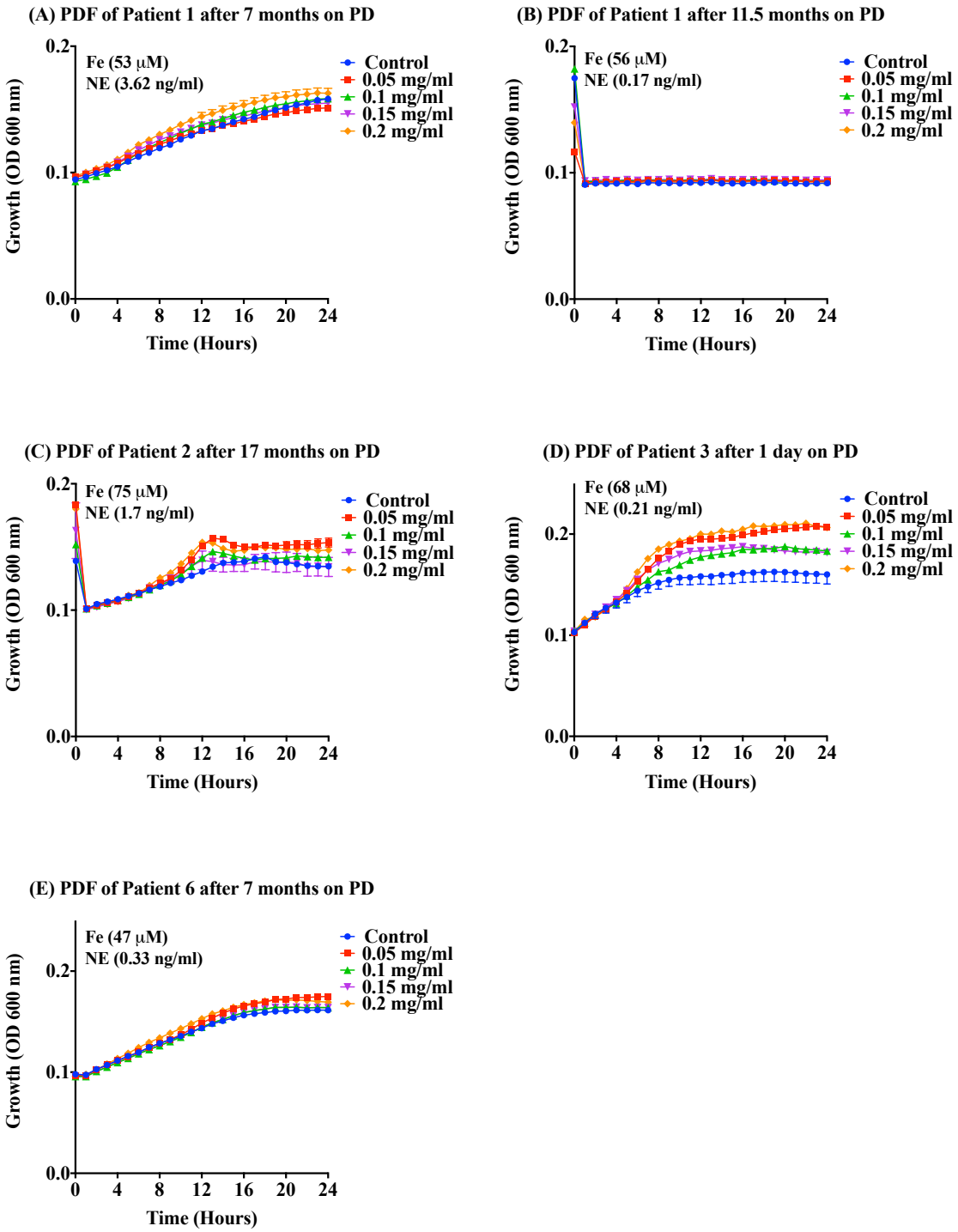


Figure 3.39 Effect of deferoxamine on *S.epidermidis* growth in PDF.
Deferoxamine was added to each PDF at 0.05, 0.1, 0.15 and 0.2 mg/ml. 'Control' refers to *S.epidermidis* growth in PDF without deferoxamine addition. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.2.4); n=3.

3.2.13 The association between PDF biochemical factor levels and staphylococcal growth

It was shown in the current chapter that there were differences in the growth patterns of staphylococci between different PDF samples obtained at the beginning of dialysis from nine PD patients. Also, it was observed that the follow-up PDF samples from the same patient had dissimilar effects on staphylococcal growth. The biochemical parameters measured in this chapter were pH, glucose, levels of iron (Fe^{+2} + Fe^{+3}), catecholamine stress hormones (NE, Adr and Dop), protein and transferrin between PDF sampling. All were found to be capable of participating in stimulating staphylococcal growth in PDF, and possibly also increasing patient susceptibility to infection by making the dialysate more supportive to the bacteria. Statistical analysis was therefore performed to determine if a significant association existed between staphylococcal growth and the studied biochemical parameters in PDF. Univariable linear regression analysis was performed to explore the relationship between *S.aureus* or *S.epidermidis* growth (as a dependent variable) and one independent variable at a time (i.e. pH, glucose, iron, catecholamine stress hormones, protein or transferrin levels).

For both bacteria, neither the pH nor the glucose level had a significant association with staphylococcal growth in the PDF ($P > 0.05$) (Figures 3.40 and 3.41). Thus, they did not seem to have an effect on staphylococcal growth in PDF. The area under the curve (AUC) of *S.epidermidis* growth was not associated with the total iron and protein levels in all the PDF samples (initial and follow-up) ($P > 0.05$) (Figures 3.42 and 3.44). By analysing only the initial PDF samples (obtained at the start of PD), the association was also shown to be insignificant between the AUC of *S.aureus* growth and the total iron and protein levels ($P > 0.05$). However, those two variables (total iron and protein) became more important with the amount of time spent on dialysis and were significantly associated with *S.aureus* growth in the PDF, which could predict the AUC of *S.aureus* growth ($P < 0.05$). The catecholamine stress hormones levels seemed to have no significant influence on *S.epidermidis* growth across all the PDF samples ($P > 0.05$). In contrast, their levels were significantly associated with *S.aureus* growth in the initial PDF samples only ($P < 0.05$) (Figure 3.43). The effect of catecholamine stress hormones levels on *S.aureus* growth was less over time of sampling, which suggests that there were patient factors released into the PDF that interfered with the effect of the catecholamine stress hormones. Interestingly, the transferrin level results were consistent for both bacteria when the initial

PDF samples were analysed, and for both a significant association was found between the growth of *S.aureus* or *S.epidermidis* and the transferrin levels in the PDF samples ($P < 0.05$) (Figure 3.45). Thus, the higher the level of transferrin, the greater the staphylococcal growth in the PDF. *S.aureus*, but not *S.epidermidis* growth was also associated positively and significantly with transferrin levels over time ($P < 0.05$). So, the transferrin level was directly associated with *S.aureus* growth, and its effect was maintained over time.

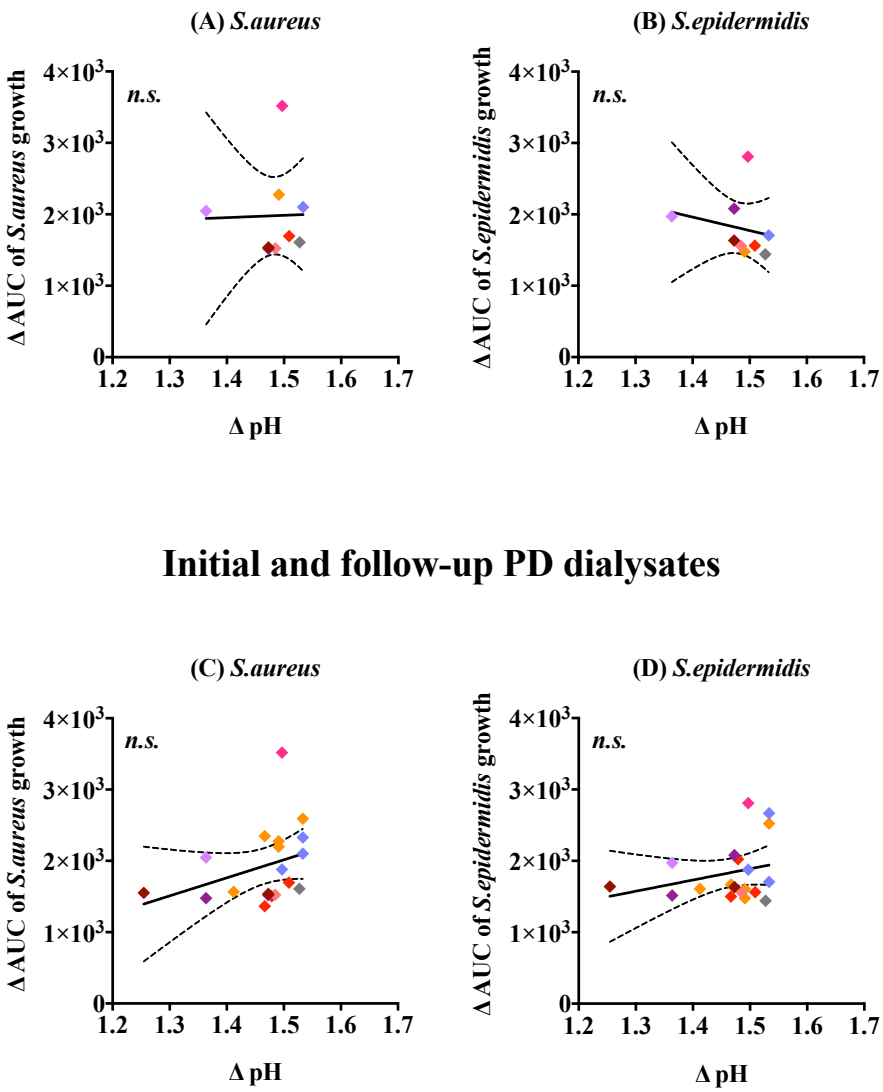


Figure 3.40 Linear regression analysis of the association between pH levels and *S.aureus* or *S.epidermidis* growth in PD dialysates.
Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of each variable (Δ pH or Δ AUC) was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Each patient was assigned a different colour, so different colours refer to different patients.

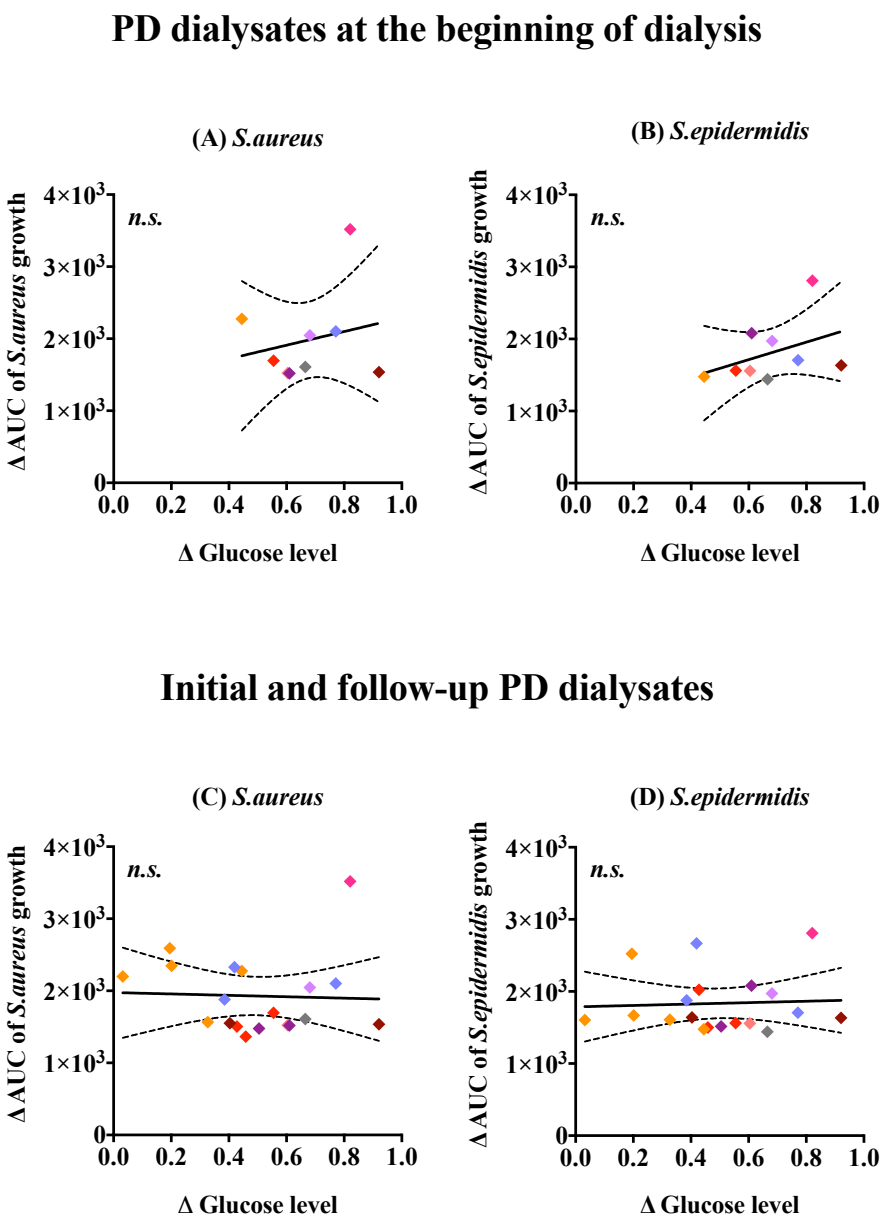


Figure 3.41 Linear regression analysis of the association between glucose levels and *S.aureus* or *S.epidermidis* growth in PD dialysates.

Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of each variable (Δ glucose or Δ AUC) was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Each patient was assigned a different colour, so different colours refer to different patients.

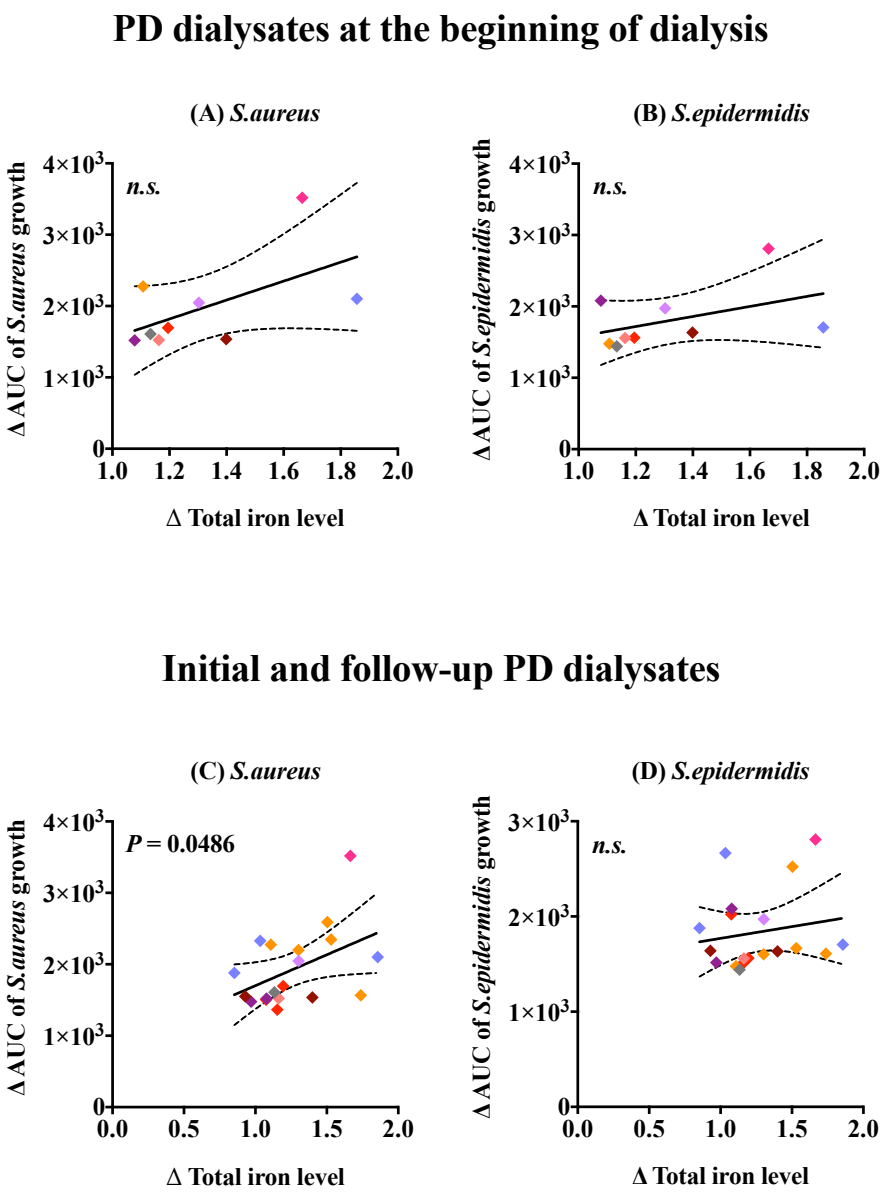


Figure 3.42 Linear regression analysis of the association between total iron (Fe^{+2} + Fe^{+3}) levels and *S.aureus* or *S.epidermidis* growth in PD dialysates.
Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of each variable (Δ total iron or Δ AUC) was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Each patient was assigned a different colour, so different colours refer to different patients.

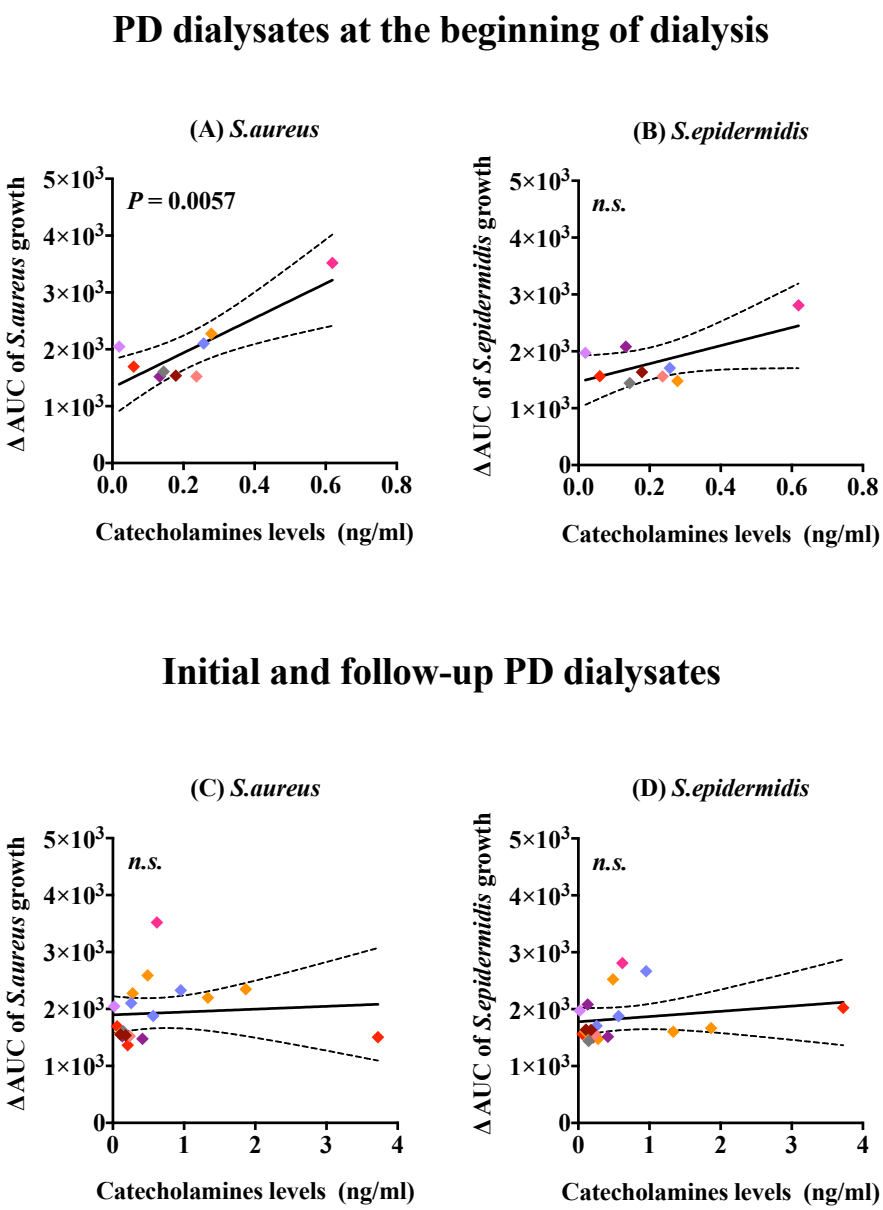


Figure 3.43 Linear regression analysis of the association between catecholamine stress hormones levels (NE + Adr + Dop) and *S.aureus* or *S.epidermidis* growth in PD dialysates.
The sum of NE, Adr and Dop was calculated for each PDF sample, then used in the analysis. Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of Δ AUC was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Each patient was assigned a different colour, so different colours refer to different patients.

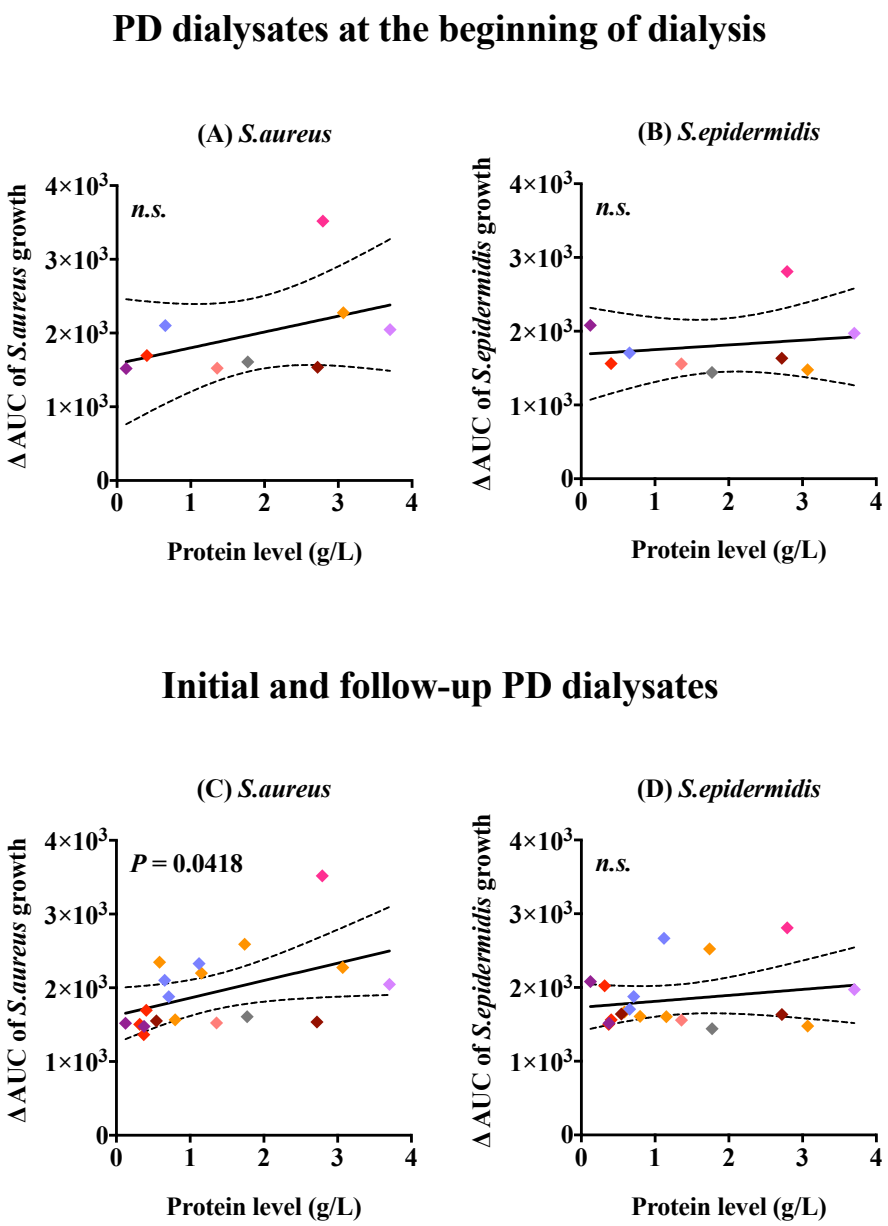


Figure 3.44 Linear regression analysis of the association between protein levels and *S.aureus* or *S.epidermidis* growth in PD dialysates.

Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of Δ AUC was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Each patient was assigned a different colour, so different colours refer to different patients.

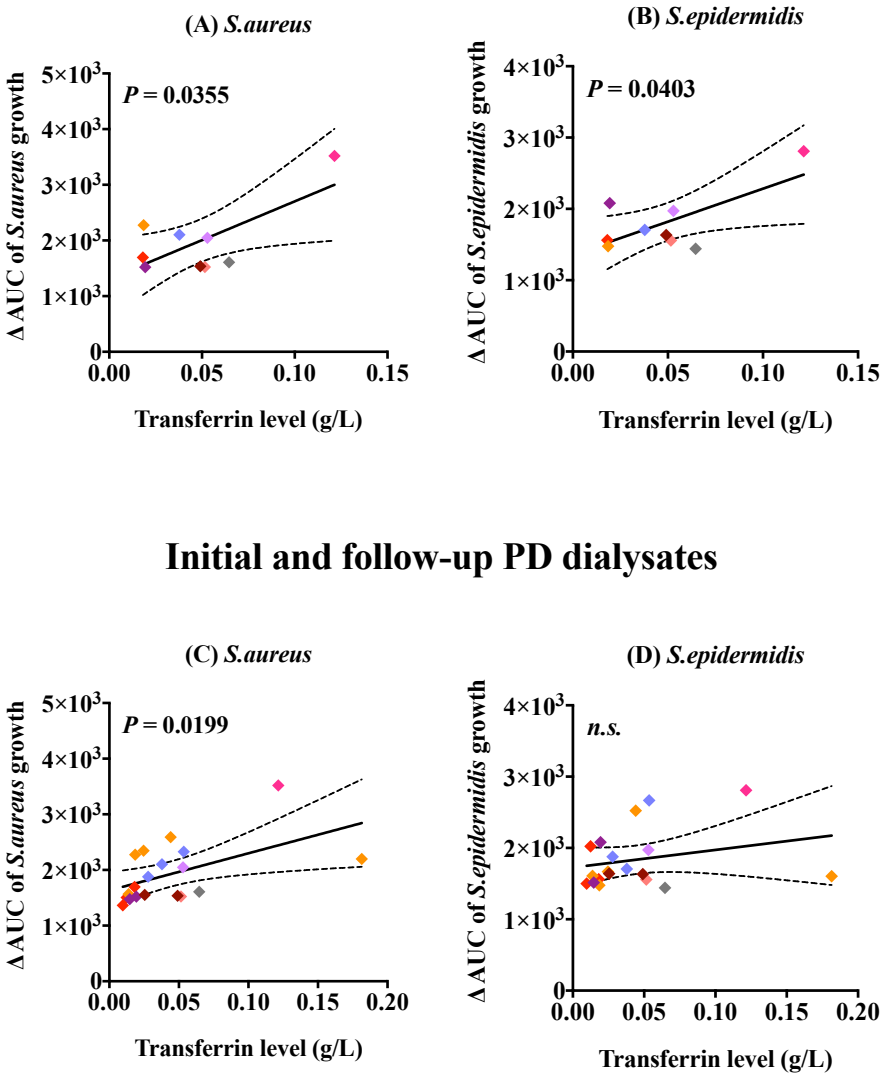


Figure 3.45 Linear regression analysis of the association between transferrin levels and *S.aureus* or *S.epidermidis* growth in PD dialysates.

Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of Δ AUC was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Each patient was assigned a different colour, so different colours refer to different patients.

3.3 Discussion

Peritonitis continues to be a significant complication of PD despite improvements and innovations in patient education, in the PD-connecting system, in the dialysis procedures and in prophylaxis (Davenport, 2009; Sweny *et al.*, 2003). Repeated peritonitis typically results in peritoneal membrane failure and necessitates PD termination and a switch to haemodialysis (Ellam & Wilkie, 2011; Li, 2001). Therefore, this study aimed to identify risk factors present within PDF that might be responsible for development of infectious peritonitis in PD patients. Comprehensive PDF protein and metabolite characterisation studies were performed on PDF from a range of PD patients to further understanding of the host-related components in PDF which may play a role in stimulating bacterial growth and virulence, and to explain any dissimilarities between patient infection rates.

The loss of protein into PDF has been found to be one of the obvious limitations of PD (Blumenkrantz *et al.*, 1981). This agrees well with the results observed in the current study. PD patients lose substantial amounts of protein into their PD dialysates. Inter- and intra-patient variations in the protein concentrations in the PDF were observed in the present study. The reasons for the differences in the quantity of protein loss into PDF have been highlighted (Dulaney & Hatch, 1984). Firstly, the amount of protein loss depends on the frequency and duration of PD (Giangrande *et al.*, 1980; Katirtzoglou *et al.*, 1980). It also depends on the components used in the commercial PDS and the permeability characteristics of the peritoneum (Twardowski *et al.*, 1981). The protein concentration in the serum and the patient's clinical status are other variables that can influence the amount of protein lost into PDF (Blumenkrantz *et al.*, 1981b; Katirtzoglou *et al.*, 1980). It was noticed that, at the beginning of dialysis, the APD patient (Patient 6) had the lowest protein concentration in her PDF (0.13 g/L) in comparison to the other patients who used CAPD (average 2.06 g/L). This result disagreed with the results reported by other investigators (Tjiong *et al.*, 2007; Blumenkrantz *et al.*, 1981; Dulaney & Hatch, 1984), as they found no differences in the protein amount in the PDF of APD patients versus CAPD patients. In a study conducted by Westra *et al.* (2007), the amount of protein lost in PDF during night-time cycling in eight APD patients was 5.6 ± 0.5 g/exchange, which was higher than the amount of protein determined in the PDF (obtained at the beginning of dialysis) of the APD patient (Patient 6) (1.5 g/exchange). On the other hand, the mean protein quantity in the PDF of the CAPD patients collected at the start of their dialysis was 2.06 ± 1.2 g/L. Most of the available studies reported that

the amount of protein lost in the PDF of CAPD patients ranged between 5 g/day and 15 g/day (Dulaney & Hatch, 1984; Blumenkrantz *et al.*, 1981; Popovich *et al.*, 1978; Giangrande *et al.*, 1980; Katirtzoglou *et al.*, 1980). However, in this present study, the number of exchanges that CAPD patients did per day was unavailable, which make it impossible to accurately predict the amount of protein lost per day; thus, it was difficult to compare this present study's findings with the results reported in other studies.

Proteomic profiling showed that the PD patients generally had the same protein profile in their PD dialysates as seen by 8% and 12% SDS-PAGE gels. In the current report, 18 proteins were identified in the patient PDF samples that were of plasma origin. The identified proteins can be classified into seven categories according to their biological functions: (1) acute phase proteins, such as alpha-1 antitrypsin, albumin and transferrin; (2) complement factors, including C3; (3) apolipoproteins, such as apolipoprotein A-I; (4) coagulation proteins, including fibrinogen; (5) extracellular matrix proteins, such as fibronectin; (6) vitamin binding proteins, such as retinol-binding protein; and (7) enzymes, such as tRNA-dihydrouridine synthase. The majority of the identified proteins were basically involved in innate immune defence, which might be linked to a systemic deficiency of the immune defence proteins, which could then increase the patient's susceptibility to infection. Across all the PDF samples (initial and follow-up), the most abundant protein was albumin, which is a plasma protein that has a role in the preservation of colloid osmotic pressure (Don & Kaysen, 2004). This finding was in agreement with the results reported in other studies, which showed that albumin was the most abundant protein lost in PDF; in fact, the loss of albumin accounted for 50 to 79% of the total protein lost (Blumenkrantz *et al.*, 1981; Dulaney & Hatch, 1984; Levy *et al.*, 2015). Apart from tRNA-dihydrouridine synthase, the proteins identified in the PDF in the current study were previously observed by other investigators (Wen *et al.*, 2013; Wang *et al.*, 2010; Oliveira *et al.*, 2014; Cuccurullo *et al.*, 2011). The tRNA-dihydrouridine synthase detection in the PDF samples has not been identified in previous studies.

I also examined potential factors in commercially available PDS that might affect the growth of the most predominant peritonitis-causing bacteria, *S.aureus* and *S.epidermidis*. The PD patients involved in this study used either Dianeal PD4 glucose solutions (1.36%, 2.27% or 3.86%) produced by Baxter Healthcare Ltd. or Bicavera glucose solution (1.5%) provided by Fresenius Medical Care Ltd. *in vitro* growth assays demonstrated that staphylococcal growth was greater when cultured in the Bicavera dialysis solution than the Dianeal PD4 solutions. The available data indicated that there were differences in the

buffering agents used in those PD solutions: the Bicavera PDS used sodium bicarbonate as a buffer, and the pH was neutral at 7.4 (according to the manufacturer's manual), whereas the Dianeal PD4 glucose solutions used sodium lactate as a buffer, and the pH was acidic at 5.5. Staphylococci are classified as bacteria which grow best in a neutral pH (Todar, 2006), and therefore their growth was better in neutral bicarbonate-buffered PDS than acidic lactate-buffered PDS. Neutral bicarbonate-buffered PDS has been shown to be more biocompatible *in vivo* and associated with less peritonitis and exit-site infection rates in PD patients than acidic PDS (Montenegro *et al.*, 2007; Furkert *et al.*, 2008). Although the commercial PDS, particularly the lactate-buffered PDS, did not stimulate staphylococcal growth, these solutions are adapted when they come into contact with host-related factors and become more convenient for bacterial growth (Verbrugh *et al.*, 1984). The results shown in this chapter indicated that staphylococci grew well in PDF obtained from a range of PD patients. Such findings are in agreement with many studies, which have shown that PDS converts from non-supportive medium (before dialysis exchange) to supportive medium (after dialysis exchange) for staphylococcal growth (Verbrugh *et al.*, 1984; Williams *et al.*, 1995; McDonald *et al.*, 1986; Sheth *et al.*, 1986). Bacteria may cause infectious peritonitis when they gain access to the peritoneal cavity either via the PD implemented catheter or via a perforation of the gastrointestinal tract (Williams *et al.*, 1995; Ahmetagic *et al.*, 2013). Bacteria adapt, in terms of both growth and virulence, as they respond to the host environment signals within the PDF in the peritoneal cavity (Williams *et al.*, 1995). Therefore, it was important to identify the host-associated factors that may increase PD patient susceptibility to peritonitis. Interestingly, in this study bacterial growth behaviour was found to be dissimilar in PDF samples obtained from different patients as well as in PDF samples derived from the same patient over time, indicating that there were inter- and intra-patient differences in the biochemical characteristics of the PDF. This study examined biochemical factors in the PDF of PD patients toward understanding how differences affect risk of peritonitis.

The first factor investigated in this study was pH (hydrogen ion concentration). The pH of a medium has been shown to be one of the environmental factors that influence bacterial growth (Todar, 2006; Baker *et al.*, 2011). Most peritonitis-causing bacteria, including staphylococci, are neutrophilic and thrive in optimum environments where pH is between 6 and 8 (Todar, 2006). As discussed earlier in this section, the Dianeal PD4 glucose solutions (which were more frequently used by the PD patients in this study) were acidic with a pH of 5.5. However, after the PD exchange, the pH of the PDF shifted to

basic. Physiologically speaking, after infusion of the acidic PDS into the peritoneal cavity, the pH of the PDS reached equilibrium (Tobudic *et al.*, 2011). A study conducted by Duwe *et al.* (1981) investigated changes in pH after administration of acidic PDS into patient abdomens and found that the pH of PDS increased rapidly to ~6.8 within the first 30 min of infusion. Moreover, after 1 hour of PDS administration, the pH equilibrated at ~7.2. Such changes in pH may result from use of lactate as a PDS buffering agent. Lactate in PDS is in fact metabolised to form bicarbonate, which then normalises the acid-base status (Levy *et al.*, 2015).

Another factor investigated in this study was the glucose levels in PDF. Most bacteria are heterotrophic, which means they obtain carbon from organic compounds, such as sugars, as an energy source (Todar, 2006). The PDS is composed mainly of glucose as an osmotic agent, and various groups have reported that glucose is rapidly absorbed by the peritoneal membrane, which leads to a fall in osmosis effectiveness over extended exchange periods (Hain & Kessel, 1987; Levy *et al.*, 2015; Chan *et al.*, 2003; Grodstein *et al.*, 1981). Glucose measurements in this report supported this concept, as glucose concentrations were lower in PDF than in PDS, and PDF glucose levels continued to decrease as patient on dialysis time increased.

Because iron is an essential nutritional element for bacterial growth (Fishbane, 1999), its level was investigated in the PDS and PDF. It is evident from the data in the current chapter that the glucose PD solutions had significant levels of iron. This is because the PD solution is in fact a mixture of high salt content and glucose (Milačič & Benedik, 1999). According to the manufacturers' manual, the fresh PD solution consisted of 1.36% to 3.85% glucose, 132 to 134 mmol/L of sodium, 1.25 to 1.75 mmol/L of calcium, 0.25 to 0.5 mmol/L of magnesium, 95 to 104.5 mmol/L of chloride and a buffer (40 mmol/L of lactate or 34 mmol/L of hydrogen carbonate) all of which contain traces of other metals including iron, copper and zinc. Few investigators have looked for trace metal contamination in fresh PD solutions, however Milačič & Benedik (1999) determined the iron content in the PD4 glucose solutions, which are the same solutions examined in this study. The iron levels detected by Milačič & Benedik (1999) were 0.13 μM, 0.1 μM and 0.2 μM for the 1.36%, 2.27% and 3.85% glucose PD solutions, respectively, which are much lower than the concentrations found in this study (the range was from 36.74 μM to 60.74 μM). It was expected, then, that iron would be detected in the PDF obtained after the dialysis exchange. In the majority of the PDFs analysed, the total iron level in the PDF was higher than that in the fresh glucose PDS, which suggests the origin for the

increased iron was the PD patient. It has been proposed by Ščančar *et al.* (2003) that the loss of trace elements into the PDF is a process induced by losing metal-binding proteins during the dialysis exchange. The mean iron level in the PDF of 12 CAPD patients determined by Ščančar *et al.* (2003) was 0.26 μM, which also conflicts with the mean iron level observed in this study (55 μM). The methodology utilised to measure iron levels in the glucose PDS and PDF in both studies (Milačič & Benedik, 1999; Ščančar *et al.*, 2003) was electrothermal and flame atomic absorption spectrometry, which is a different methodology from that used in the current study (an iron assay kit). It may be argued that a different methodology could provide different measurements.

The literature on the levels of Fe^{+2} and Fe^{+3} in the PDF is scarce. In the current study, Fe^{+2} (non-transferrin bound iron, also termed free iron), as well as Fe^{+3}, which exists in combination with molecules such as transferrin (Gomme *et al.*, 2005; Modun *et al.*, 1994; Parkkinen *et al.*, 2002), were detected at variable levels in the range of PDF samples. Under normal circumstances, serum transferrin is usually partially saturated with Fe^{+3} (25% to 35%) to maintain the level of Fe^{+2} at around 10^{-18} M, which is too low to enable microbial growth (Gomme *et al.*, 2005). In comparison to normal conditions, the mean Fe^{+2} level in the PDF samples was detected at 2.9×10^{-5} M, which is higher than the free iron level in the circulation. It is also more than the minimum iron level required by pathogenic bacteria to grow (4×10^{-7} to 4×10^{-6} M) (Park *et al.*, 2005). Some investigators have reported that staphylococcal growth in body fluids, cells or tissues is induced by the presence of excessive free iron (Weinberg, 2009; Sunder-Plassmann *et al.*, 1999). Given all of the above considerations, in the iron context PDF is not a bacteriostatic medium, which means that it might increase a patient's susceptibility to peritoneal infection.

Some investigators detected the presence of transferrin in PDF by using either sequencing or Western blotting techniques (Oliveira *et al.*, 2014; Modun *et al.*, 1994). Such findings agree with the results of the current study, as the transferrin presence across all the PDF samples was observed by sequencing method, confirmed by Western blotting technique, and its precise level was also determined using the ELISA method. Transferrin is an iron-binding protein which has a major presence in plasma (Afzali & Goldsmith, 2004). It is a key part of the innate immunity as it makes extracellular body fluids bacteriostatic due to its ability to bind free iron and thus inhibit the growth of bacteria (Afzali & Goldsmith, 2004). Transferrin is arranged into two homologous lobes (the *N*-lobe and *C*-lobe), and each lobe has a high affinity single ferric iron (Fe^{+3}) binding site; thus, transferrin binds two atoms of Fe^{+3} per molecule of protein (Lacey *et al.*, 2001; Parkkinen *et al.*, 2002;

Gomme *et al.*, 2005). Catecholamine stress hormones are a group of tyrosine-derived effectors, which are chemically characterised by having a catechol and an opposing amine side chain (Freestone *et al.*, 2008b). Catecholamine stress hormones are released in response to stress either physiologically or psychologically (Collins & Bercik, 2009). Catecholamines were also detected, for the first time, in the dialysates of PD patients. To the best of my knowledge, no studies have looked at the elimination of the catecholamine stress hormones into the PDF.

The available data in the present chapter indicated inter- and intra- patient variances in transferrin as well as catecholamine stress hormones concentrations in the PDF. The most abundant catecholamine stress hormone in the PDF samples was NE, which was also detected across all PDF samples. From a clinical point of view, NE is the principal neurotransmitter released by the sympathetic nerves and it serves as an index for sympathetic outflow (Aneman *et al.*, 1996). This could explain why the NE level is the highest in PDF samples in the current study. Various researchers (Sandrini *et al.*, 2010; Anderson & Armstrong, 2008; Freestone *et al.*, 2000; Freestone *et al.*, 2002; Freestone *et al.*, 2007; Freestone *et al.*, 2008; Neal *et al.*, 2001) have demonstrated that the bacterial growth is significantly stimulated in the presence of transferrin by catecholamine-iron provision from the transferrin. The mechanism for iron removal from transferrin was then investigated by Sandrini *et al.* (2010), and the catechol part of the NE was found to complex with the Fe^{+3} sequestered by transferrin and reduce it to Fe^{+2}. The resultant NE-Fe^{+2} compound then dissociates and was internalised by bacteria (Sandrini *et al.*, 2010). Urea polyacrylamide gel investigations of the iron-binding status of transferrin shown in this report indicated that the transferrin detected within PDF is saturated with Fe^{+3}. Also, the simultaneous presence of iron-saturated transferrin and the catecholamine NE in the PDF could lead to NE-Fe^{+2} complex formation, which subsequently reduce the iron binding capacity of transferrin. It is, therefore, possible to argue that the co-localisation of the catecholamines and iron-saturated transferrin in PDF weakens the bacteriostatic nature of PDF and results in what could be regarded as a highly nutritious bacterial growth medium.

It was appropriate to consider use of an iron chelator, which could bind free iron in PDF and restore bacteriostatic conditions there. It was found that deferoxamine was able to increase iron uptake of staphylococci in PDF and was not suitable as a PDF bacterial growth inhibitor. These data are incompatible with the results of Van Asbeck *et al.* (1983). Van Asbeck *et al.* (1983) have demonstrated that the addition of deferoxamine

(0.2 – 0.4 mg/ml) can reduce the growth of staphylococci. In fact, Van Asbeck *et al.* (1983) have examined the effect of deferoxamine on staphylococcal growth in nutrient broth media that contained much lower total iron (10 μM) than that in the PDF samples analysed for the present study (mean 60 μM). It might be proposed from these data that the level of total iron may influence the inhibitory effect of deferoxamine on staphylococcal growth in PDF. Alternately, it is possible the Staphylococci are using the deferoxamine as a siderophore, in which case it would increase rather than decrease growth. In contrast to deferoxamine, incorporation of apo-transferrin did inhibit growth of *S.epidermidis*, and on some occasions, that of *S.aureus* as well. The bacteriostatic effect of apo-transferrin on *S.epidermidis* was in agreement with the results of Von Bonsdorff *et al.* (2003) study, who have reported that the addition of apo-transferrin at 0.1 mg/ml, the same concentration examined in the current study, inhibited *S.epidermidis* growth in patient serum. The limited effect of apo-transferrin on *S.aureus* growth in the present study might be due to the fact that *S.aureus* contains more iron uptake mechanisms than does *S.epidermidis* (Skaar & Schneewind, 2004). In the future, it might be useful to examine the effect of other iron chelators such as deferiprone and deferasirox (Weinberg, 2009).

The study sought to identify biochemical parameters that could independently predict a risk of infectious peritonitis in PD patients. Univariable linear regression analysis identified two parameters as possible risk factors for *S.aureus* growth in PDF: transferrin and catecholamine stress hormones. Data from Patient 4 and others showed that the higher the levels of transferrin and catecholamine stress hormones, the greater the degree of *S.aureus* growth in the sample PDF, findings which supported the study hypothesis. While the level of transferrin continued to effect *S.aureus* growth in PDF over time, the effect of catecholamine stress hormone levels on *S.aureus* growth was lost, which suggests there were factors released into the PDF that interfered with the action of catecholamine stress hormones. Four of the PD patients took either an alpha- or a beta-adrenergic blocker, or a combination of both alpha- and beta-adrenergic blockers as hypertension management drugs, and these may have been the source of the observed interference. A previous *in vitro* study by Freestone *et al.* (2008) have demonstrated that alpha-adrenergic but not beta-adrenergic blockers have the ability to inhibit NE-induced staphylococcal growth. Freestone's study also showed that both alpha- and beta-adrenergic blockers slightly inhibit staphylococcal growth in response to Dop. Accordingly, in the current study, some patients' continuous use of these blockers could account for the inhibition of catecholamine stress hormones in PDF.

From a biological point of view, *S.aureus* is a more aggressive pathogen than *S.epidermidis* and contains a greater array of host iron-uptake systems (Skaar & Schneewind, 2004). One iron-uptake system bacteria employ involves secreting siderophores, low molecular weight molecules with a high affinity for Fe^{+3} (Beasley & Heinrichs, 2010). *S.aureus* produces three types of siderophores: staphyloferrin A, staphyloferrin B and aureochelin. In contrast, *S.epidermidis* produces only one type of siderophore: staphyloferrin A (Skaar & Schneewind, 2004; Beasley & Heinrichs, 2010). *S.aureus*, but not *S.epidermidis*, also can access human heme iron present within hemoproteins through iron-regulated cell determinants (Skaar & Schneewind, 2004; Beasley & Heinrichs, 2010). These features could explain differences between *S.aureus* and *S.epidermidis* growth responses to different biochemical parameters in PDF. After *S.epidermidis*, which causes PD peritonitis, *S.aureus* is the next most common bacteria; however, PD-related *S.aureus* peritonitis is responsible for a significantly higher patient hospitalisation rate, a higher catheter removal rate and a higher mortality rate (Barretti *et al.*, 2012).

In summary, data presented herein show that the transferrin level could be a stimulatory factor for *S.aureus* growth in PDF. This finding could lead to the development of a simple dipstick prognostic test to identify patients with high transferrin levels and who may become more susceptible to developing *S.aureus* infection. Using of such a test could have the potential to reduce a great deal of PD patient suffering and even save lives.

CHAPTER 4 Investigation of PDF exposure on the virulence of *S.aureus* and *S.epidermidis*

4.1 Introduction

One of the key routes for infectious peritonitis is from the normal flora that resides on the skin (skin contaminants) via the PD catheter (Levy *et al.*, 2015). The skin contaminants are microbes, which are introduced accidently into the body at the time of connection of the PD catheter to the PDS bag or by an infected exit-site (Levy *et al.*, 2015; Ellam & Wilkie, 2011). Many studies have been conducted to identify the causative microbiological agents responsible for peritonitis during PD treatment (Castrale *et al.*, 2010; Thirugnanasambathan *et al.*, 2012; Kofteridis *et al.*, 2010; Pajek *et al.*, 2011; Vikrant *et al.*, 2013). Coagulase-negative staphylococci (mainly *S.epidermidis*) and *S.aureus* were microbiota most commonly responsible for peritonitis (Castrale *et al.*, 2010; Thirugnanasambathan *et al.*, 2012; Kofteridis *et al.*, 2010; Pajek *et al.*, 2011; Vikrant *et al.*, 2013); therefore, these two bacteria were the test microbes used in this part of my study.

S.epidermidis becomes an opportunistic pathogen in immunocompromised patients or when translocated from its original niche (skin and mucus membranes) to a new place (the peritoneal cavity, in the case of PD) (Otto, 2008; Otto, 2009). *S.epidermidis* has been shown to be the most predominant bacterium responsible for infection on indwelling medical devices, such as PD catheters (Otto, 2009; Dasgupta, 2002). The virulence of this bacterium depends mainly on its ability to produce a biofilm on plastic surfaces (e.g. PD catheters), which makes the extermination of the infection difficult, and in some cases, requires PD catheter removal. Hence, biofilm production becomes a central concern for PD patients (Otto, 2009; Dasgupta, 2002). *S.aureus* is the next most common bacterium, which causes a more severe PD peritonitis (Barretti *et al.*, 2012). Like *S.epidermidis*, *S.aureus* has the capacity to produce biofilm on plastic surfaces (Barretti *et al.*, 2012). It has been shown (Lyte *et al.*, 2003; Neal *et al.*, 2001) that catecholamine hormones and inotropes (including noradrenaline) can stimulate *S.epidermidis* growth and biofilm formation on intravenous catheter material in blood or plasma. Because catecholamine hormones were detected at variable levels in PDF samples (Figure 3.16), it was necessary to examine whether the biofilm produced by staphylococci was also affected by the catecholamine hormones levels in PDF.

Chapter 3 demonstrated that PDF (which is the fluid collected after the PD exchange and has been modified with host-protein molecules, hormones and other factors), in contrast to PDS (which is the solution that is infused into the peritoneal cavity and contains

glucose, electrolytes and buffer), was markedly more supportive for *S.aureus* and *S.epidermidis* growth. Also, it was shown that the PDF of different PD patients contained iron, catecholamine hormones and iron-saturated transferrin, which altogether could be the factors that stimulate staphylococcal growth in PD patients. Since these factors had an impact on *S.aureus* and *S.epidermidis* growth, it was necessary to examine if virulence of these two bacteria were also influenced by PDF. Therefore, the current chapter aims to investigate the virulence characteristics of *S.aureus* and *S.epidermidis* after exposure to PDF.

4.2 Results

4.2.1 Biofilm formation

Biofilm formation is one of the principal virulence strategies by which *S.aureus* and *S.epidermidis* establish infection in patients who have indwelling medical devices (e.g. a PD catheter); this which makes the extermination of infection difficult and in some cases requires device removal (Dasgupta, 2002; Otto, 2009). As shown previously in Figure 3.2, PD dialysates derived from different patients had a differential impact on *S.aureus* and *S.epidermidis* growth. Therefore, the effects of PD dialysates on the ability of staphylococci to produce biofilms were investigated.

The initial stages of biofilm formation (i.e. attachment) by staphylococci cultured in PD dialysates were examined using a photometric crystal violet assay, as described by Tendolkar *et al.* (2004). Later stages in the development of the biofilm were examined using light microscopy. Biofilm formation by *S.aureus* (strain T288354) and *S.epidermidis* (strain Tü3298) were examined, as these two strains were used in the growth studies (Section 3.2.3). An additional *S.epidermidis* (strain RP62A) was also examined as it is known to be a slime-producing strain (Lyte *et al.*, 2003). Briefly, ~10^7 CFU/ml of staphylococcal culture was dispensed into wells of a flat-bottomed polystyrene 96-well microtiter plate, and then incubated at 37°C for 24 hours. Next, 200 µl of 0.2% aqueous crystal violet solution was added and the plates were re-incubated and washed as described in Section 2.2.3.1. The aqueous crystal violet bound to the attached staphylococci was extracted by adding 95% ethanol and absorbance was taken at 595 nm. It has been shown that glucose availability in a growth medium can significantly enhance staphylococcal biofilm formation (Stepanović *et al.*, 2000). Additionally, because the PD patients used variable glucose formulations in their PD solutions (Table 3.2), it was useful

first to examine whether different glucose concentrations in PDS had an impact on staphylococcal biofilm production. Four different glucose formulations in the PDS were analysed: 1.36%, 1.5%, 2.27% and 3.86% (Figure 4.1). On the whole, there were clear variations in how *S.aureus* and *S.epidermidis* (both strains) attached to the polystyrene culture tray in different PD solutions. The highest glucose formulation in the PDS (3.86%) had the highest enhancement effect on *S.aureus* attachment, whereas the greatest attachment by *S.epidermidis* (both strains) was in *S.epidermidis* incubated in 2.27% glucose PDS. For both species, the lowest attachment was quantified when 1.36% glucose PDS was used as a growth medium. Thus, variable glucose percentages in the PDS did influence the attachment patterns of *S.aureus* and *S.epidermidis*.

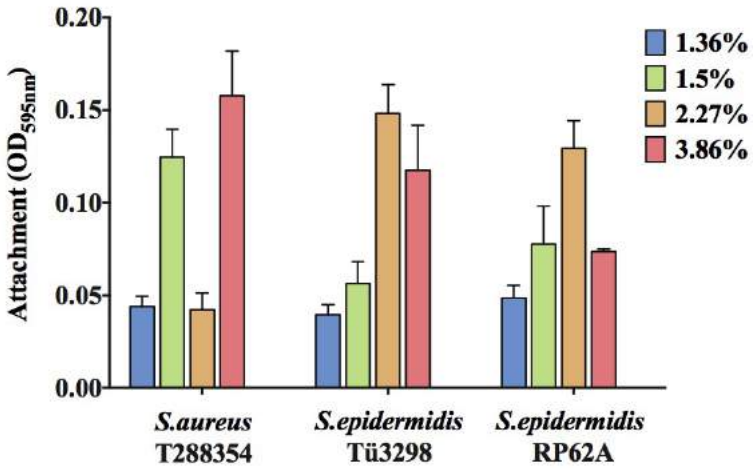


Figure 4.1 Attachment of *S.aureus* and *S.epidermidis* cultured in different glucose formulations of PD solutions.
The degree of biofilm formation (attachment) was measured using the crystal violet attachment assay (Section 2.2.3.1). OD readings of staphylococci were subtracted from the media control readings (the crystal violet binding to the wells containing only PD solution); n=3.

Comparison studies between PD dialysates and the corresponding PD solutions were conducted using the PDF collected from nine PD patients at the beginning of their dialysis (Table 3.2). Generally speaking, *S.epidermidis* strain RP62A was by far the most responsive bacterium in terms of biofilm production in PD dialysates. With the exception of the PDFs of Patients 1, 5 and 7, there were no major differences in the attachment of *S.aureus* in the PDF, compared to the corresponding PD solution (Figure 4.2 A). The PDF of Patient 1 markedly stimulated the attachment of *S.aureus*, compared to the corresponding PDS. However, *S.aureus* attachment in the PDF of Patients 5 and 7 was markedly lower than that in the PDS used by both patients, which was 1.5% glucose PDS. *S.epidermidis* strain Tü3298 showed markedly more attachment in the PD solution of Patients 1, 2 and 6 than in their PDFs (Figure 4.2 B). However, no major variation in the attachment of *S.epidermidis* strain Tü3298 was observed among the PDFs of Patients 3, 4, 5, 7, 8 and 9 and their corresponding PD solutions. The PDF of Patient 4—who among the nine PD patients had the most supportive fluid for staphylococcal growth (Figure 3.2)—was also the fluid to most highly stimulate *S.epidermidis* strain RP62A attachment, followed by the PDF of Patients 1 and 6 (Figure 4.2 C). In contrast, the PDFs of the other PD patients demonstrated no major differences in the *S.epidermidis* strain RP62A attachment, compared to the corresponding PD solutions. Figure 4.2 shows that PDFs from the nine PD patients do indeed have different effects on biofilm production by the PD peritonitis bacteria.

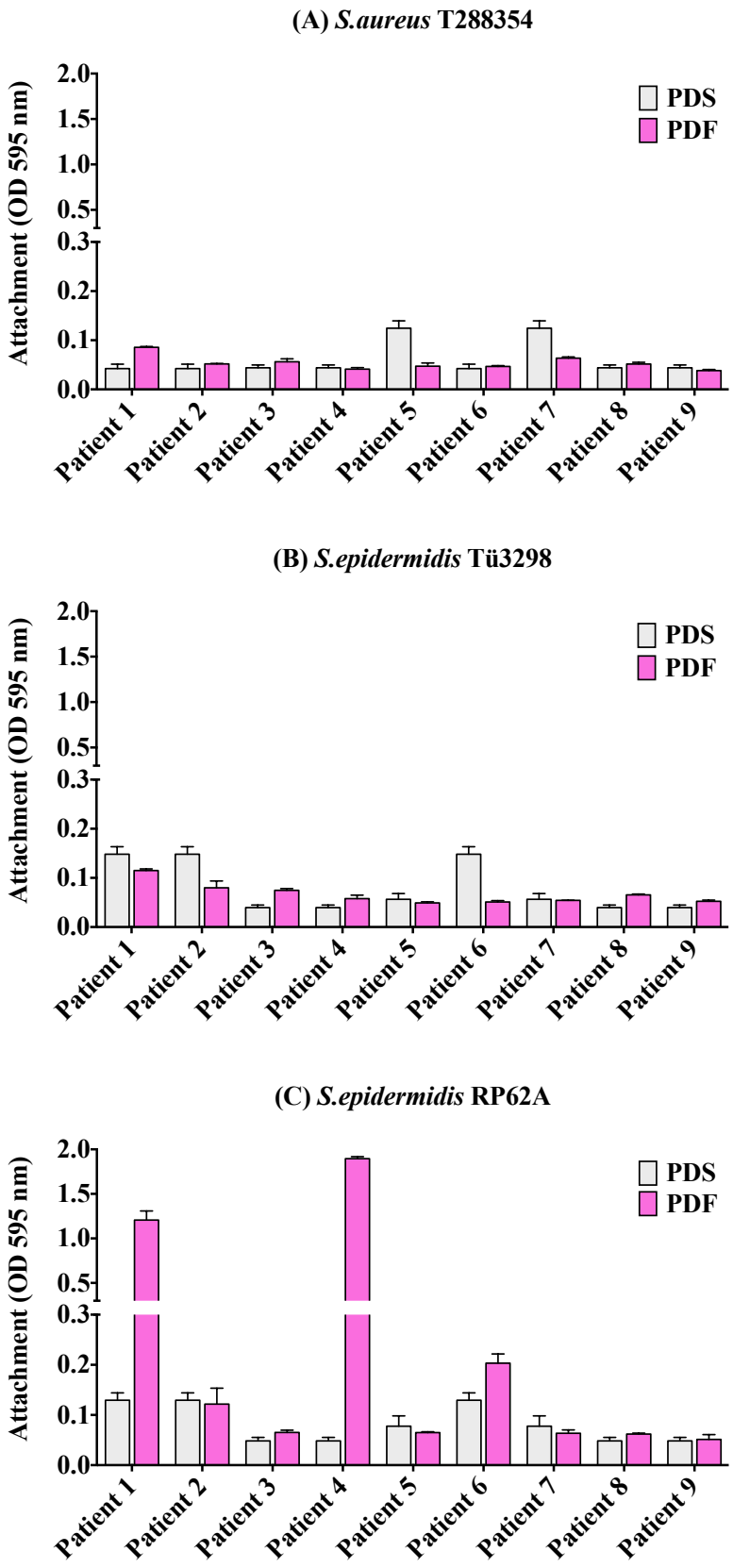


Figure 4.2 Attachment of *S.aureus* and *S.epidermidis* cultured in PDF of nine PD patients and in their corresponding PD solutions.
The degree of biofilm formation (attachment) was measured using the crystal violet attachment assay (Section 2.2.3.1). OD) readings of staphylococci were subtracted from the media control readings (the crystal violet binding to the wells containing only PD solution or PDF); n=3.

Biofilm production investigations were also conducted using follow-up PDF collected over the course of PD therapy (Table 3.2). In general, *S.epidermidis* strain RP62A was the most responsive bacterium in terms of biofilm production in PDF. The follow-up PDF of Patient 1 collected after three days and after seven months on PD therapy demonstrated clearly higher attachment by *S.aureus* and *S.epidermidis* strain RP62A than the corresponding PDS. However, the attachment of the *S.epidermidis* strain Tü3298 was markedly higher in the PDS than in the follow-up PDF (Figure 4.3). Interestingly, the follow-up PDF of Patient 2 (gathered after six months and after 24 months on PD course therapy) were fluids that had stimulated biofilm formation more strongly among the three strains of staphylococci than the corresponding PD solutions (Figure 4.4). There were no major variations in *S.aureus* attachment in the follow-up PDF of Patient 3 and the corresponding PD solutions. However, the different strains of *S.epidermidis* showed different patterns in terms of bacterial attachment. For instance, the PDF collected after 11 months on PD course was found to stimulate *S.epidermidis* strain RP62A attachment more than the corresponding PD solution. However, the same fluid showed clearly less attachment by *S.epidermidis* strain Tü3298 than the corresponding PDS (Figure 4.5). Similar to the PDFs of Patient 3, the follow-up PDF of Patients 6 and 8 showed no major differences in *S.aureus* attachment, compared to the corresponding PD solutions (Figures 4.6 A and 4.7 A). On the other hand, the PDFs of Patient 6 (collected after 22 days and after seven months on PD course therapy) and of Patient 8 (gathered after six months on PD course therapy) demonstrated marked increases in *S.epidermidis* strain RP62A attachment than the corresponding PD solutions (Figures 4.6 C and 4.7 C). Hence, within the same patient, the patterns of attachment in PDF were variable among the three strains of staphylococci.

Patient 1

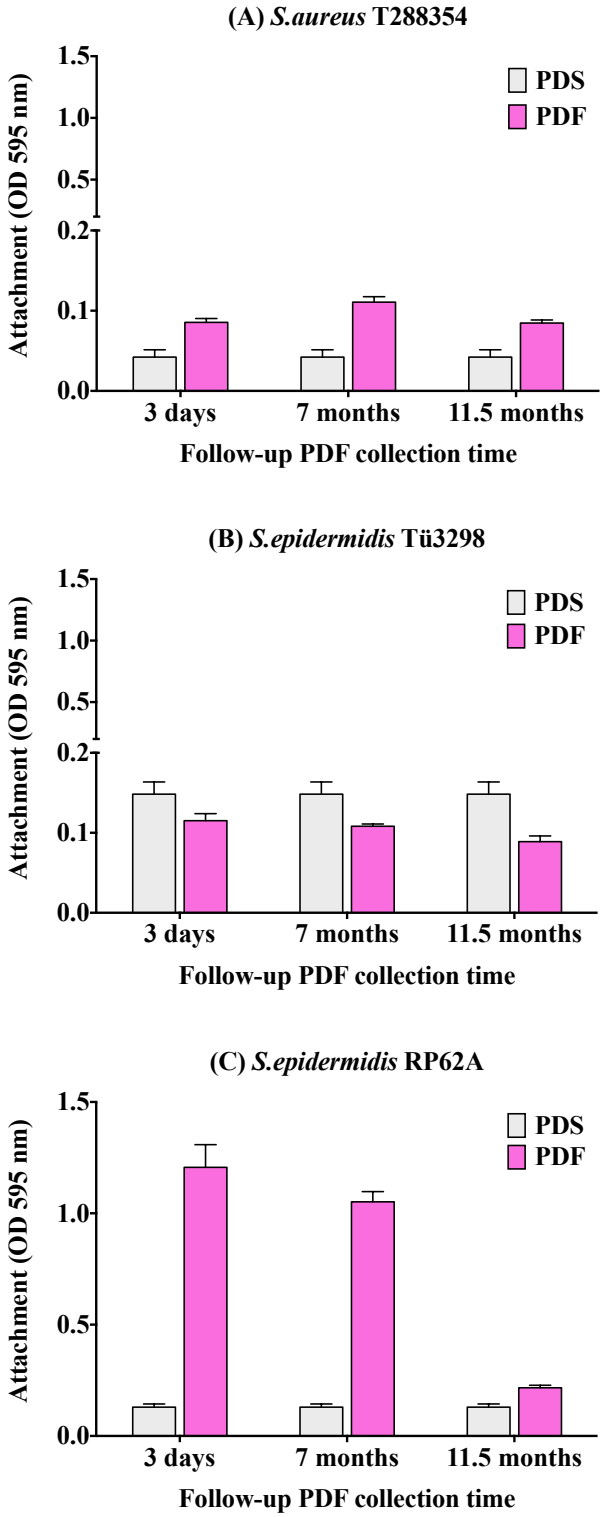


Figure 4.3 Attachment of *S.aureus* and *S.epidermidis* cultured in the PDFs of Patient 1 and corresponding PD solutions.
The degree of biofilm formation (attachment) was measured using the crystal violet attachment assay (Section 2.2.3.1). OD readings of staphylococci were subtracted from the media control readings (the crystal violet binding to the wells containing only PD solution or PDF); n=3.

Patient 2

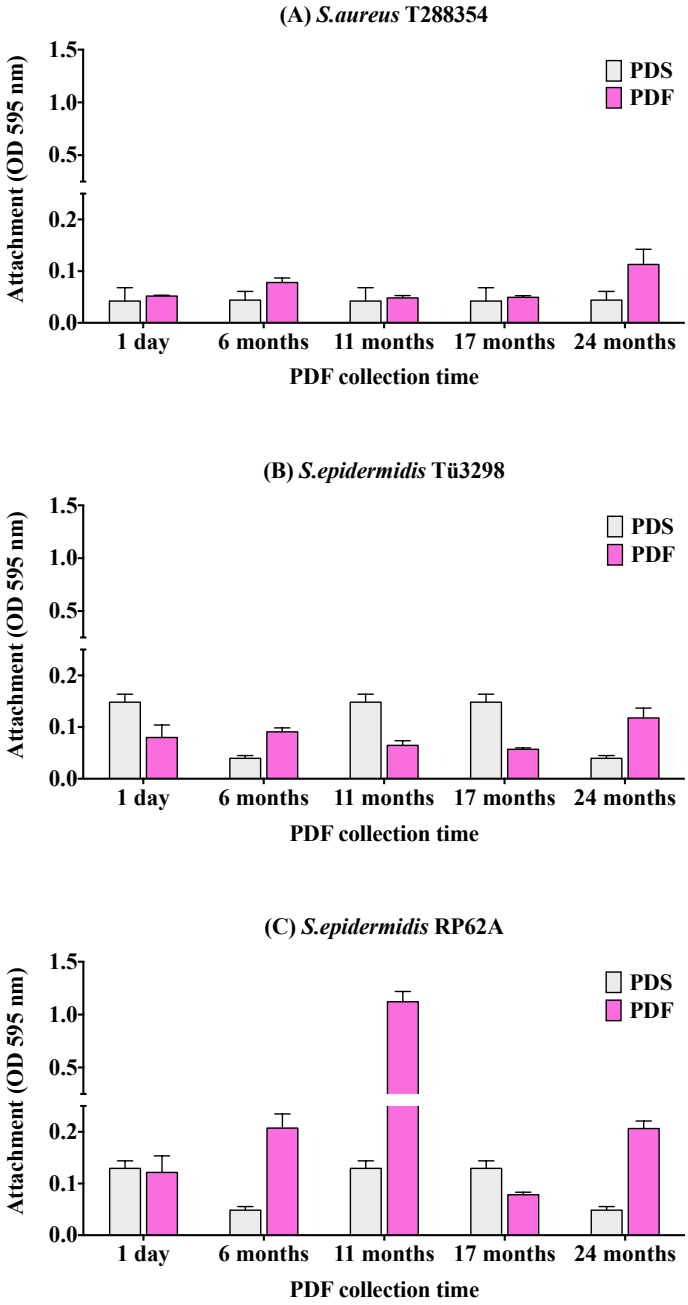


Figure 4.4 Attachment of *S.aureus* and *S.epidermidis* cultured in the PDFs of Patient 2 and corresponding PD solutions.
The degree of biofilm formation (attachment) was measured using the crystal violet attachment assay (Section 2.2.3.1). OD readings of staphylococci were subtracted from the media control readings (the crystal violet binding to the wells containing only PD solution or PDF); n=3.

Patient 3

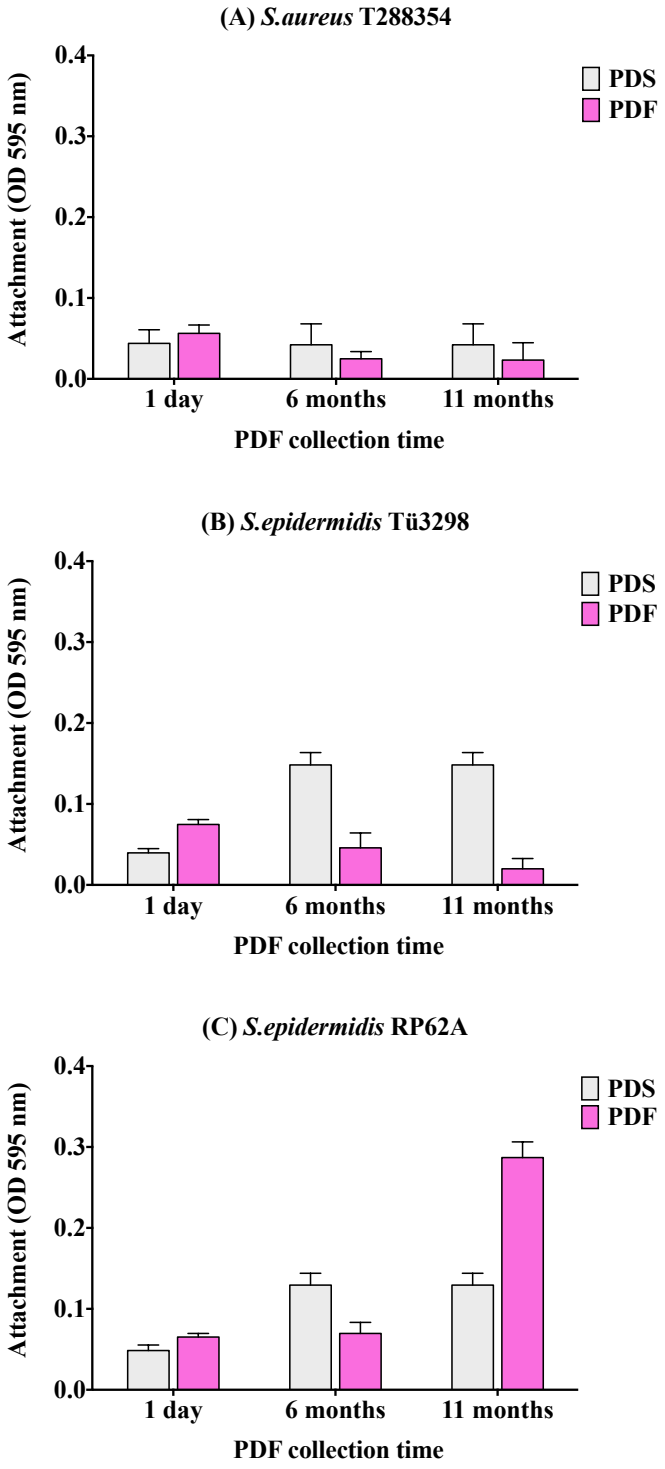


Figure 4.5 Attachment of *S.aureus* and *S.epidermidis* cultured in the PDFs of Patient 3 and corresponding PD solutions.
The degree of biofilm formation (attachment) was measured using the crystal violet attachment assay (Section 2.2.3.1). OD readings of staphylococci were subtracted from the media control readings (the crystal violet binding to the wells containing only PD solution or PDF); n=3.

Patient 6

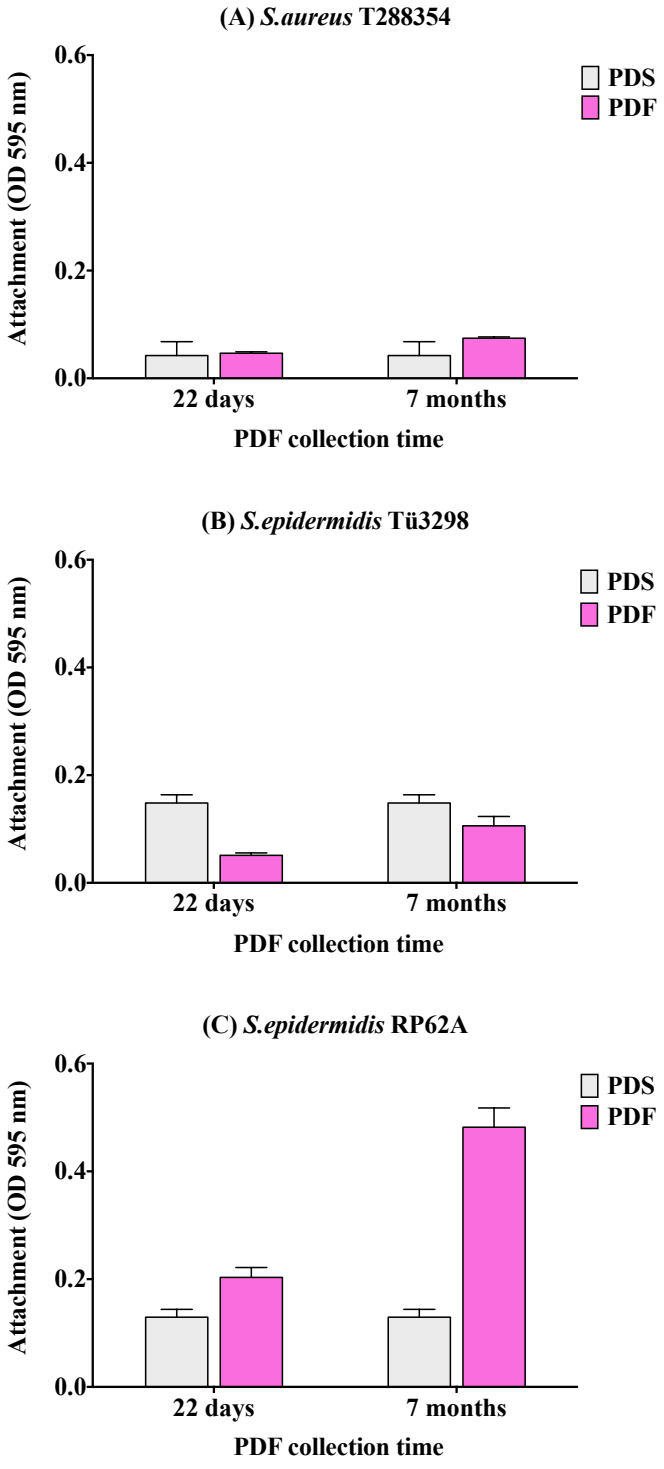


Figure 4.6 Attachment of *S.aureus* and *S.epidermidis* cultured in the PDFs of Patient 6 and corresponding PD solutions.
The degree of biofilm formation (attachment) was measured using the crystal violet attachment assay (Section 2.2.3.1). OD readings of staphylococci were subtracted from the media control readings (the crystal violet binding to the wells containing only PD solution or PDF); n=3.

Patient 8

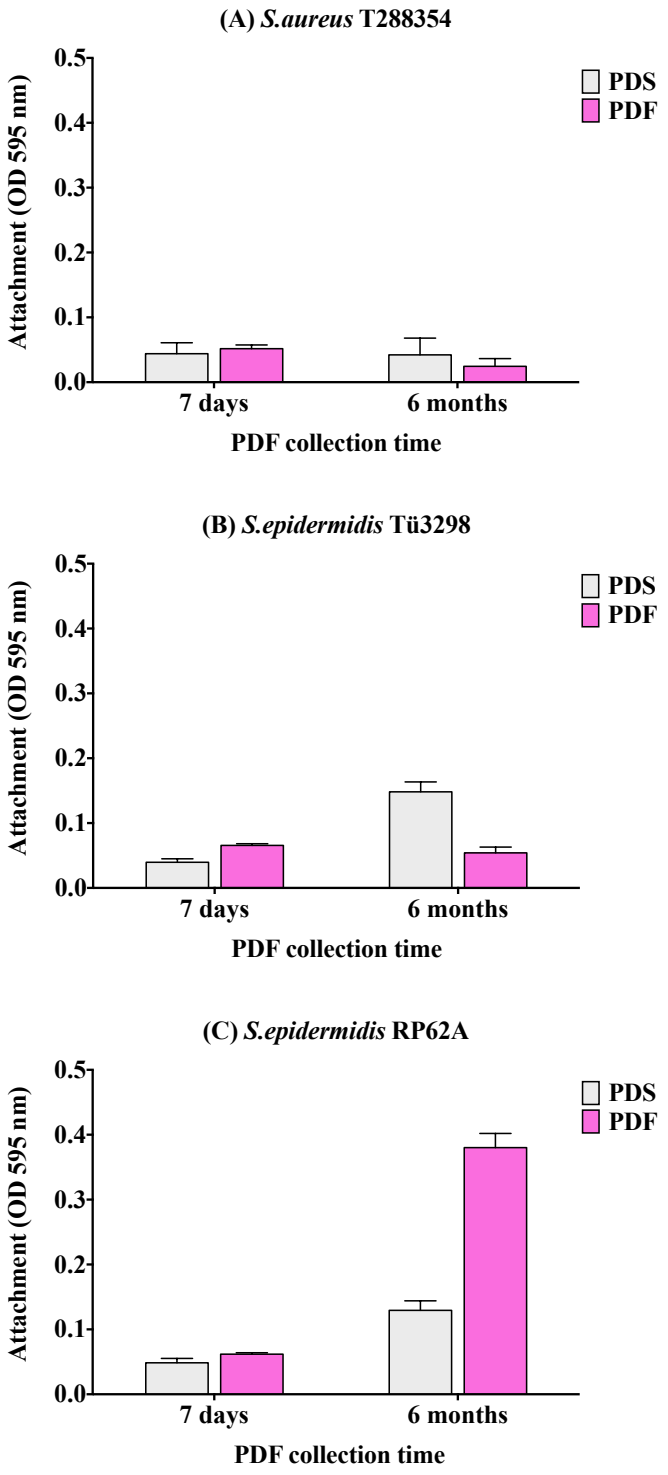


Figure 4.7 Attachment of *S.aureus* and *S.epidermidis* cultured in the PDFs of Patient 8 and corresponding PD solutions.
The degree of biofilm formation (attachment) was measured using the crystal violet attachment assay (Section 2.2.3.1). OD readings of staphylococci were subtracted from the media control readings (the crystal violet binding to the wells containing only PD solution or PDF); n=3.

4.2.1.1 Microscopic examination of biofilm formation by staphylococci in PDS and PDF

Basically, ~10^7 CFU/ml of staphylococcal cultures were pipetted into PDS or PDF in the wells of a 24-well plate. The plates were incubated as described in Section 2.2.3.1.1 and examined under a microscope using a 40× objective lens for biofilm formation by staphylococcal cultures in different PDS or PDF samples. Direct microscopic analysis of staphylococcal cultures in different glucose formulations in PD solutions (1.36%, 1.5%, 2.27% and 3.86%) demonstrated, as expected, no biofilm production by *S.aureus* or *S.epidermidis* (Figure 4.8). In contrast, there was an obvious presence of *S.aureus* and *S.epidermidis* biofilm in a majority of PD dialysates (Figure 4.9). The PDFs were previously shown to be more supportive of staphylococcal growth than the PDS (Figure 3.2). Thus, while the glucose in the PDS did enhance the initial attachment of staphylococci to polystyrene as shown in Figure 4.1, because the PDF contained more growth-stimulating factors than the PDS, the bacteria grew better and formed microcolonies, which could then develop into a multilayered biofilm as shown in Figure 4.9.

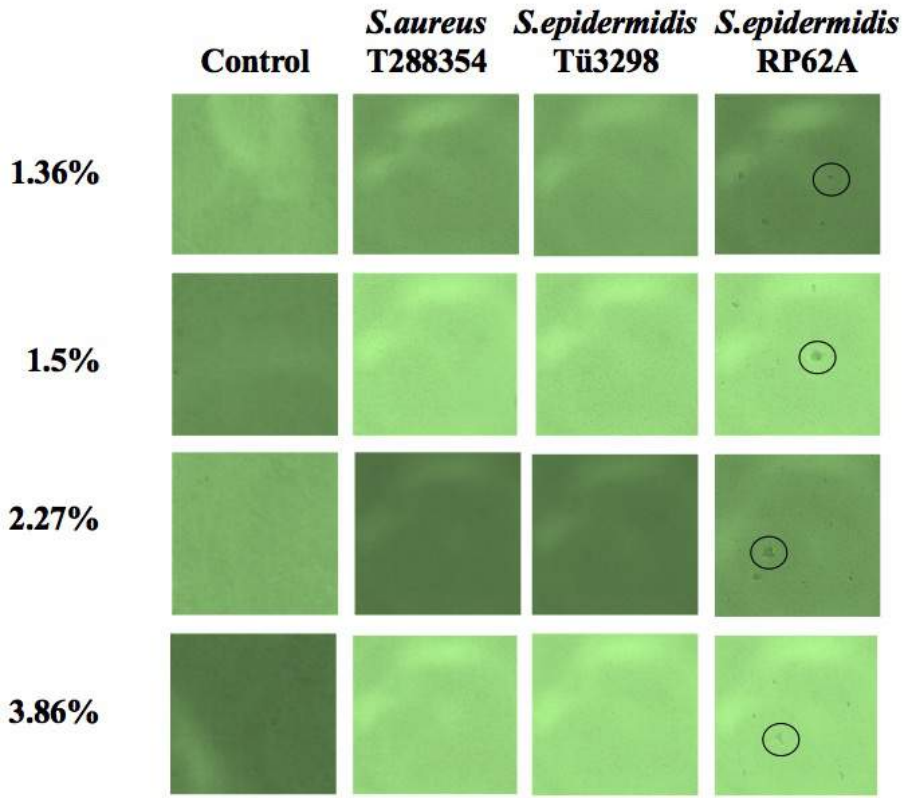


Figure 4.8 Microscopic analysis of biofilm formation by different strains of staphylococci in PD solutions.

Approximately 10^7 CFU/ml of staphylococci were incubated in PDS for 24 hours and photographs of staphylococcal cultures were taken using an inverted microscope at 40× magnification, as described in Section 2.2.3.1.1. No biofilm was formed by staphylococci in glucose-based PD solutions. Few aggregations of *S.epidermidis* strain RP62A were present, as shown in the figure (black circles). 'Control' refers to the PDS without bacteria.

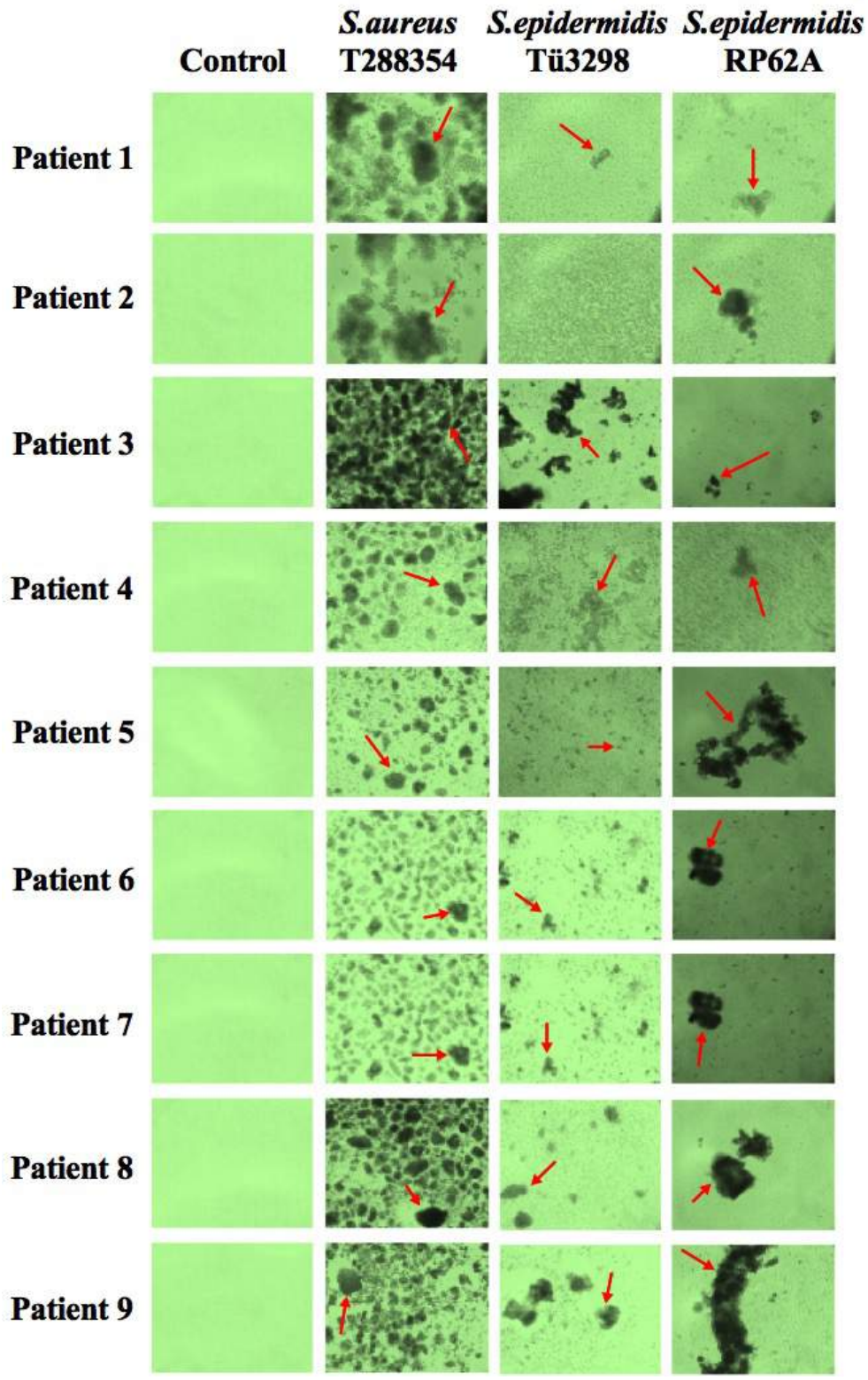


Figure 4.9 Microscopic analysis of biofilm formation by different strains of staphylococci in the PD dialysates of nine PD patients.
Approximately 10^7 CFU/ml of staphylococci were incubated in PDF for 24 hours and photographs of staphylococcal cultures were taken using an inverted microscope at 40× magnification, as described in Section 2.2.3.1.1. The biofilm formed by staphylococci in PD dialysates can be seen, as shown in the figure (red arrows). 'Control' refers to the PD dialysate without bacteria.

4.2.2 Haemolytic activity

S.epidermidis produces a toxin known as *N*-formylated alpha-helical peptide delta toxin that is encoded by the *hld* gene, which leads to erythrocyte lysis by forming pores in the erythrocyte's cytoplasmic membrane (Vuong & Otto, 2002). *S.aureus* synthesises a 33-kDa protein alpha-toxin, which has a role in the virulence and pathogenesis of *S.aureus* in terms of pore formation and erythrocyte haemolysis (Bhakdi & Tranum-Jensen, 1991; Lowy, 1998). Investigations were therefore carried out to determine whether PD dialysates stimulated the haemolytic activity of staphylococci.

The haemolytic activity of *S.aureus* (strain T288354) and *S.epidermidis* (strain Tü3298) in the PD dialysates was examined using the method described by Molnar *et al.* (1994). Briefly, ~10^8 CFU/ml of staphylococcal cultures were incubated overnight in PDF samples. Then, a serial two-fold dilution of the staphylococcal suspension was performed in sterile PBS across 12 wells of a round-bottomed 96-well micro-titre plate followed by the addition of a suspension of sheep RBCs to each well, as described in Section 2.2.3.2. The potential haemolytic activity of the PDF samples without bacteria was also assessed. The haemolytic activity results of staphylococci in the PDF samples are summarised in Tables 4.1 and 4.2, and representative examples are shown in Figure 4.10. To evaluate the validity of the results and to avoid identifying false positive haemolysis, the erythrocytes were diluted in PBS only and showed there was no haemolytic activity (Figure 4.10 row A). In contrast, *S.aureus* cultured in TSB (Table 2.3) was used as a positive control and provided complete haemolysis of all the RBCs after 24 hours incubation (Figure 4.10 row B).

A comparative analysis demonstrated that *S.aureus* was able to produce more haemolysin in both the initial PDFs (collected at the beginning of dialysis) and in the follow-up PDFs than *S.epidermidis*. In a majority of PDFs, the haemolytic activity of *S.aureus* was observed after 30 min of incubation with variations between different PD dialysates. Furthermore, not unexpectedly the haemolytic activity of *S.aureus* increased over time, reaching its greatest level (20,480 HU/ml) after 24 hours of incubation in most PDF samples. In contrast, the highest level of haemolysin produced by *S.epidermidis* after 24 hours did not exceed 80 HU/ml. No haemolytic activity was recognised in the PD dialysates in the absence of staphylococci (Figure 4.10 row C).

Table 4.1 A comparison of the haemolytic activity of *S.aureus* (strain T288354) and *S.epidermidis* (strain Tü3298) in the PDF collected from nine PD patients at the beginning of their dialysis.

Patient ID	Haemolytic activity of *S.aureus* (HU/ml)				Haemolytic activity of *S.epidermidis* (HU/ml)			
	30 min	90 min	4 hours	24 hours	30 min	90 min	4 hours	24 hours
Patient 1	10	20	40	10,240	10	20	40	40
Patient 2	0	0	0	20,480	0	0	0	0
Patient 3	10	10	40	20,480	0	0	10	40
Patient 4	10	10	80	20,480	0	0	10	20
Patient 5	10	20	80	20,480	10	10	20	40
Patient 6	10	10	20	20,480	10	10	20	20
Patient 7	10	10	80	20,480	0	0	10	20
Patient 8	10	10	20	20,480	0	0	0	20
Patient 9	10	10	10	20,480	0	0	0	20

The haemolytic activity assay was performed as described in Section 2.2.3.2. The number of haemolytic units (HUs) was calculated in all wells until 50% haemolysis was reached, and was expressed as HU/ml. The readings were taken after 30 min, 90 min, 4 hours, and 24 hours.

Table 4.2 A comparison of the haemolytic activity of *S.aureus* (strain T288354) and *S.epidermidis* (strain Tü3298) in follow-up PDF samples gathered from five PD patients.

Patient ID	PDF collection time	Haemolytic activity of *S.aureus* (HU/ml)				Haemolytic activity of *S.epidermidis* (HU/ml)			
		30 min	90 min	4 hours	24 hours	30 min	90 min	4 hours	24 hours
Patient 1	**3 days**	10	20	40	10,240	10	20	40	40
	7 months	10	10	20	20,480	10	10	10	20
	11 months	10	20	40	20,480	10	20	40	40
Patient 2	**1 day**	0	0	0	20,480	0	0	0	0
	6 months	40	40	320	20,480	0	0	0	0
	11 months	20	20	80	20,480	10	20	20	40
	17 months	40	40	640	20,480	0	0	10	40
	24 months	40	40	1,280	20,480	0	0	0	10
Patient 3	**1 day**	0	0	10	20,480	0	0	0	0
	6 months	10	20	80	20,480	10	20	40	40
	11 months	20	20	40	20,480	10	10	20	40
Patient 6	**22 days**	10	10	20	20,480	10	10	20	20
	7 months	10	10	10	20,480	0	0	10	40
Patient 8	**7 days**	10	10	20	20,480	0	0	0	40
	6 months	10	20	40	20,480	10	20	40	80

The haemolytic activity assay was performed as described in Section 2.2.3.2. The number of haemolytic units (HUs) was calculated in all wells until 50% haemolysis was reached, and was expressed as HU/ml. The readings were taken after 30 min, 90 min, 4 hours, and 24 hours.

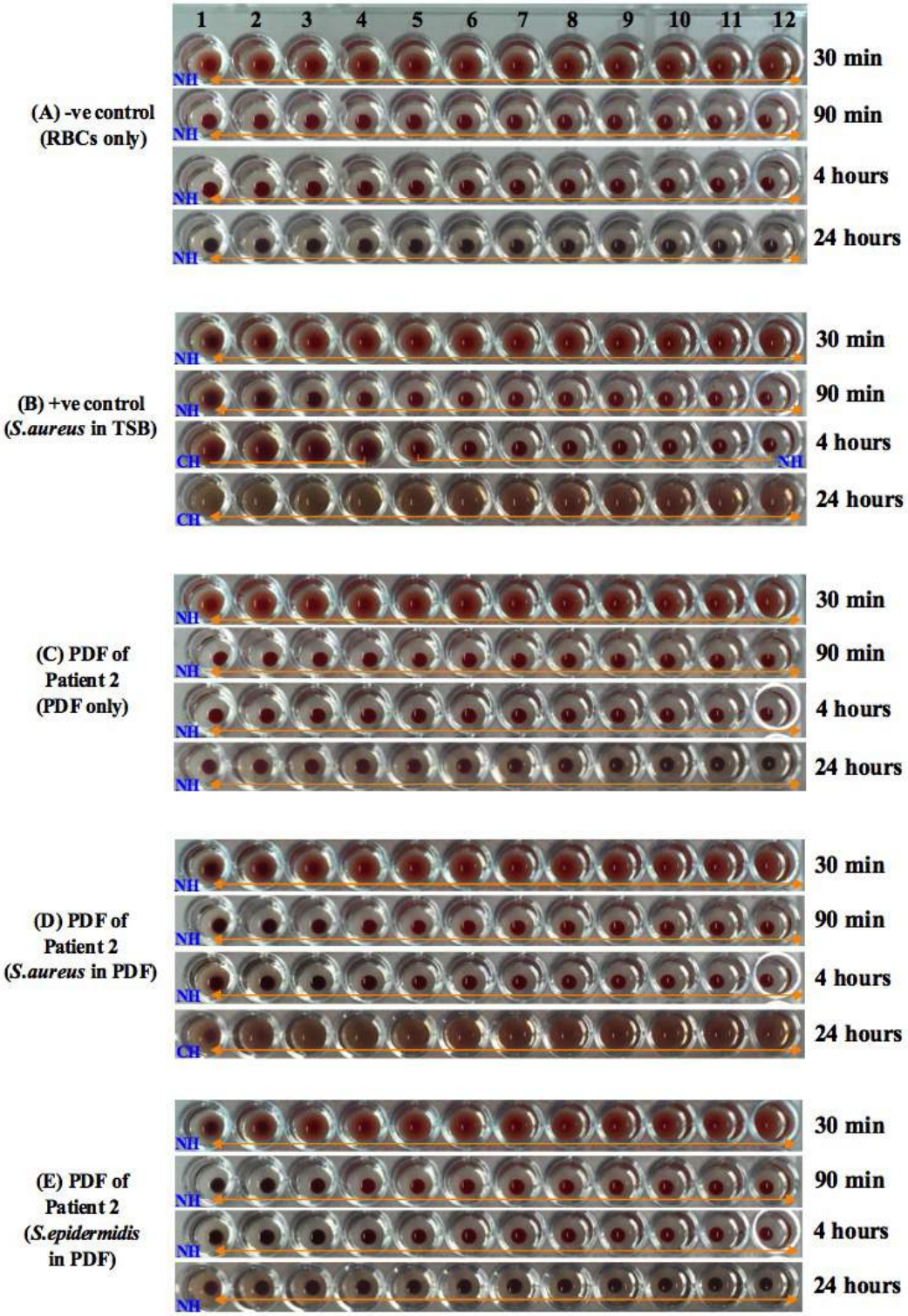


Figure 4.10 A representative example of the haemolytic activity assays.
Row A represents a two-fold serial dilution of RBCs in PBS to verify the autohaemolytic activity of the erythrocytes used in the assay. Row B illustrates the haemolytic activity of *S.aureus* (strain T288354) grown in TSB (Table 2.3) and then serially diluted in PBS. This condition was used as a positive control in the haemolytic activity assay. Row C shows that the PDF of Patient 2 collected after 1 day on PD course therapy (without staphylococci) produced no haemolytic (NH) activity. Row D demonstrates complete haemolysis (CH) of *S.aureus* (strain T288354) in the PDF of Patient 2 after 24 hours of incubation. Row E indicates that NH activity was produced in the PDF of Patient 2 by *S.epidermidis* (strain Tü3298) even after 24 hours of incubation.

4.2.3 Proteolytic activity

S.aureus and *S.epidermidis* produce a range of proteases that have a role in bacterial virulence and pathogenesis (Dubin *et al.*, 2001; Potempa & Travis, 2000). Extracellular protease production leads to tissue damage by degrading host-matrix proteins (Vuong & Otto, 2002). In this study, a proteolysis assay was used to examine the biological action of staphylococcal protease production following exposure to PDF. To conduct the assay, casein was used as a substrate in skimmed milk agar (SMA), as described by Kanekar *et al.* (2002) and Morris *et al.* (2012). Briefly, ~10^8 CFU/ml of *S.aureus* (strain T288354) or *S.epidermidis* (strain Tü3298) cultures were incubated overnight in PDF samples at 37°C. Next, the cultures were centrifuged to separate the supernatant, which contained exogenous proteases, from the staphylococcal pellets, which contained endogenous proteases (Section 2.2.3.3). PDF samples without staphylococci were also examined for their potential proteolytic activity. *S.aureus*, cultured in TSB (Table 2.3), was used as a positive control.

When using the initial PDF samples collected from the nine PD patients at the onset of their dialysis, as well as the follow-up samples, neither exogenous nor endogenous proteolytic activity by *S.epidermidis* was detected even after 48 hours incubation. In contrast, *S.aureus* produced both types of proteolytic activity in all of the PDF samples tested. In terms of a comparison between PDF samples between PD patients at the commencement of their dialysis, the majority stimulated more *S.aureus* endogenous than exogenous proteolytic activity (Figure 4.11). The highest level of both kinds of *S.aureus* protease activity was observed in the PDF of Patient 7, followed by that of Patient 6, which was as high as the level seen in the positive control (*S.aureus* in TSB). PDF samples without bacteria produced no proteolytic activity on SMA (Figure 4.12). In most of the follow-up PDF samples, the degree of endogenous proteolytic activity was higher than that of the exogenous activity (Figures 4.13, 4.14 and 4.15). In addition, there were no major variations between the proteolytic activity of *S.aureus* in the initial PDF and that of the follow-up ones. Despite differences between the exogenous and endogenous proteolytic activity, the *S.aureus* bacterium was able to produce protease enzyme when exposed to PDF samples.

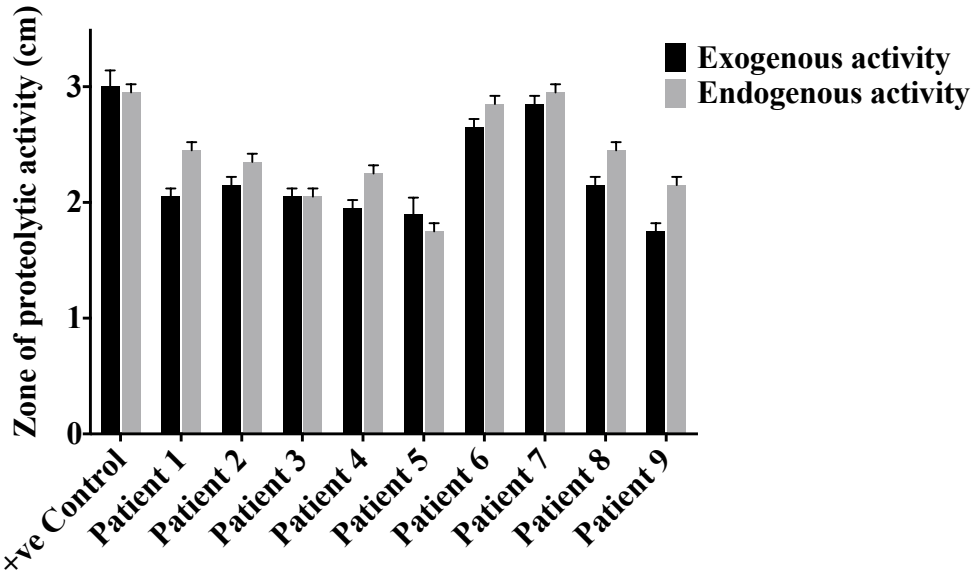


Figure 4.11 *S.aureus* endogenous and exogenous proteolytic activity in PDF samples of nine PD patients at the onset of dialysis.
10 µl of the filter-sterilised supernatant and washed pellet of *S.aureus*, which were exposed to different PDF samples, were inoculated onto SMA. The proteolytic activity of *S.aureus* was evaluated by measuring the diameter (cm) of the clear zones, which indicated proteolytic activity around the bacterial colonies (Section 2.2.3.3). *S.aureus*, cultured in TSB (Table 2.3), was used as a positive control.

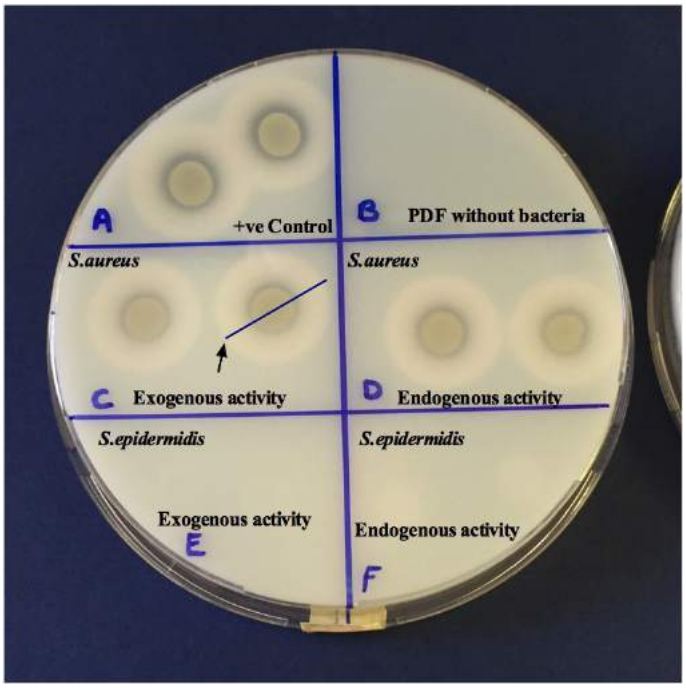


Figure 4.12 A representative example of detecting proteolytic activity on SMA.
The SMA plate was prepared as described in Section 2.2.3.3. (A) The positive control, which was *S.aureus* that was cultured in TSB (Table 2.3) and showed proteolytic activity. (B) The PDF sample without bacteria, which demonstrated no proteolytic activity. (C) and (D) show the exogenous and endogenous proteolytic activity of *S.aureus* incubated in PDF; (E) and (F) indicated no proteolytic activity being produced by *S.epidermidis*. The proteolytic activity of *S.aureus* was evaluated by measuring the diameter (black arrow) of the clear zones, which indicated proteolytic activity around the bacterial colonies.

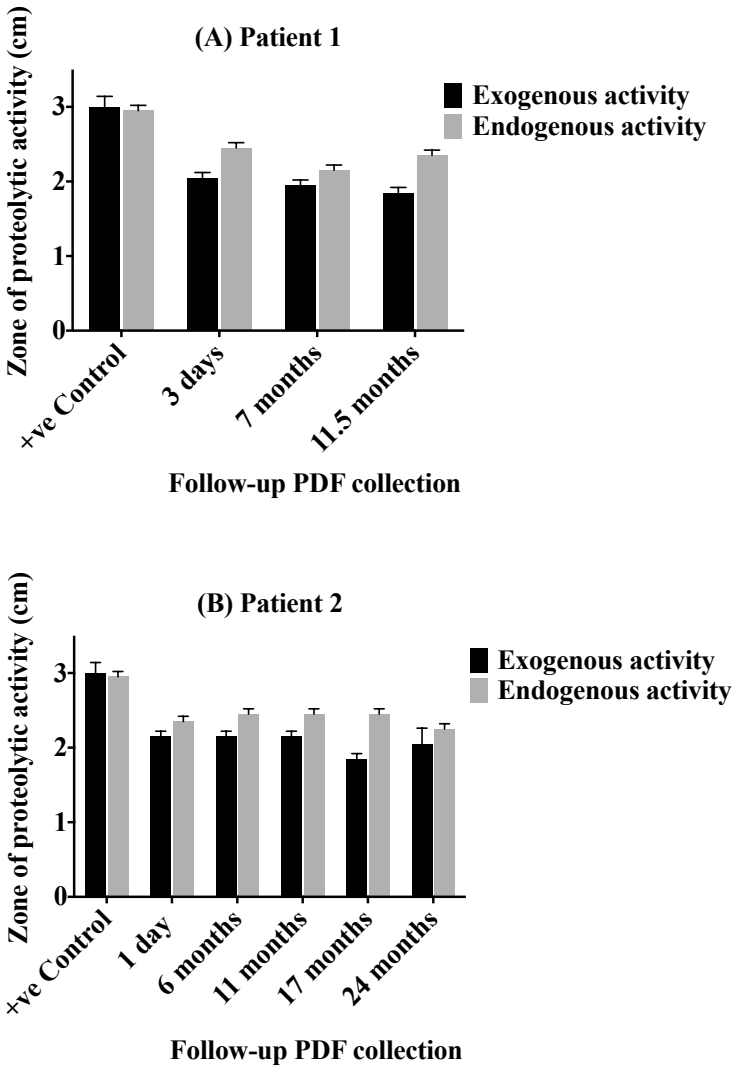


Figure 4.13 *S.aureus* proteolytic activity in follow-up PDFs of Patients 1 and 2.
10 µl of the filter-sterilised supernatant and washed pellet of *S.aureus*, which were exposed to different PDF samples, were inoculated onto SMA. The proteolytic activity of *S.aureus* was evaluated by measuring the diameter (cm) of the clear zones, which indicated proteolytic activity around the bacterial colonies (Section 2.2.3.3). *S.aureus*, cultured in TSB (Table 2.3), was used as a positive control.

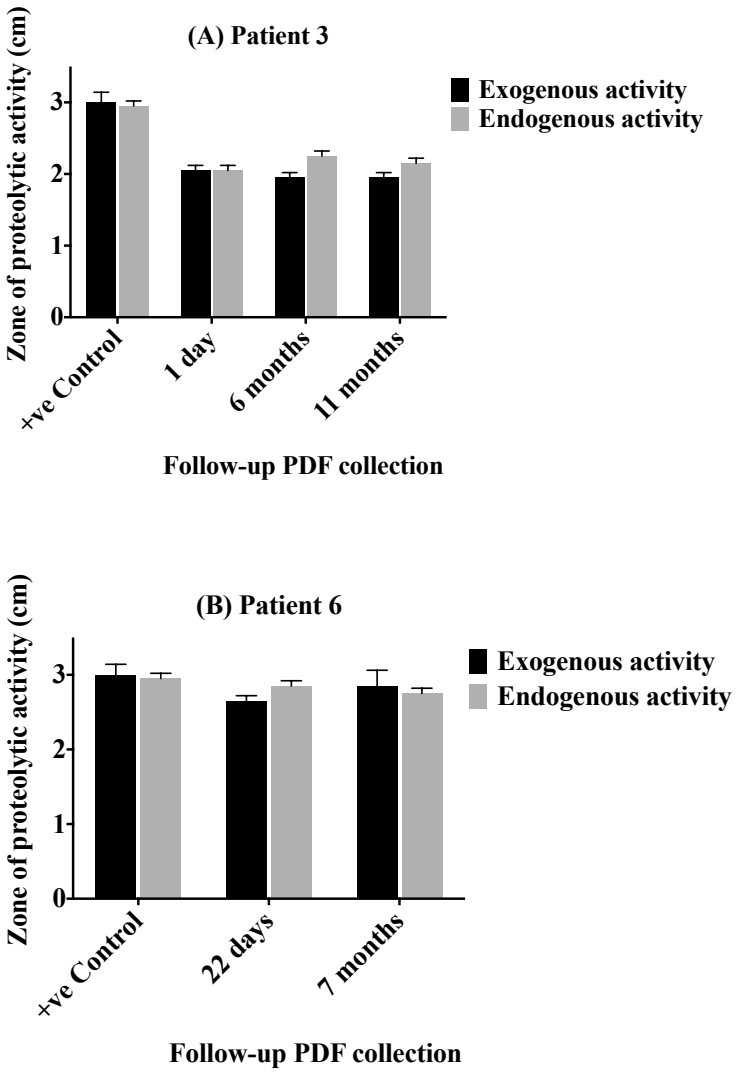


Figure 4.14 *S.aureus* proteolytic activity in follow-up PDFs of Patients 3 and 6.
10 µl of the filter-sterilised supernatant and washed pellet of *S.aureus*, which were exposed to different PDF samples, were inoculated onto SMA. The proteolytic activity of *S.aureus* was evaluated by measuring the diameter (cm) of the clear zones, which indicated proteolytic activity around the bacterial colonies (Section 2.2.3.3). *S.aureus*, cultured in TSB (Table 2.3), was used as a positive control.

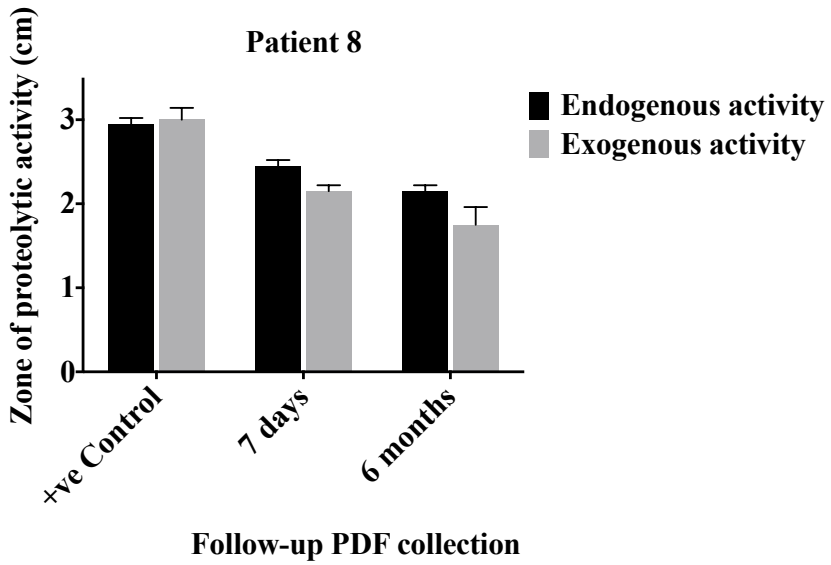


Figure 4.15 *S.aureus* proteolytic activity in follow-up PDFs of Patient 8.
10 µl of the filter-sterilised supernatant and washed pellet of *S.aureus*, which were exposed to different PDF samples, were inoculated onto SMA. The proteolytic activity of *S.aureus* was evaluated by measuring the diameter (cm) of the clear zones, which indicated proteolytic activity around the bacterial colonies (Section 2.2.3.3). *S.aureus*, cultured in TSB (Table 2.3), was used as a positive control.

4.3 Discussion

It was demonstrated in the previous chapter that the simultaneous presence of catecholamines and the iron-saturated transferrin in PDF had an impact on *S.aureus* and *S.epidermidis* growth. It was necessary therefore to examine whether the virulence of these two bacteria also was influenced by PDF.

The first virulence determinant examined was biofilm production. The term *biofilm* refers to the accumulation of bacterial cells on plastic surfaces, a process that enables bacteria to be resistant to antibiotics as well as to the host's immune system (Dasgupta, 2002; Otto, 2009). The results of this study indicated that in contrast to a glucose PD solution, the PD dialysate did enhance initial attachment and overall biofilm formation. This enhancement effect may be due to the presence of growth and biofilm-stimulating factors (iron-saturated transferrin and catecholamines) in PDF. Freestone *et al.* (2000, 2008) and Lyte *et al.* (2003) have reported that catecholamine inotropes not only stimulate the growth of staphylococci but also may affect bacterial biofilm development on biomaterials by removing iron from transferrin and subsequent use by bacteria. The data from Patient 4 were consistent with Freestone *et al.* (2000, 2008) and Lyte *et al.* (2003) findings, as the PDF from this patient, who had the highest levels of catecholamines, also stimulated the greatest amount of staphylococcal attachment. Another host-associated factor, which could increase biofilm formation on PD catheters, is the presence of fibrinogen (Otto, 2009; Vuong & Otto, 2002). Otto (2009) has demonstrated that biofilm formation is initiated by the direct attachment of free-floating (planktonic) bacteria to the catheter surface, followed by bacterial attachment to attached host-matrix proteins such as fibrinogen. Interestingly, the proteomic studies analysed in Chapter 3 (Table 3.5), revealed that all PD patients enrolled in this study lost considerable amounts of fibrinogen into their PD dialysates. It is therefore possible that the release of transferrin, catecholamines and fibrinogen into PDF could enhanced staphylococci attachment to the PD catheter and thereby participated in biofilm development.

Further virulence assays were performed for staphylococci exposed to PD dialysate to determine their haemolytic and proteolytic activities. The obtained results were dissimilar between the two species of staphylococci. PDF did not enhance the haemolytic activity of *S.epidermidis*, which was weakly haemolytic compared to *S.aureus*. Similarly, PDF did not stimulate the proteolytic activity of *S.epidermidis* in any of the PDF samples analysed. In contrast and not unexpectedly, *S.aureus* was strongly haemolytic and

proteolytic in PDF. From a biological point of view, *S.epidermidis*, which is a commensal expresses a very limited number of extracellular proteases and toxins compared to *S.aureus* (Vuong & Otto, 2002). Two types of proteases have been reported for *S.epidermidis*: cysteine protease and metalloprotease, both of which have a role in tissue damage (Vuong & Otto, 2002). In terms of haemolytic activity, *S.epidermidis* produces only one type of haemolytic toxin: the *N*-formylated alpha-helical peptide delta toxin, which leads to erythrocyte lysis by forming pores in the erythrocyte cytoplasmic membrane (Vuong & Otto, 2002). On the other hand, *S.aureus* produces three types of haemolytic toxins: haemotoxins, cytolysins and haemolysins, all of which play a role in destroying host tissue (Kerro Dego *et al.*, 2002). *S.aureus* also produces a wide range of extracellular proteases, including a metalloprotease, seven serine proteases and two cysteine proteases (Drapeau, 1978; Reed *et al.*, 2001; Filipek *et al.*, 2003). The presence of these multiple haemolytic toxins and extracellular proteases could explain differences in the haemolytic and proteolytic activities of *S.aureus* and *S.epidermidis* when exposed to PDF.

Collectively, these studies showed that the most important aspect of *S.aureus* and *S.epidermidis* virulence, biofilm formation, is enhanced by the presence of host-factors in PDF. Neither bacteria's haemolytic or proteolytic activities were greatly stimulated by PDF, but the virulence factor activities were still being expressed when the bacteria were incubated in PDF, suggesting that haemolytic and proteolytic activities could be involved in PD peritonitis.

CHAPTER 5 Profiling changes in PDF during health and infectious peritonitis: a case study

5.1 Introduction

Infectious peritonitis is a clinically significant condition considered to be a leading complication of PD treatment modality (Ellam & Wilkie, 2011; Zarrinkalam *et al.*, 2001; Kerschbaum *et al.*, 2012). It is also a contributing factor to the high mortality and morbidity rates observed in PD patients (Ellam & Wilkie, 2011; Zarrinkalam *et al.*, 2001; Kerschbaum *et al.*, 2012; Li, 2001). One of the main catastrophic peritonitis complications is repeated peritonitis, which results in long-term ultrafiltration failure and necessitates PD termination and switching to haemodiaysis (Ellam & Wilkie, 2011; Zarrinkalam *et al.*, 2001; Kerschbaum *et al.*, 2012; Li, 2001).

Patient 6, who was one of the PD patients recruited to the current study (Table 3.1) and whose PDF due to family connections was available on a very regular basis, will be intensively studied in this chapter. I greatly appreciate her donation of her PDFs to this study. She is 54 years old, diabetic and hypertensive. She used beta-blockers as hypertension management drugs. The cause of her renal failure was hypertensive nephrosclerosis. She was placed on APD as an initial RRT and she commenced her dialysis in January 2014. In less than nine months from the start of the APD course, she suffered three confirmed cases of infectious peritonitis followed by an exit-site infection. As a result, her PD therapy was terminated in February 2015 and she transferred to haemodialysis. Hence, the whole period spent on APD was 14 months.

This was a unique study that presents a very unusual opportunity to characterise the PDF in both the healthy and infected states. It was therefore the overall objective of this chapter to investigate differences in the PDF before, during and after episodes of peritonitis. The first question that was exciting to investigate is "What was the bacteria responsible for the episodes of peritonitis? Was it from the gut or from the skin microflora?" Moreover, because protein loss into PDF is one of the main disadvantages of the PD therapy (Blumenkrantz *et al.*, 1981), it was therefore important to examine whether the protein loss or types of proteins lost during peritonitis was different from that before or after peritonitis. It was also decided to study if changes existed in the protein profile of the PDF before, during and after peritonitis. Were there particular protective proteins that disappeared in PDF during peritonitis? Another central objective of this chapter was to study changes in transferrin, iron and catecholamine stress hormones (NE, Adr and Dop) levels in PDF collected before, during and after peritonitis. The growth response was also analysed for bacteria causing most PD patient infections, *S.aureus* and *S.epidermidis*, in

non-infected normal PDF and peritonitis PDF. The last aim of the current chapter was to analyse differences in the uptake of radioactivity by staphylococcal cells in non-infected normal PDF and peritonitis PDF by using ^{55}Fe complexed with transferrin. These comparative investigations were addressed by subjecting the follow-up PDFs of Patient 6 to a comprehensive analysis using weekly PDFs—and later in the study following episodes of peritonitis daily PDFs.

5.1.1 Timelines of infectious peritonitis

Within four months of dialysis initiation, Patient 6 suffered from mild diarrhoea and stomach ache, with a slightly high temperature of 37.5°C. On the following day, her body temperature rose to 38.7°C and she suffered severe abdominal pain. The PDF collected that night was turbid. She was admitted to Leicester General Hospital and the diagnosis was confirmed as a poly-microbial gut-associated peritonitis leading to antibiotic prescription and recovery. The second episode of peritonitis was from the same origin and occurred five weeks after the first one. It developed very quickly, as the day-time PDF was clear, without signs of infection, while the night-time PDF (on the same day) showed the characteristic turbidity along with rapid development of abdominal pain and fever. Patient 6 was again admitted to the hospital and recovered. Two months after the second episode of peritonitis, Patient 6 again suffered from peritonitis and she was admitted to the same hospital. Her vital signs and PDF characteristics were similar to those in the first and second episodes of peritonitis, as was also the gut as the source of infection. Consequently, within nine months of APD therapy duration, Patient 6 experienced three confirmed cases of infectious peritonitis. In addition to the episodes of peritonitis, Patient 6 developed an exit-site infection starting three weeks after the third episode of peritonitis. Oral ciprofloxacin was prescribed and vancomycin was introduced into her body intravenously during the episodes of peritonitis, whereas erythromycin and fusidic acid cream were prescribed for her to treat the exit-site infection combined with a ciprofloxacin oral antibiotic course. For Patient 6, PDF samples were collected at the point of discovery of the peritonitis, and as soon as possible after admission and antibiotic treatment. However, accessing the PDF samples while hospitalised was not always possible, and so there were some gaps in sample collection immediately post infection diagnosis.

5.1.2 Scenario applied to collecting follow-up PDFs

At the beginning of Patient 6 dialysis, it was decided to analyse PDF twice weekly. However, immediately after she experienced the first episode of peritonitis, the decision was made to collect daily PDFs rather than weekly to take any opportunity in characterising the PDF before and during peritonitis in case the patient experienced another episode in the future (Figure 5.1). The PDFs were aliquoted under aseptic conditions into sterile tubes and kept at -20°C until analysed.

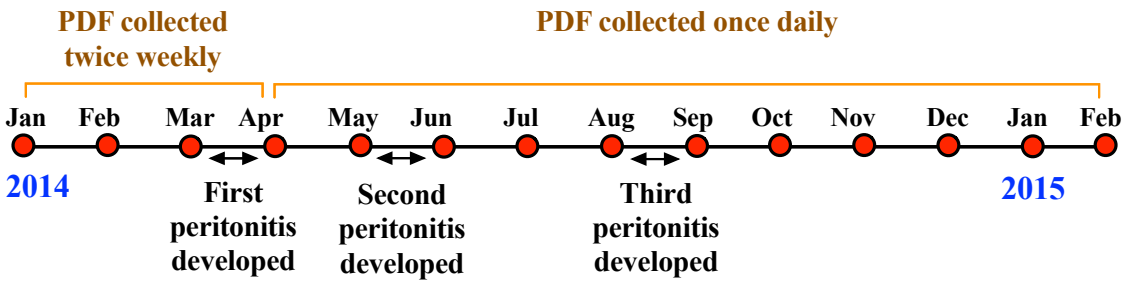


Figure 5.1 Timetable of Patient 6 infectious peritonitis development.
Patient 6 started APD in Jan 2014 and due to episodes of peritonitis her dialysis was terminated in Feb 2015. Between Mar & Apr and May & Jun 2014 she experienced peritonitis, whereas between Aug and Sep 2014 she suffered peritonitis as well as exit-site infection. From Jan to Apr the PDF samples were collected twice weekly. Following that, the PDF samples were picked up from the patient on a daily basis.

5.1.3 Types of PDS used during APD

Patient 6 largely used a glucose-based PDS and occasionally an icodextrin-based PDS over the dialysis duration. The PD solutions were provided by Dr. Jonathan Barratt, Leicester General Hospital. Icodextrin is a high molecular weight glucose polymer (average M.W 17,000 Da) that acts as an osmotic agent alternative to glucose (Hain & Kessel, 1987; Levy *et al.*, 2015). Compared to glucose, icodextrin achieves more sustained, positive net ultrafiltration rates over long exchange periods. In addition, it causes lower deterioration in the peritoneal membrane function (Hain & Kessel, 1987; Levy *et al.*, 2015). The concentration of glucose used in the APD PDS was 22.7 g/L of glucose, whereas the concentration of icodextrin was 75 g/L of icodextrin. Both the glucose and icodextrin PD solutions were manufactured by Baxter Healthcare Ltd., UK. The actual solute composition of the glucose PDS was described previously in Table 2.2. However, Table 5.1 provides information about the actual solute composition of the 7.5% icodextrin PDS (as according to the manufacturer's manual).

Table 5.1 Solute composition of the 7.5% icodextrin PDS.

Component	Concentration
Icodextrin	75 g/L
Sodium chloride	5.4 g/L
Calcium chloride	0.257 g/L
Magnesium chloride	0.051 g/L
Sodium lactate	4.5 g/L
pH	5 to 6

5.2 Specific methods

5.2.1 Microscopic examination of PDF

One ml of the PDF was pipetted into the wells of a 24-well plate (Nunc™ Cell Culture Treated, Thermo Fisher Scientific). Then, the plates were examined for the presence of WBCs and bacteria under the microscope using a 40x objective lens. Photographs were taken of the appearance of the PDF using an inverted microscope, which was connected to DeltaPix InSight Software.

5.2.2 Observation of WBCs

If the PDF indicated the presence of WBCs as described in the preceding methodology (Section 5.2.1), Wright's staining protocol was performed on the PDF to provide an overview of the types of WBCs present. First, a smear of the PDF was fixed onto a clean glass microscopic slide and allowed to air-dry completely. Afterwards, Wright's staining procedures were performed as described by Yam *et al.* (1971):

1. Following fixation, each slide was covered with 1.0 ml of Wright's stain solution for 1-3 min.
2. Approximately 2.0 ml of distilled water was added to each slide for 2-6 min.
3. The slides were then washed with distilled water until a slight pinkish-red colour appeared on the edges.
4. Finally, the slides were dried at RT, then examined using 100x oil immersion bright field objectives.

5.2.3 Identification of unknown bacteria that cause infectious peritonitis

5.2.3.1 Bacterial culture media

In addition to the TSB and TSA, which were prepared according to the methodology described previously in Table 2.3, three types of growth media were used. Those were a brain heart infusion (BHI) broth, a MacConkey agar and a citrate agar. They were purchased from Oxoid, UK and prepared according to the manufacturer's instructions (Table 5.2).

Table 5.2 Preparation of bacterial growth media.

Media	Preparation
BHI broth	3.7 g of BHI medium were dissolved in 100 ml of distilled water.
MacConkey agar	5.2 g of MacConkey medium were mixed with 100 ml of distilled water.
Citrate agar	2.3 g of citrate medium were added to 100 ml of distilled water.

The bacterial culture media were prepared, and then sterilised by autoclaving at 121°C, 15 psi for 15 min. After sterilisation, broth (liquid) media were kept at RT until used, whereas agar media were cooled to around 50°C, poured into sterile plastic Petri dishes at ~20 ml per Petri dish and kept at 4°C until utilised.

5.2.3.2 Extraction and isolation of unknown bacteria from peritonitis PDF

PDFs that were collected from the patient at the days of peritonitis and showed turbidity were centrifuged for 10 min at 1,734×g. The pellets were re-suspended in the BHI broth medium. The re-suspended pellets were then incubated at 37°C in a shaking incubator overnight. Afterward, a streak plate isolation technique was used to produce pure colonies as described by Baker *et al.* (2011).

5.2.3.3 Identification of Gram-negative bacilli by API 20 Enterobacteriaceae kit

Gram-negative bacillus bacteria, which were isolated from the peritonitis PDFs, were identified using an analytical profile index (API) kit specially designed to identify Enterobacteriaceae and other non-fastidious Gram-negative bacilli (API® BioMerieux SA). To prepare the bacterial suspension, a single colony was inoculated into 3 ml of sterile water. The API 20 E strip contained 20 microtubes comprised of dehydrated substrates. Each microtube was inoculated with a bacterial suspension that re-constituted the medium. Afterward, the strips were incubated at 37°C for 24 hours. After incubation, the strips were read by referring to the reading table provided with the kit to generate a

7-digit profile number. With the assistance of *apiweb*^{TM} identification software, the 7-digit number was manually entered to provide the bacterial species name.

5.3 Results

5.3.1 Macroscopic and microscopic examinations of PDF

As mentioned in Chapter 1 (Section 1.5.2.2), cloudy PDF is a principal sign of acute infectious peritonitis (Levy *et al.*, 2015). Upon macroscopic investigation, the PDFs obtained from Patient 6 on days that coincided with peritonitis demonstrated significant turbidity (Figure 5.2 A). However, in non-infected normal PDFs taken when the patient underwent routine dialysis without signs or symptoms of peritonitis the PDFs were clear. Microscopic examinations of the turbid infected PDFs indicated the presence of bacterial aggregations as well as a huge number of WBCs, whereas the non-infected normal PDFs demonstrated very few WBCs and no bacteria (Figure 5.2 B, C and D). The most predominant WBCs detected were neutrophilic granulocytes, the presence of which was an indication of acute bacterial infection (Brinkmann *et al.*, 2004) (Figure 5.2 E). Further tests were also performed to identify bacteria that caused peritonitis and this is analysed in Section 5.3.2.

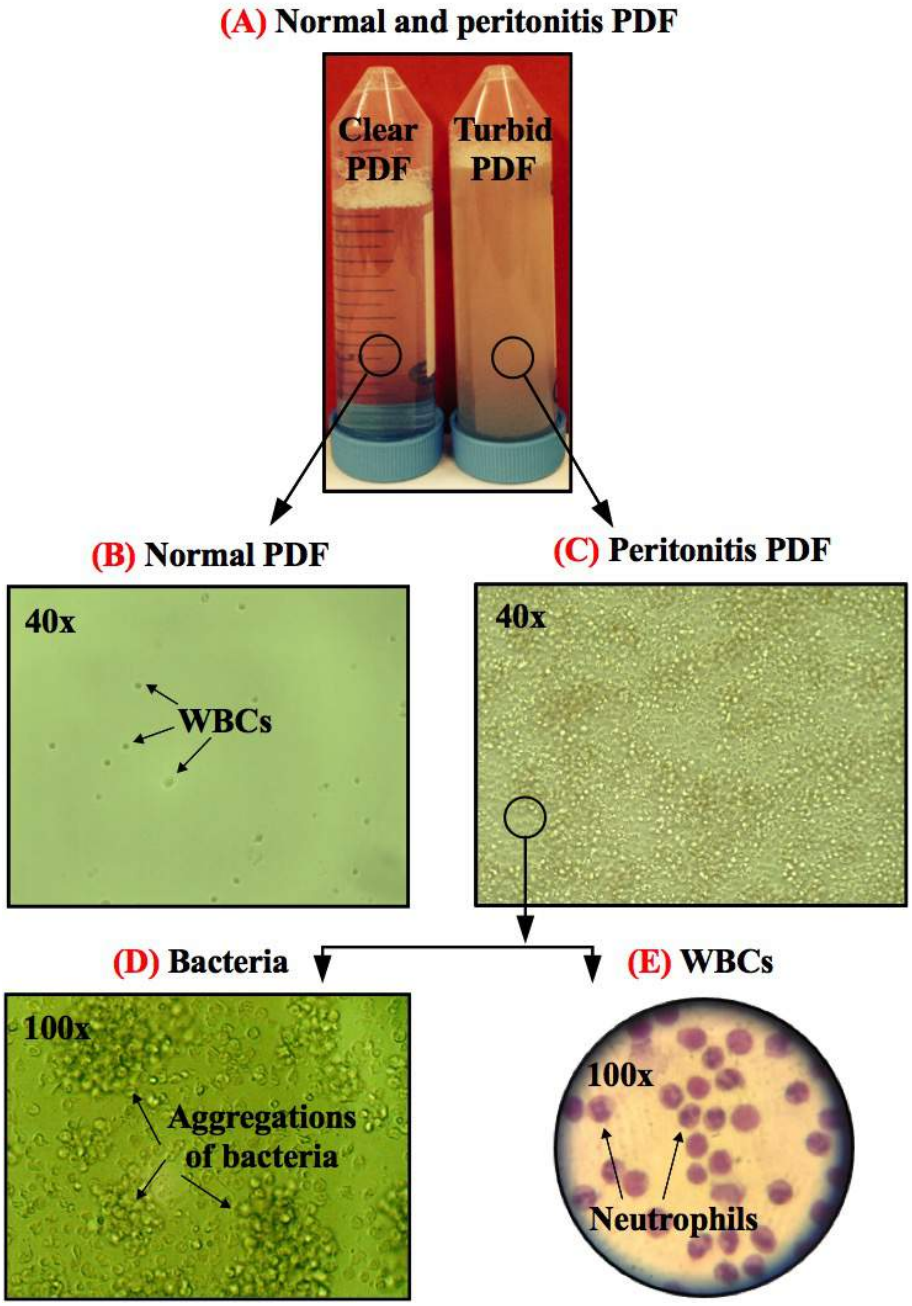


Figure 5.2 Macroscopic and microscopic appearance of non-infected normal and peritonitis PDF. (A) The non-infected normal (clear) and peritonitis (turbid) PDF samples in 50 ml sterile falcon tubes. (B) Appearance of the non-infected normal PDF under the microscope (40x magnification), which demonstrated the presence of a limited number of WBCs. (C) Appearance of the peritonitis PDF under the microscope (40x magnification), which showed abundant WBCs and bacteria. (D) 100x magnification of the turbid PDF showed a population of bacterial cells. (E) Wright's stain of the peritonitis PDF showed neutrophilic granulocytes.

5.3.2 Identification of peritonitis causing bacteria

Gram staining procedures are considered to be valuable diagnostic tools in clinical and research situations as they distinguish between Gram-positive and Gram-negative bacteria (Levy *et al.*, 1988). Therefore, Gram staining was utilised in this study to detect the presence of bacteria in the peritonitis PDFs collected during the first and second peritonitis. The Gram staining protocol was performed on the isolated bacterial colonies to identify their Gram reactions and later to confirm the purity of bacteria for further examination. The results of using this protocol to the peritonitis PDFs from the first and second peritonitis were similar. As demonstrated in Figure 5.3, two groups of bacteria with different morphologies were identified: Gram-positive (blue cells) and Gram-negative (red cells) bacteria. Peritoneal infections were found to be polymicrobial in nature and later investigations revealed that they were of gut origin, the PD catheter had nicked the colon and released a diverse microbial population that resulted in an instant infection. The Gram-negative bacterium that appeared most frequently was isolated from the mixed culture using a streak plate isolation technique to conduct bacterial identification studies.

The unknown bacteria isolated from the first and second peritonitis PDFs were subjected to a set of microbiology biochemical tests to provide us with a general idea of what we were looking at. As a result, we observed that the bacteria from the first and second peritonitis PDFs acted as a lactose fermenter, produced mucoid pink colonies on MacConkey agar and utilised citrate as a carbon source. Accordingly, the bacteria appeared to belong to the Enterobacteriaceae family. Figure 5.4 shows the biochemical test results of the API kit applied to the unknown bacteria. The positive and negative results were analysed as described in Section 5.2.3.3 to produce a 7-digit number, which was: 5215773. This number was entered manually into the *apiweb*^{TM} database to determine the name of the mystery bacteria. Interestingly, it was found the species was *Klebsiella pneumoniae*, with a 97% probability.

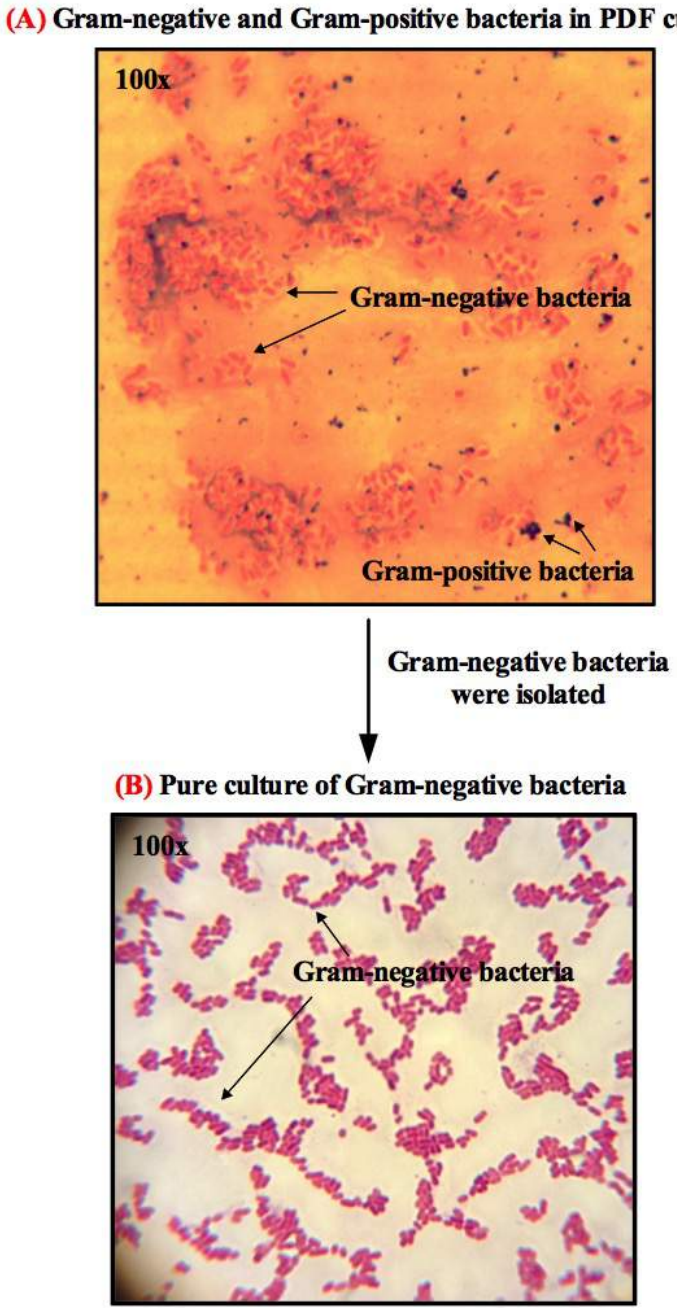


Figure 5.3 Gram staining outcomes of peritonitis PDF.
The peritonitis PDFs collected from Patient 6 during the first and second peritonitis were cultured in a highly nutritious medium (BHI) (Table 5.2). (A) Gram staining protocol was performed on the cultured peritonitis PDF and indicated the presence of both Gram-positive and Gram-negative bacteria. (B) Gram-negative bacterium was isolated using a streak plate isolation technique, and then re-stained to confirm the purity of the culture.

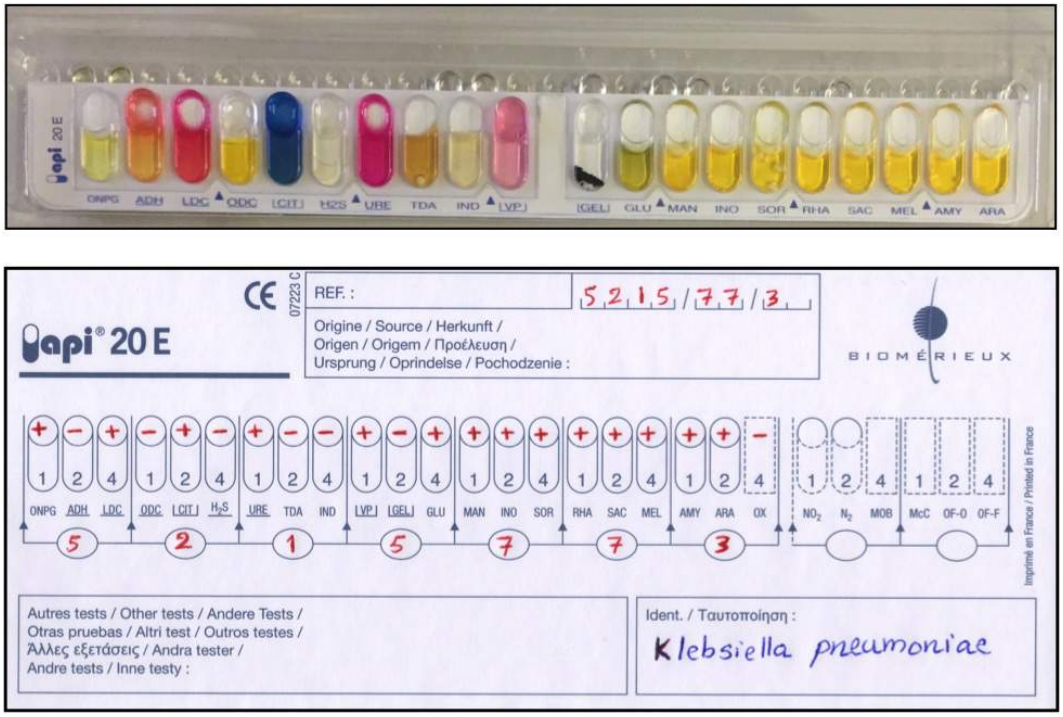


Figure 5.4 The API 20 Enterobacteriaceae identification system, which consists of a series of diagnostic tests, was used to identify the unknown bacterium isolated from the first peritonitis PDF. The test results for the unidentified bacterium isolated from the second peritonitis PDF were identical to the results from the tests of the first peritonitis PDF. In both cases, the positive and negative results were read by referring to a table provided with the kit, which produced the seven-digit number 5215773. This number was entered manually into the *apiweb*^{TM} database, which identified the unknown bacterium at the genus and species level as *Klebsiella pneumoniae*.

5.3.3 Investigation of protein level in PDF during health and infectious peritonitis

Protein loss into PDF is one of the major complications of PD therapy (Blumenkrantz *et al.*, 1981), and it was hypothesised that it associates with an increased susceptibility to development of peritoneal infection. Accessibility of PDF from Patient 6 before, during and after episodes of peritonitis was a golden opportunity to assess the changes that may occurred to the protein level in the PDF. A Bio-Rad protein assay kit was used to measure the protein level in the PDF (Section 2.2.1.6). Since the peritonitis PDFs contained a huge number of WBCs and bacteria, the PDFs were initially filter sterilised through a 0.2-μm pore size syringe unit filter and then assessed for the protein level.

5.3.3.1 Trend in the protein level in PDF over 14 months on APD

The duration that the patient spent on the APD was 14 months starting from January 2014 and terminating in February 2015 (Figure 5.1). Over this period, 258 PDFs were obtained including those when the patient was suffering from peritonitis. Generally speaking, the protein level in the PDF markedly varied over the dialysis time (Figure 5.5). The mean protein level in the PDF over the course of dialysis was 0.365 ± 0.289 g/L, with a highest value after seven months and lowest after 11 months on APD of 3.802 ± 0.013 g/L and 0.0248 ± 0.001 g/L, respectively. Interestingly, the greatest protein level was detected in the third peritonitis PDF, whereas the lowest protein level was observed in non-infected normal PDF (obtained after 11 months on APD).

5.3.3.2 Protein level in PDF during infectious peritonitis

There were marked changes in the protein level in the PDFs collected during the first, second and third peritonitis. As shown in Figure 5.5, there was an increase in the protein level in the PDFs over the three peritonitis episodes. For instance, the protein level in the first peritonitis PDF was 0.967 ± 0.001 g/L and clearly rose to 1.251 ± 0.001 g/L during second episode. It then reached its greatest level in the third peritonitis PDF at 3.802 ± 0.013 g/L. In contrast to the episodes of peritonitis, the protein level in PDF obtained during the exit-site infection was considerably lower than during the episodes of peritonitis. Overall, consistent increases in the protein level in the PDF were observed following each episode of peritonitis.

Comparative studies were also performed to investigate changes in the protein profile in non-infected normal PDFs (gathered before and after peritonitis), and in the peritonitis PDFs (obtained on the days of peritonitis). It was apparent that during each episode of peritonitis the protein level in the PDF reached its highest level compared to that in the PDF before and after peritonitis. For instance, the protein level in the PDF collected one day before the third peritonitis was 0.095 ± 0.001 g/L, however, it rose sharply to 3.802 ± 0.013 g/L on the day of occurrence, and then noticeably dropped to 0.544 ± 0.001 g/L five days afterwards (Figure 5.5).

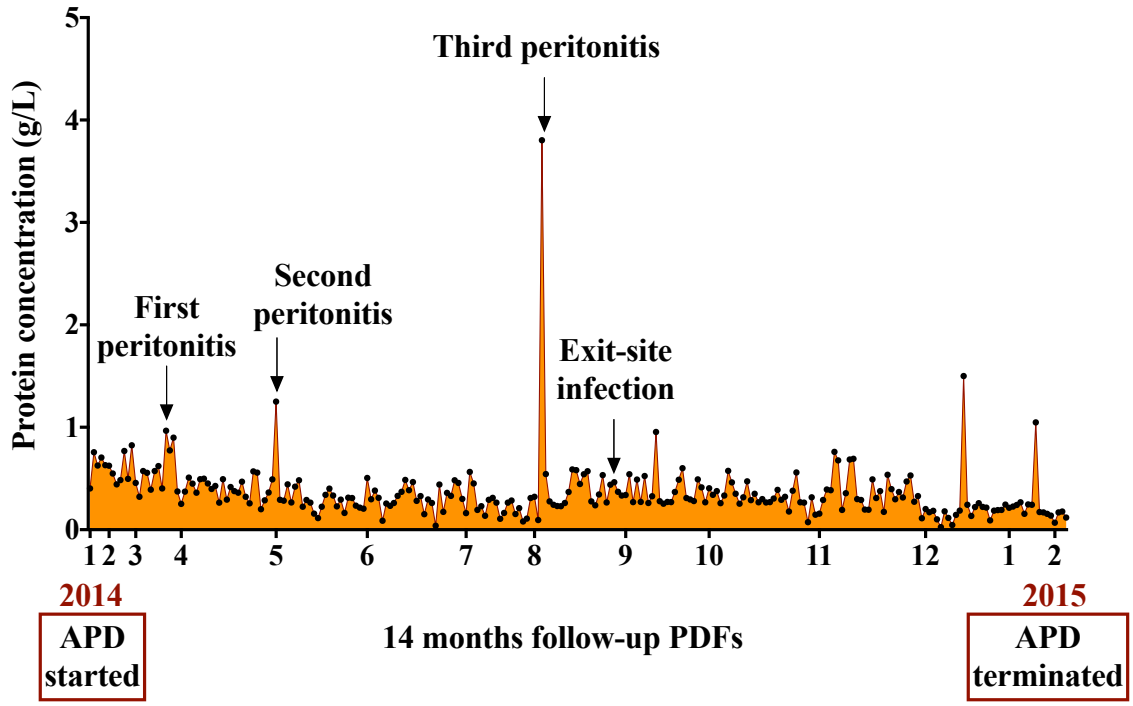


Figure 5.5 Protein concentration in PDFs collected over 14 months on APD.
Numbers at the bottom of the graph refer to the calendar months. Patient 6 began APD in January 2014 and ceased in February 2015. There were 258 PDFs collected during these 14 months and they were analysed for protein concentration using the Bio-Rad protein assay kit (Section 2.2.1.6).

5.3.3.3 Protein lost in PDF during infectious peritonitis

As Patient 6 used APD, 12 L of the PDS were used per exchange. Accordingly, the amount of protein lost by the patient during a dialysis exchange can be determined. Over the 258 PDFs analysed, the mean protein loss during the dialysis exchange was determined at 4.382 ± 3.472 g/exchange. The highest protein loss analysed was detected in the third peritonitis PDF (45.628 ± 0.161 g/exchange), while the lowest protein loss was observed in non-infected normal PDF (0.2982 ± 0.014 g/exchange) (obtained after 11 months on APD). Table 5.3 gives information about the protein loss into the PDF

during the dialysis exchange before, during and after the three episodes of peritonitis as well as detailing the exit-site infection.

Table 5.3 Mean protein level and the determined protein loss in the PDF of Patient 6 before, during and after the three episodes of peritonitis as well as the exit-site infection.

	PDF collection time	Protein level (g/L)	Protein loss (g)/APD exchange
First peritonitis	6 days before peritonitis	0.621 ± 0.003	7.453 ± 0.039
	2 days before peritonitis	0.403 ± 0.002	4.841 ± 0.025
	Day of peritonitis	**0.967 ± 0.001**	**11.599 ± 0.007**
	1 day after peritonitis	0.775 ± 0.002	9.295 ± 0.028
	2 days after peritonitis	0.899 ± 0.002	10.788 ± 0.021
Second peritonitis	2 days before peritonitis	0.363 ± 0.005	4.358 ± 0.061
	1 day before peritonitis	0.492 ± 0.003	5.902 ± 0.005
	Day of peritonitis	**1.251 ± 0.001**	**15.014 ± 0.010**
	7 days after peritonitis	0.293 ± 0.003	3.512 ± 0.005
	8 days after peritonitis	0.284 ± 0.004	3.406 ± 0.002
Third peritonitis	4 days before peritonitis	0.322 ± 0.002	3.860 ± 0.025
	1 day before peritonitis	0.095 ± 0.001	1.143 ± 0.008
	Day of peritonitis	**3.802 ± 0.013**	**45.628 ± 0.161**
	5 days after peritonitis	0.544 ± 0.001	6.533 ± 0.008
	6 days after peritonitis	0.277 ± 0.001	3.328 ± 0.006
Exit-site infection	2 days before infection	0.266 ± 0.002	3.192 ± 0.022
	1 day before infection	0.439 ± 0.003	5.264 ± 0.039
	Day of exit-site infection	**0.464 ± 0.009**	**5.574 ± 0.107**
	1 day after infection	0.370 ± 0.003	4.440 ± 0.032
	2 days after infection	0.334 ± 0.005	4.005 ± 0.005

5.3.4 Investigation of PDF protein profiles

It was shown in Figure 5.5 that the trend of the protein lost into the PDF fluctuated during the 14 months of dialysis treatment. Furthermore, on the days of the three episodes of peritonitis the protein lost into the PDF was clearly higher compared with the days before or after the episodes of peritonitis. These results prompted further analysis on whether Patient 6 lost different types of proteins into the PDF during her 14 months of dialysis treatment, and so SDS-PAGE comparison studies were performed as described in Section 2.2.1.7.

5.3.4.1 Protein profiles of PDF during health and infectious peritonitis

The first PDF collected from the patient when she did her first dialysis treatment in January 2014 and the last PDF obtained from her before she terminated her treatment in February 2015 were examined. Since the patient spent 14 months on dialysis, an individual PDF obtained from her at the beginning of each calendar month was also analysed. In total, 14 non-infected normal PDFs were assessed for changes in the protein profile. The three percent of SDS-PAGE gels (8%, 12% and 15%) demonstrated that Patient 6 released the same type and number of proteins in the non-infected normal PDFs during the 14 months of dialysis treatment (Figure 5.6). However, the protein profiles had a markedly different appearance during peritoneal infection, which is investigated in Section 5.3.4.2.

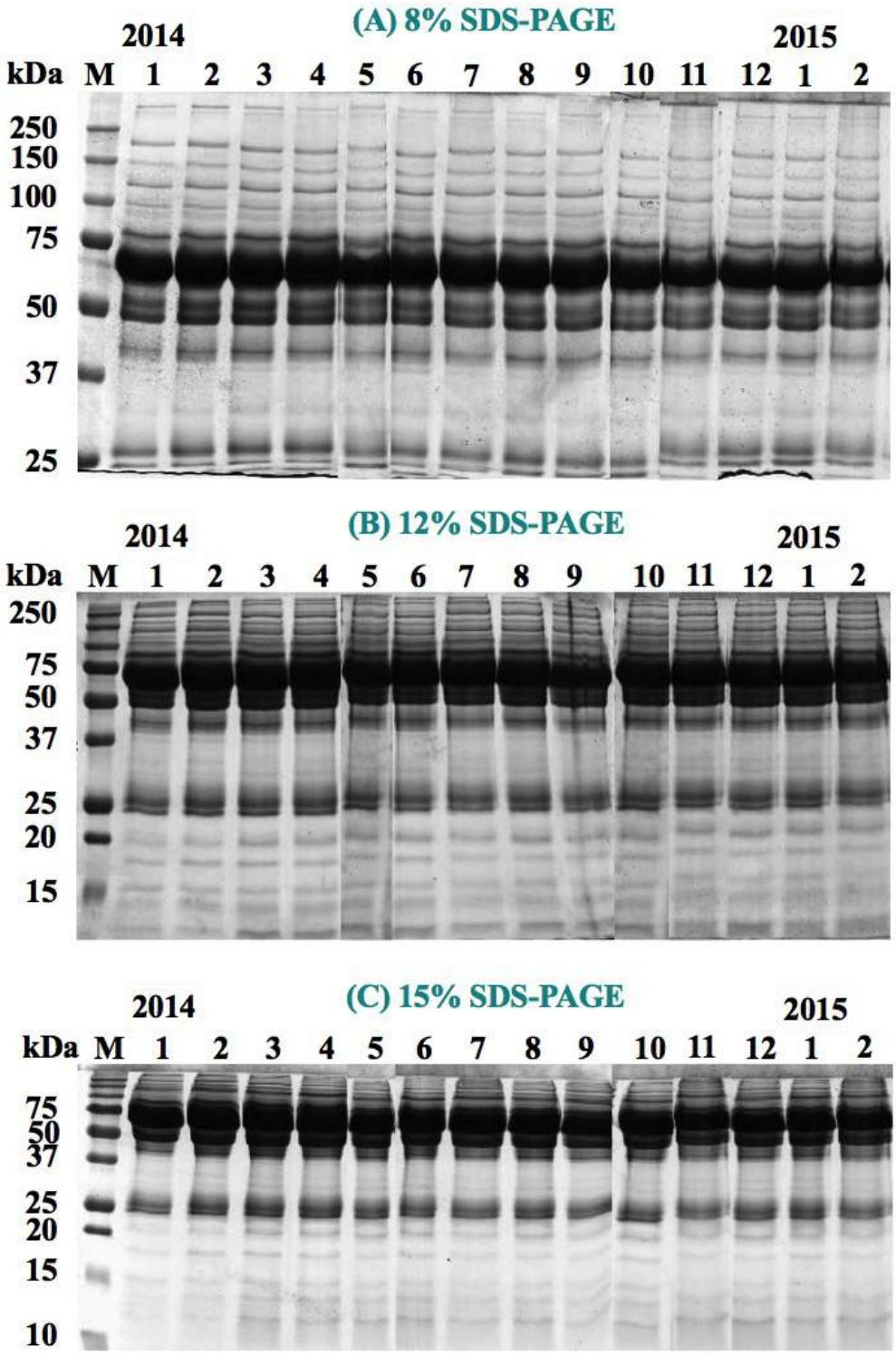


Figure 5.6 Comparative protein profiles of non-infected normal monthly PDFs obtained from Patient 6 during 14 months of dialysis treatment using SDS-PAGE gels.
Numbers at the top of each gel refer to the calendar months. Non-infected normal PDFs including the first PDF collected when she did her first dialysis therapy in January 2014 and the last PDF obtained before she switched to haemodialysis in February 2015. Non-infected normal monthly PDFs gathered at the start of each calendar month were also analysed. A constant quantity of 20 µg of protein of each PDF was loaded per lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7). The M.W of the PDF proteins was estimated using protein molecular weight markers (M). 'kDa' (kilodalton).

5.3.4.2 Protein profiles of PDF during infectious peritonitis

On the whole, marked changes were seen in the protein patterns in the first and second peritonitis PDFs compared with the non-infected normal PDFs (obtained before peritonitis). On the 8% SDS-PAGE gels, protein numbers 1, 2 (> 250 kDa), 3 (163 kDa), 4 (140 kDa), 5 (128 kDa) and 6 (100 kDa) (Figures 5.7 A and 5.8 A) almost disappeared in the first and second peritonitis PDFs compared with the non-infected normal PDFs, which suggests that these proteins were released in response to the peritonitis. Furthermore, the 15% SDS-PAGE gels (Figures 5.7 C and 5.8 C) indicated the presence of a novel protein with M.W 24 kDa (number 19) and proteins of 15 kDa, 12.5 kDa and 12 kDa (numbers 20, 21 and 22, respectively) in the first and second peritonitis PDFs. The first peritonitis PDF showed a higher level of protein number 12 (41 kDa) compared to the non-infected normal PDF (Figure 5.7 A).

The PDF collected the day after the first peritonitis had approximately the same profile as the peritonitis dialysate. However, two days later as the infection resolved, the proteins released into the PDF were similar to those before peritonitis. Since the PDF obtained after the second peritonitis was accessible only after seven days of peritonitis when the patient had recovered from it, it represented the same protein profile as the non-infected normal one. Proteins number 7 (80 kDa) and 8 (70 kDa) showed changes in protein levels in the first and second peritonitis PDFs compared to the non-infected normal ones. On the other hand, proteins number 9 (63 kDa), 10 (50 kDa), 11 (45 kDa), 13 (25 kDa), 14 (24 kDa), 15 (23 kDa), 16 (12 kDa), 17 (19 kDa) and 18 (16 kDa) demonstrated no marked differences in the protein levels between the non-infected normal PDFs and peritonitis PDFs. During the third peritonitis the protein profile was similar to that before and after peritonitis as shown in Figure 5.9. The exit-site infection tended to have no influence on the protein profile of the PDF, which is probably because this was a localised skin infection (Figure 5.10).

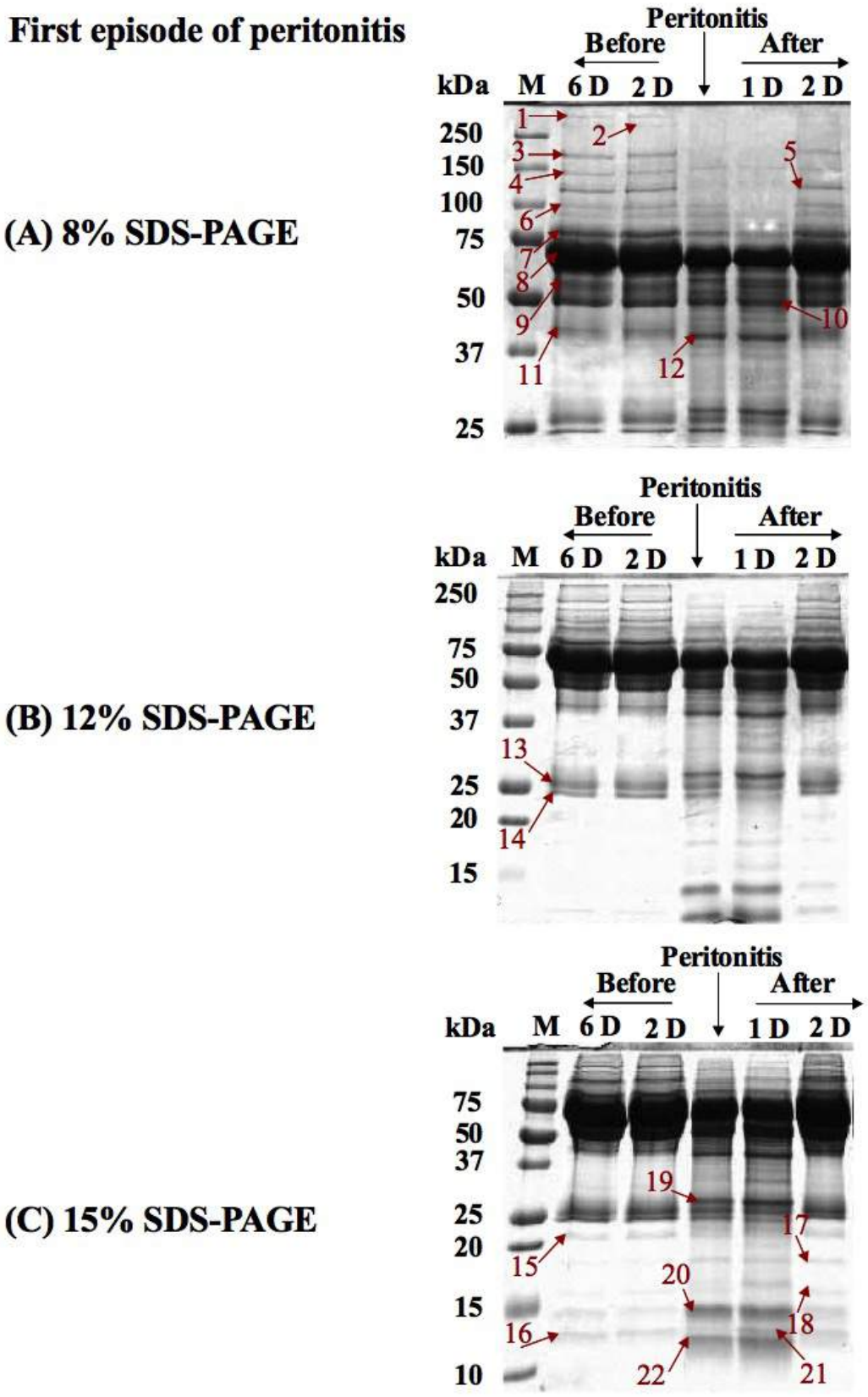


Figure 5.7 Comparative protein profiles of PDF before, during and after the first peritonitis using SDS-PAGE gels.

Key: D refers to day; kDa (kilodalton) refers to the M.W of the protein. A constant quantity of 20 µg of protein of each PDF was loaded per lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7).

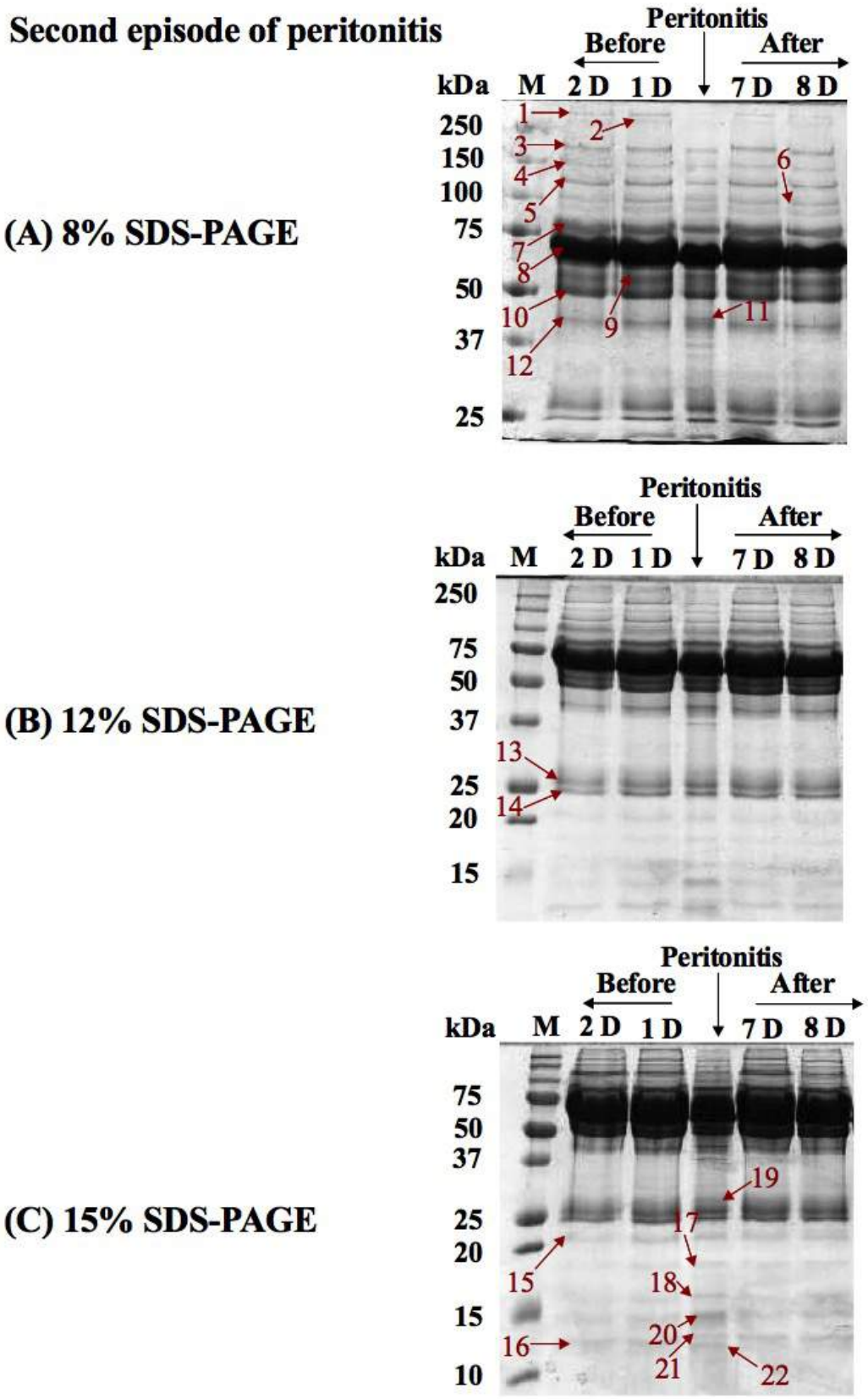


Figure 5.8 Comparative protein profiles of PDF before, during and after the second peritonitis using SDS-PAGE gels.

Key: D refers to day; kDa (kilodalton) refers to the M.W of the protein. A constant quantity of 20 µg of protein of each PDF was loaded per lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7).

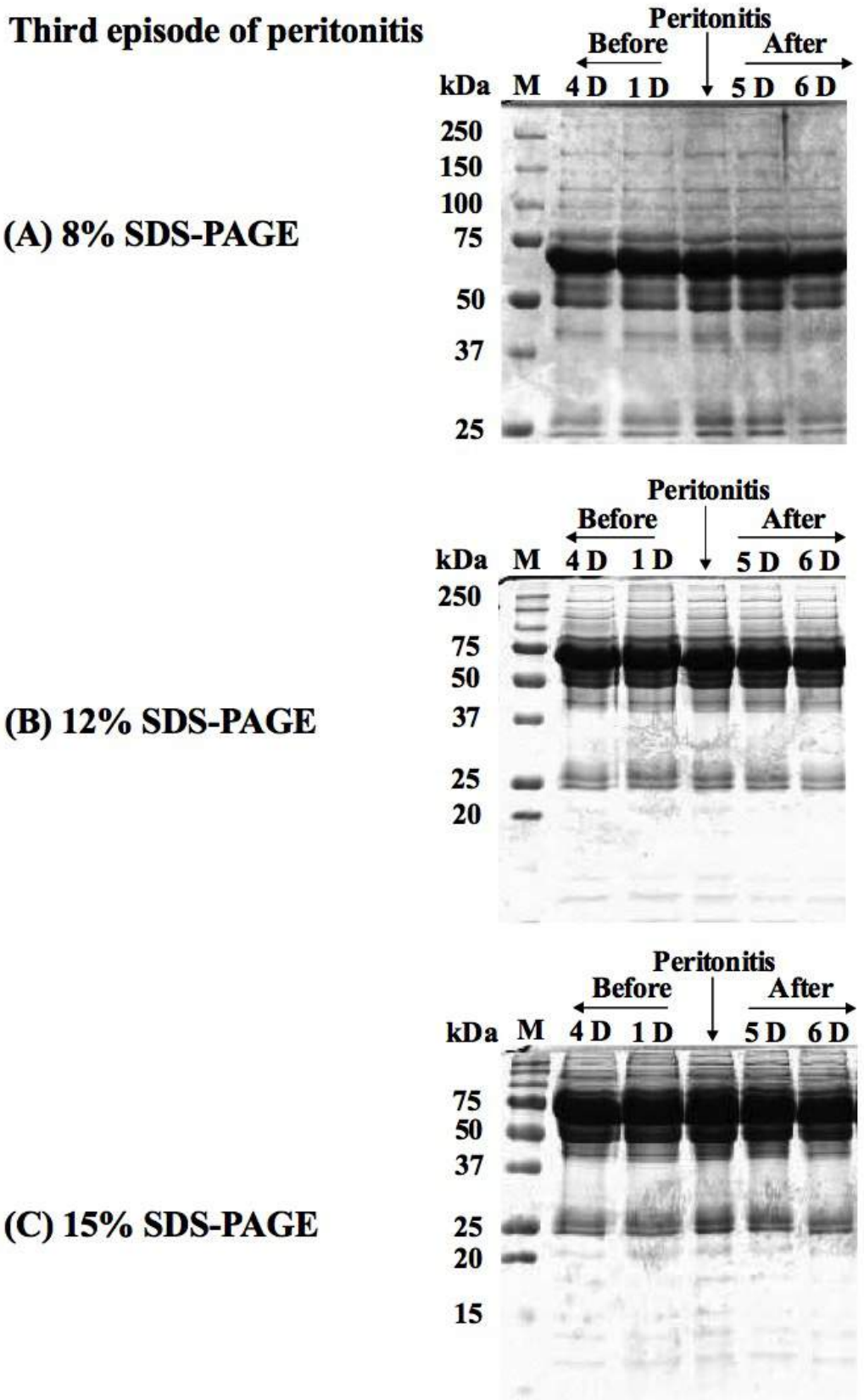


Figure 5.9 Comparative protein profiles of PDF before, during and after the third peritonitis using SDS-PAGE gels.

Key: D refers to day; kDa (kilodalton) refers to the M.W of the protein. A constant quantity of 20 µg of protein of each PDF was loaded per lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7).

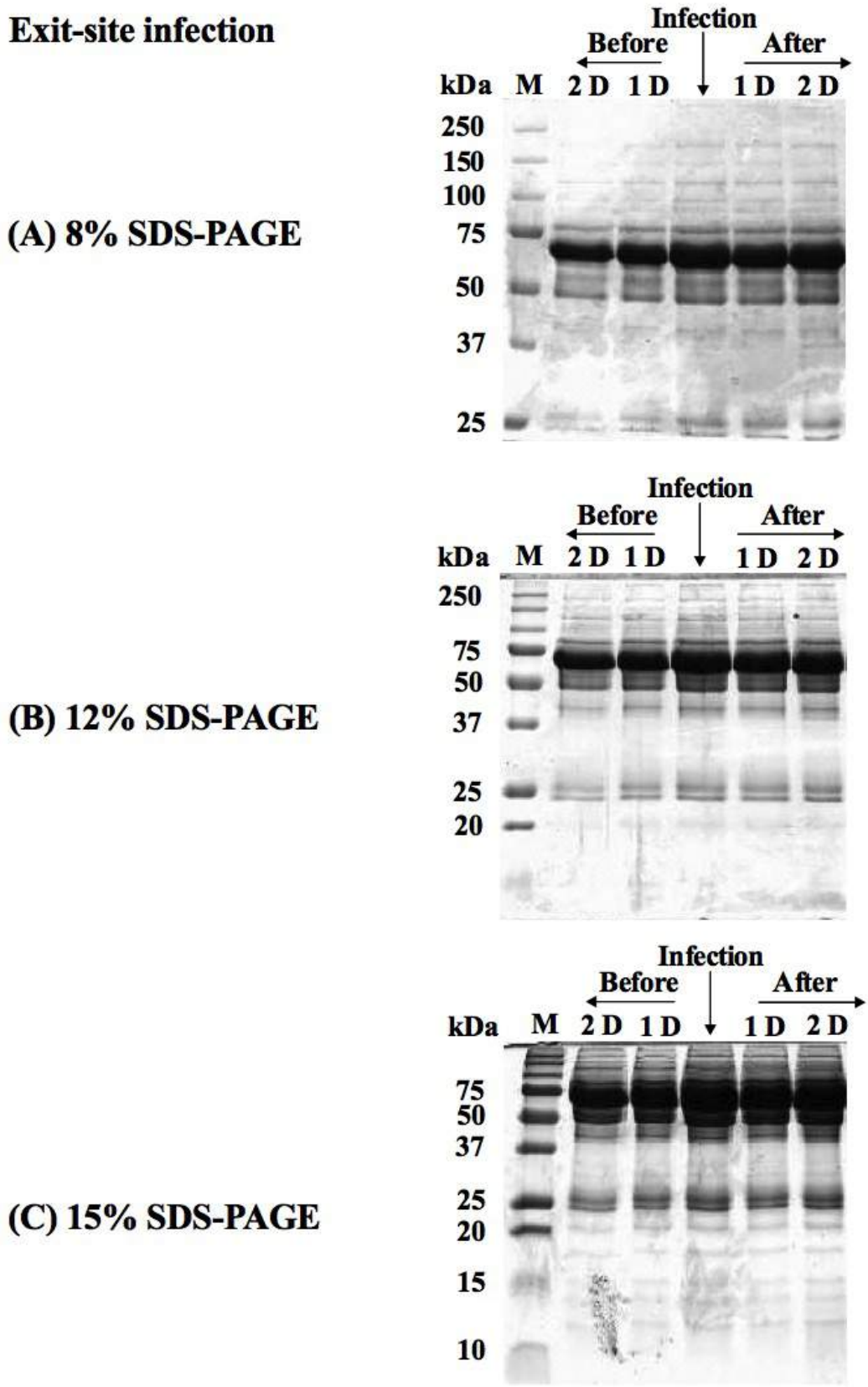


Figure 5.10 Comparative protein profiles of PDF before, during and after the exit-site infection using SDS-PAGE gels.

Key: D refers to day; kDa (kilodalton) refers to the M.W of the protein. A constant quantity of 20 µg of protein of each PDF was loaded per lane. The PDF protein separation was performed using a mini protein separation system at a constant 25 mA per gel (Section 2.2.1.7).

5.3.4.3 PDF protein changes during infectious peritonitis

Proteins with a M.W > 100 kDa (numbers 1 to 6 in Figures 5.7 A and 5.8 A) almost disappeared during the first and second peritonitis. It was therefore investigated whether these proteins played a defensive role during infection, and so these six proteins were sequenced using the LC-MS with the support of the PNACL department as described in Section 2.2.1.8. Proteomic sequencing data of the six proteins indicated that the proteins were from the immune system (Table 5.4).

Table 5.4 Identity and biological roles of the six proteins shown in Figures 5.7 A and 5.8 A that were of lower levels during the first and second peritonitis.

Protein ID	Protein identity	M.W (kDa)	Biological roles
1	**Apolipoprotein B-100**	**515**	Plays a role as a protector against invasive infection. It could work against a quorum sensing system of *S.aureus*; a system responsible for up regulating genes involved in bacterial invasiveness (Peterson *et al.*, 2008).
2	**Fibronectin**	**262**	Plays a role in wound healing. It is also used by the fibronectin-binding proteins of *S.aureus* to mediate attachment to host cells and to fibronectin-coated biomaterials (Menzies, 2003).
3	**alpha-2-macroglobulin**	**163**	Protects proteins against proteinase enzymes (Rabelink *et al.*, 1998).
4	**Ceruloplasmin**	**122**	A copper-binding protein and a serum ferroxidase enzyme, which plays a role in iron metabolism (Hellman & Gitlin, 2002).
5	**Complement component 3**	**187**	A central protein in complement system activation, which plays a role as protector against infections by promoting a local inflammatory response (Sahu & Lambris, 2001).
6	**Fibrinogen gamma chain**	**52**	Involved in the blood coagulation process (Mosesson, 2005).

The proteins showing reduced levels included apolipoprotein B-100 and fibronectin (proteins number 1 and 2, respectively). Positive acute-phase proteins were also detected, whose levels are normally elevated in response to inflammation (Gruys *et al.*, 2005). Those were alpha-2-macroglobulin, ceruloplasmin, C3 and fibrinogen gamma chain (proteins number 3, 4, 5 and 6, respectively). More details of the protein sequences are in Appendix VIII. Apart from the fibronectin, an absence or reduced levels of proteins identified during the first and second peritonitis may result from their consumption as defensive proteins against bacterial infection (Peterson *et al.*, 2008; Rabelink *et al.*, 1998; Hellman & Gitlin, 2002; Sahu & Lambris, 2001; Mosesson, 2005). Despite the role of fibronectin in wound healing (Menzies, 2003), it might be used by peritonitis bacteria to mediate attachment to host cells or to the PD catheter. A further quantitative analysis was performed on those proteins using a densitometer, which was linked to the GS-710 Quantity One Software (Section 2.2.1.9). The purpose of this analysis was to measure the relative protein level of those proteins in the PDF before, during and after the first and second peritonitis. As shown in Figure 5.11 A, four proteins disappeared completely during the first peritonitis. They were apolipoprotein B-100, fibronectin, alpha-2-macroglobuline and C3. However, the level of ceruloplasmin and fibrinogen gamma chain did decrease markedly during the first peritonitis compared with their levels before peritonitis. Two days after peritonitis, the level of proteins in the PDF were quite similar to those before peritonitis. The apolipoprotein B-100, alpha-2-macroglobuline, C3, ceruloplasmin and fibrinogen gamma chain proteins were notably present at a lower level during the second peritonitis, than the days before peritonitis. Fibronectin, in particular, apparently disappeared completely during the second peritonitis. In conclusion, protective proteins mostly disappeared during peritonitis and were possibly consumed by the patient's immune system against the polymicrobial infection.

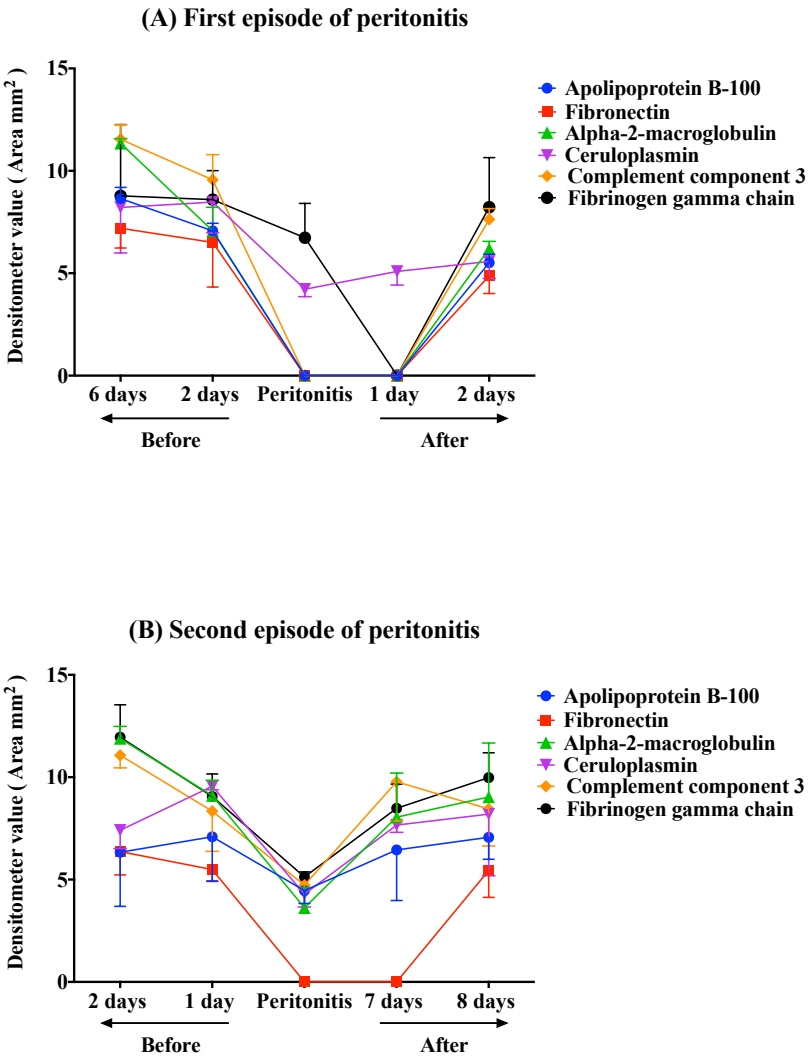


Figure 5.11 Relative protein levels of the high M.W proteins in the PDF before, during and after the first and second peritonitis.
The graphs show the quantitative data (area mm^2) of the six proteins shown in Figures 5.7 A and 5.8 A numbered 1 to 6 and identified in Table 5.4. Labelling at the bottom of each graph indicates the collection time of the PDF.

5.3.4.4 Were novel protein(s) released into PDF during infectious peritonitis that might be infection risk-associated?

Protein with M.W 24 kDa (number 19) and proteins of 15 kDa, 12.5 kDa and 12 kDa (numbers 20, 21 and 22, respectively) appeared in the PDF during the first and second peritonitis (Figures 5.7 C and 5.8 C). Protein sequencing was performed to identify these proteins. The proteins were sequenced either by LC-MS or MALDI-TOf MS as described in Section 2.2.1.8. Table 5.5 provides the identification of these proteins and their biological functions. Further details of the protein sequences are in Appendix VIII.

Table 5.5 Identity and biological roles of the proteins shown in Figures 5.7 C and 5.8 C that appeared during the first and second peritonitis.

Protein ID	Protein identity	M.W (kDa)	Biological roles
19	**Immunoglobulin lambda-2 chain C regions**	**11**	Subunit of the immunoglobulin. Immunoglobulins are antibodies that protect the human body against infection (Vidarsson *et al.*, 2014).
20	**Protein S100-A9**	**13**	Calcium-binding proteins that are secreted by neutrophilic granulocytes and act as antimicrobial agents (Gebhardt *et al.*, 2006).
21	**Protein S100-A8**	**10**	
22	**Haemoglobin subunit beta**	**15**	Part of a larger protein known as haemoglobin present within the RBCs. Haemoglobin plays a key role in oxygen transportation from the lungs to the peripheral tissues (Yoo *et al.*, 2015).

The immunoglobulin lambda-2 chain C regions, S100-A9 and S100-A8 were clearly visible during peritonitis. These proteins play a defensive role against infection (Gebhardt *et al.*, 2006; Vidarsson *et al.*, 2014). Therefore, their release during an acute infection such as peritonitis was expected. Moreover, a possible interpretation for haemoglobin appearance in the peritonitis PDF was that the haemolytic activity of the gut bacteria could lyse the RBCs, resulting in haemoglobin release (Han *et al.*, 2010). On the other hand, it was noticed that Patient 6 lost protein with M.W of 80 kDa (number 7 in Figure 5.7) in the non-infected normal PDF and peritonitis PDF, which was sequenced by MALDI-TOf MS and identified as transferrin.

An essential part of the innate immunity, transferrin is an iron-binding protein present in plasma and other tissue fluids (Afzali & Goldsmith, 2004). It makes extracellular body fluids bacteriostatic due to its ability to bind free iron and inhibit the growth of bacteria (Afzali & Goldsmith, 2004). Also, transferrin has been found to become a bacterial iron source in the presence of catecholamine stress hormones (NE, Adr and Dop) and can directly lead to bacterial infection induction (Freestone *et al.*, 2000; Sandrini *et al.*, 2010). Therefore, it was useful to evaluate the changes that may exist in transferrin levels as well as the presence of catecholamine stress hormones in the non-infected normal PDF and peritonitis PDF.

5.3.5 Comparison studies of transferrin in non-infected normal PDF and peritonitis PDF

Western blotting protocol was performed to confirm the presence of transferrin in non-infected normal PDFs and peritonitis PDFs. The PDFs were loaded on 12% SDS-PAGE gels, Western blotted onto PVDF membranes and the blots probed with anti-transferrin antibodies developed in goat (Section 2.2.1.9). The scanned images of the Western blots shown in Figures 5.12 and 5.13 confirmed the presence of transferrin in the non-infected normal PDFs (obtained before and after peritonitis) and especially in the peritonitis PDFs. Transferrin was also observed in the PDFs collected before, during and after the exit-site infection. What was very striking about the results in Figures 5.12 and 5.13 was that the transferrin levels rose markedly during infection of the peritoneal cavity.

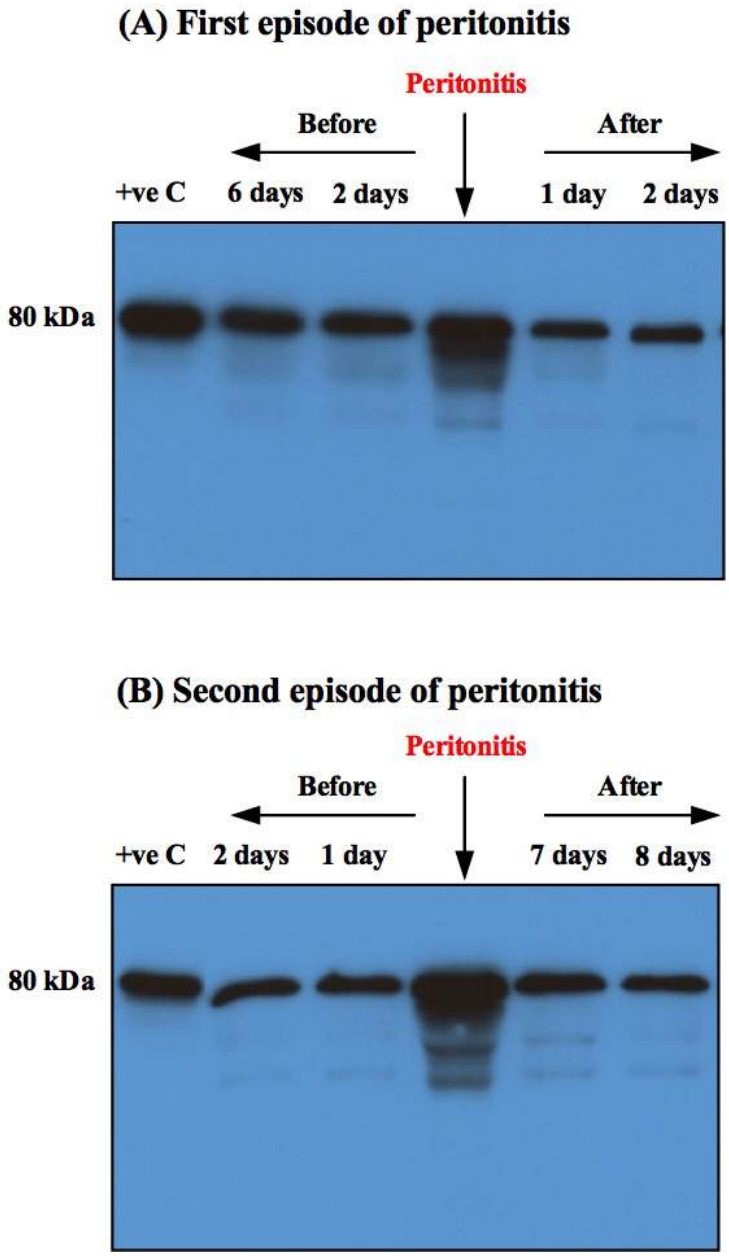


Figure 5.12 Transferrin presence in PDF of Patient 6 before, during and after the first and second peritonitis.

PDFs were collected from the patient as annotated above the blots. The proteins in PDFs were separated first on 12% SDS-PAGE gels, then Western blotted onto PVDF membranes and probed with anti-transferrin antibodies developed in goat (Section 2.2.1.9). Key: C: positive control, which was pure human transferrin (0.5 µg); 80 kilodaltons (kDa) refers to the molecular weight of transferrin. For PDF, 15 µl of each dialysate sample was loaded per well.

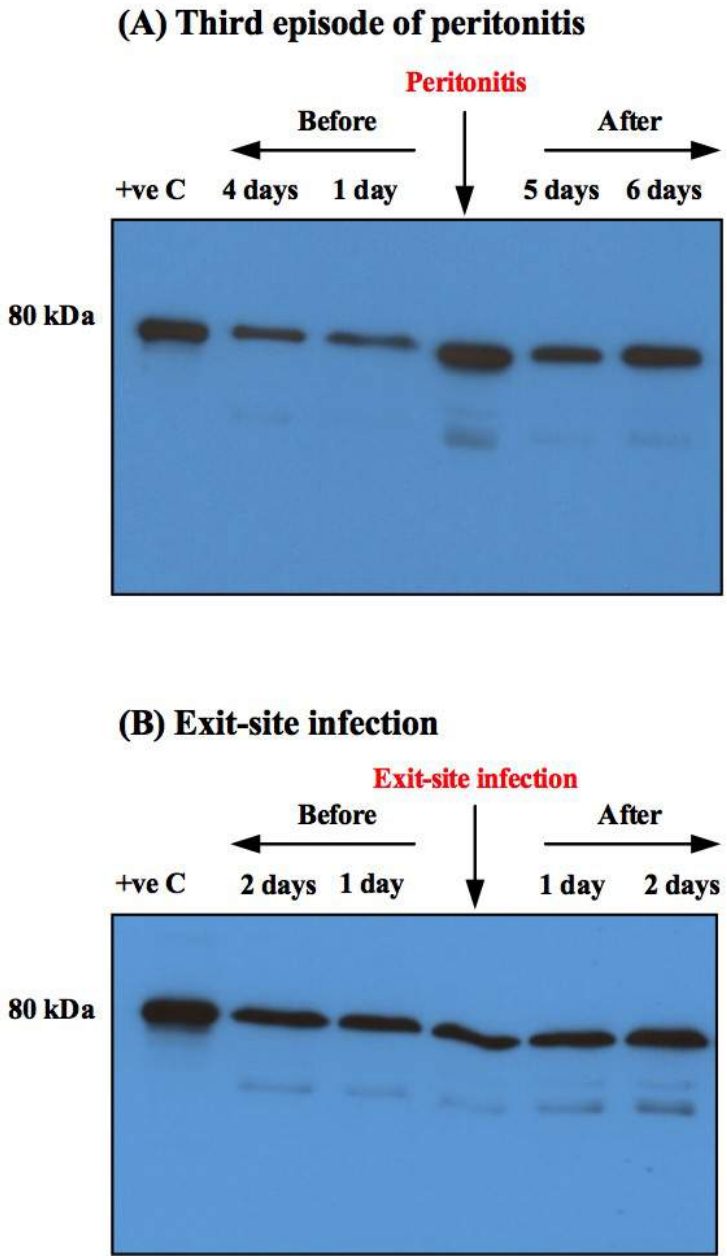


Figure 5.13 Transferrin presence in PDF of Patient 6 before, during and after the third peritonitis and the exit-site infection.
PDFs were collected from the patient as annotated above the blots. The proteins in PDFs were separated first on 12% SDS-PAGE gels, then Western blotted onto PVDF membranes and probed with anti-transferrin antibodies developed in goat (Section 2.2.1.9). Key: C: positive control, which was pure human transferrin (0.5 μg); 80 kilodaltons (kDa) refers to the molecular weight of transferrin. For PDF, 15 μl of each dialysate sample was loaded per well.

A further step was then taken to measure the level of transferrin in non-infected normal PDFs and peritonitis PDFs through the use of a commercial human transferrin ELISA kit (Section 2.2.1.10). The level of transferrin was confirmed to be the greatest in the PDFs on the days of peritonitis (Figures 5.14 and 5.15). A consistent and marked increment in the transferrin level was also recognised after each episode of peritonitis. This consistency was compatible with the clear rose in the protein level in the peritonitis PDFs (Figure 5.5). For instance, the mean transferrin level in the PDF during the first peritonitis was 0.0305 ± 0.0008 g/L (3.1% of total protein); the level rose to 0.0834 ± 0.003 g/L (6.6% of total protein) during the second peritonitis and strikingly peaked at 0.16 ± 0.005 g/L (4.2% of total protein) during the third peritonitis. In a comparison between the non-infected normal PDFs collected before the onset of peritonitis and the peritonitis PDFs, the level of transferrin noticeably increased during peritonitis, followed by a marked decrease a few days after peritonitis. The transferrin release during the exit-site infection showed at lower levels than the three episodes of peritonitis.

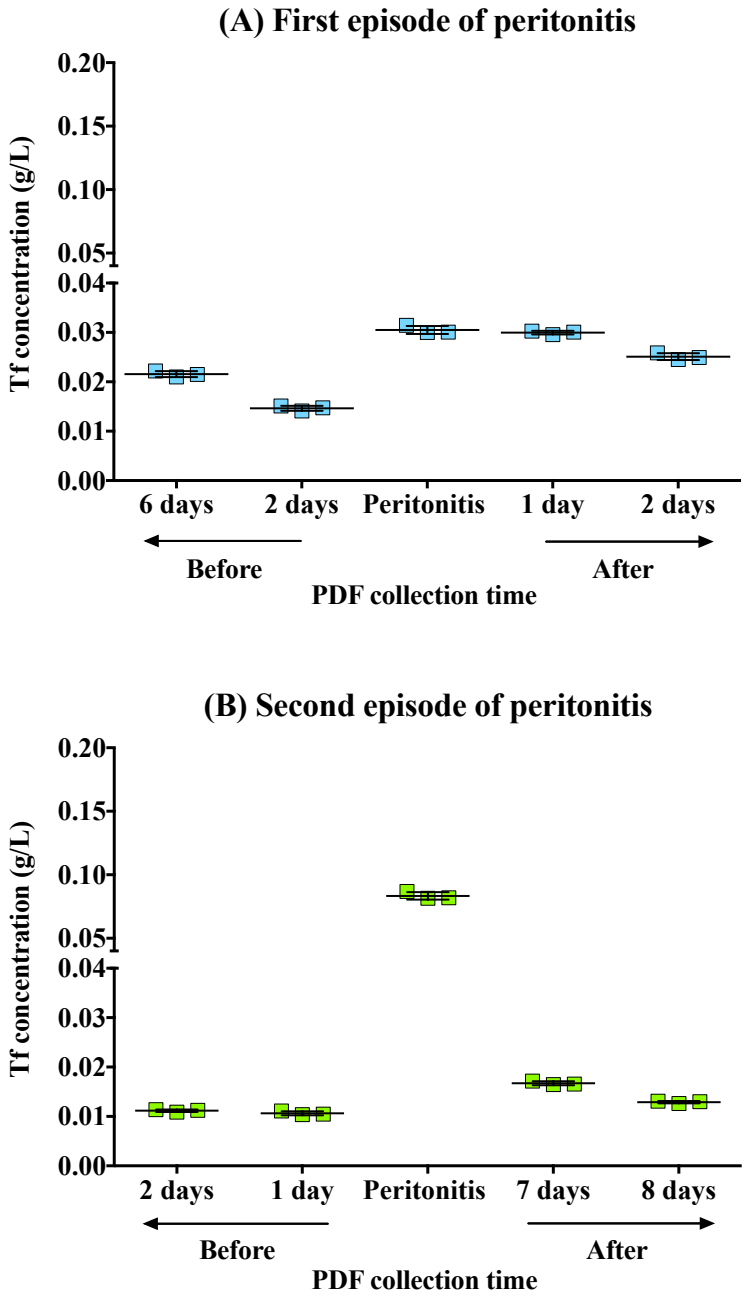


Figure 5.14 Transferrin level in PDF of Patient 6 before, during and after the first and second peritonitis.
Levels of transferrin (Tf) were measured using the human Tf ELISA kit (Section 2.2.1.10).

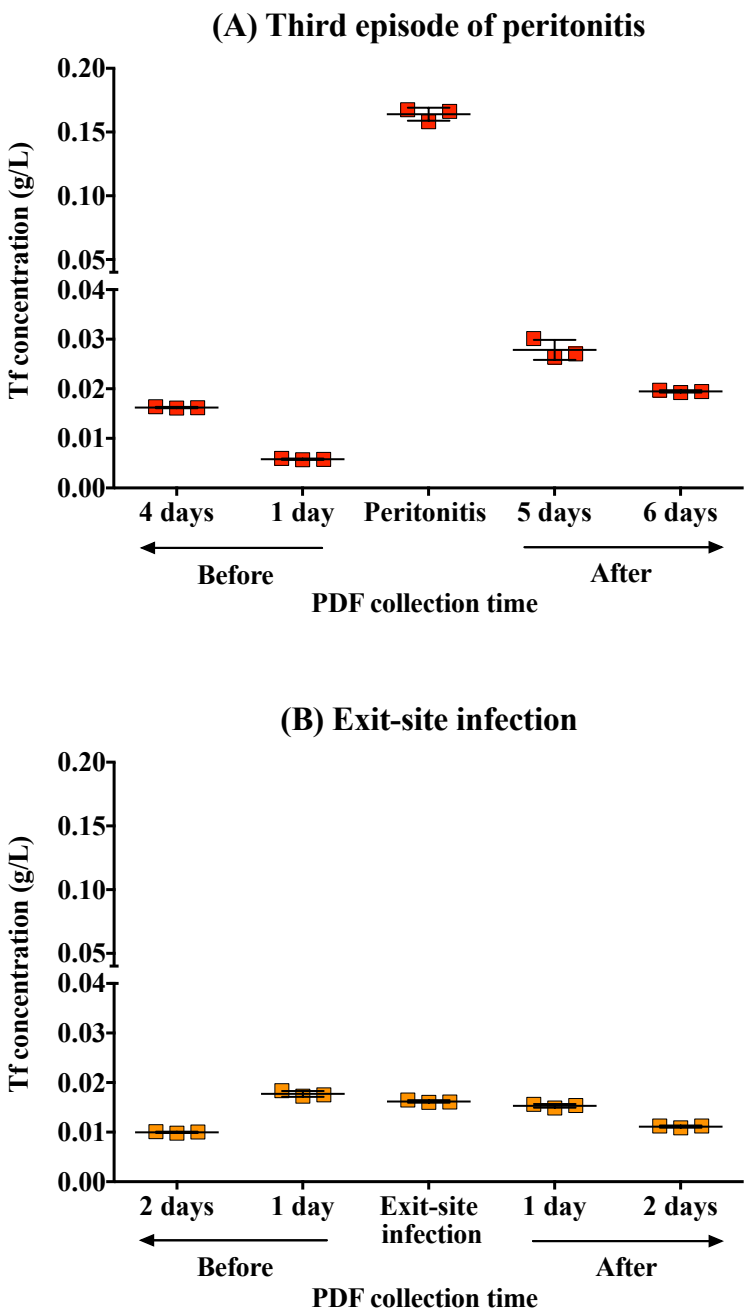


Figure 5.15 Transferrin (Tf) level in PDF of Patient 6 before, during and after the third peritonitis and exit-site infection.
Levels of transferrin (Tf) were measured using the human Tf ELISA kit (Section 2.2.1.10).

5.3.6 Catecholamine stress hormones levels in non-infected normal PDF and peritonitis PDF

Figures 5.14 and 5.15 demonstrated that the level of transferrin was markedly higher in the peritonitis PDF than the non-infected normal PDF. Therefore, it was necessary to examine whether catecholamine stress hormones showed the same pattern of variety as transferrin. Measurements of NE, Adr and Dop levels were performed using a tri-cat ELISA kit (Section 2.2.1.5). Figures 5.16 and 5.17 show that catecholamine stress hormones were in the PDFs accessed before, during and after the three episodes of peritonitis as well as the exit-site infection. There were marked amounts of NE in the first and second peritonitis PDFs compared to the non-infected normal PDFs (gathered before or after onset of peritonitis). NE level was determined at 0.12 ± 0.029 ng/ml in the non-infected normal PDF (obtained two days before first peritonitis); however, its level markedly rose and reached a peak at 0.48 ± 0.21 ng/ml in the first peritonitis PDF. The trend seen in NE level in the PDF collected before, during and after the second peritonitis was similar to the trend observed in the first peritonitis (Figure 5.16). However, the level of NE was greater in the second peritonitis PDF (0.701 ± 0.12 ng/ml) than the first peritonitis PDF (0.48 ± 0.21 ng/ml). More importantly, the greatest levels of NE in the first and second peritonitis PDFs were associated with the highest transferrin levels seen in these PDFs (Figure 5.14). In contrast, there was no NE recognised in the third peritonitis PDF (Figure 5.17 A). Adr was present in the PDFs at lower levels than NE, and the highest Adr levels were identified in non-infected normal PDFs rather than peritonitis PDFs. Marked variations were found in Adr levels in the PDFs during development of the second, but not the first or third, peritonitis. The lowest level of catecholamine stress hormone observed in the PDFs was that of Dop, although the most abundant catecholamine stress hormone in the PDFs during development of the exit-site infection was Dop (Figure 5.17 B).

In conclusion, during the three episodes of peritonitis the most abundant catecholamine stress hormone available in the PDFs was NE, whereas the least detectable one was Dop. The highest NE level was identified in the first and second peritonitis PDFs, was associated with the highest transferrin levels observed in these peritonitis PDFs.

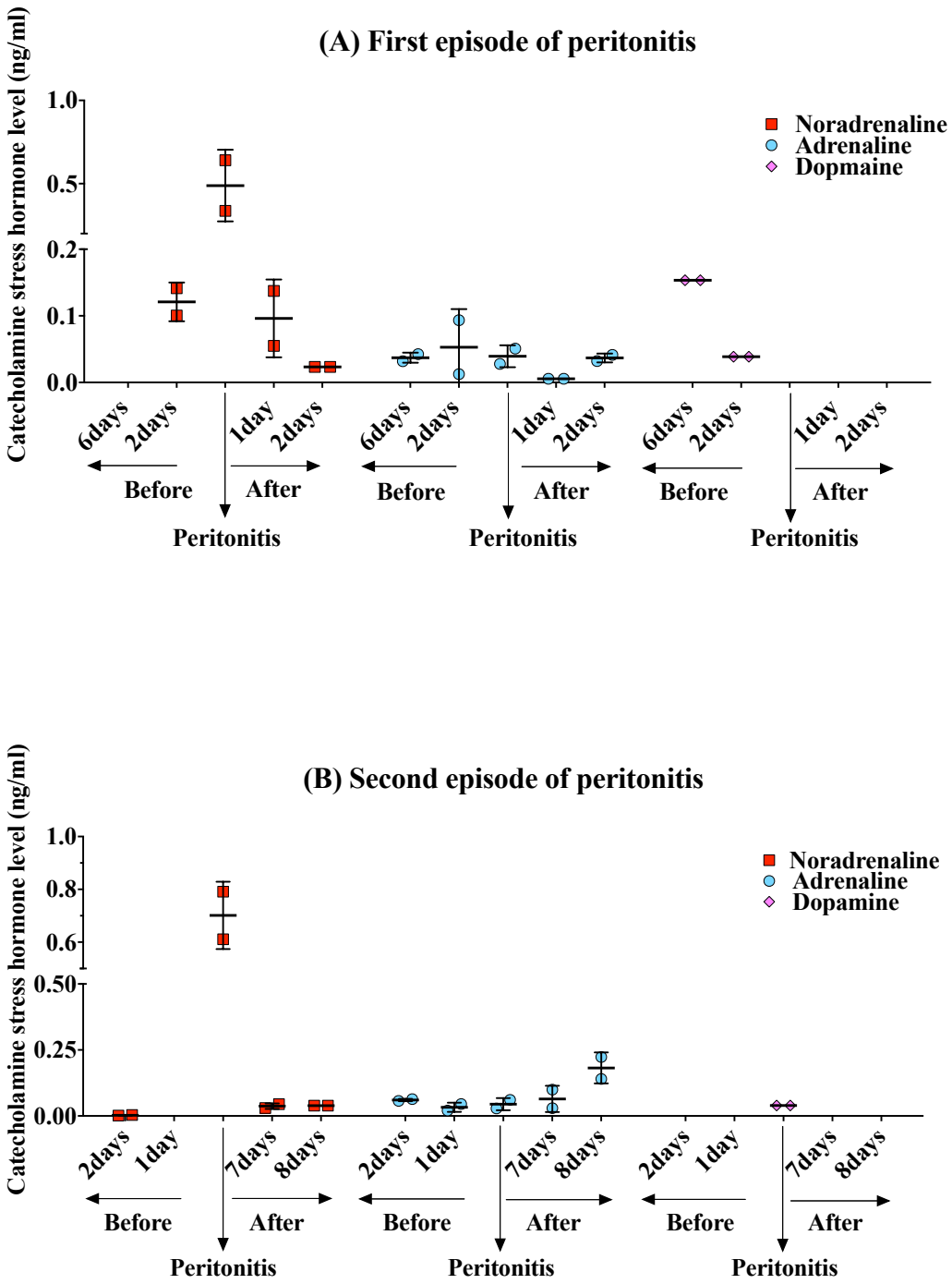


Figure 5.16 Measurements of catecholamine stress hormone levels in PDF of Patient 6 before, during and after the first and second peritonitis.
Noradrenaline, adrenaline and dopamine levels were determined using the tri cat ELISA kit (Section 2.2.1.5). Labelling at the bottom of each graph indicates the collection time of the PDF.

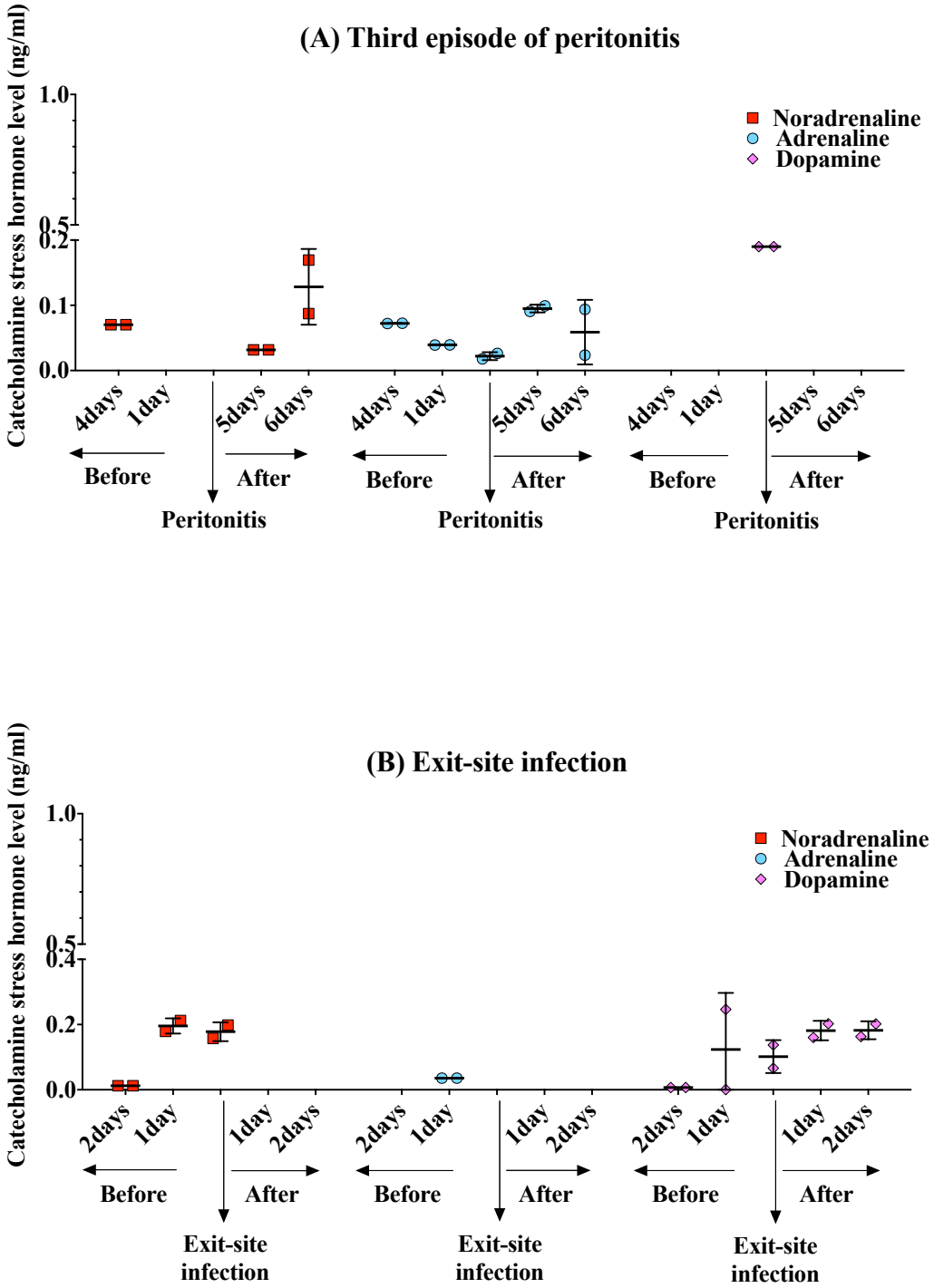
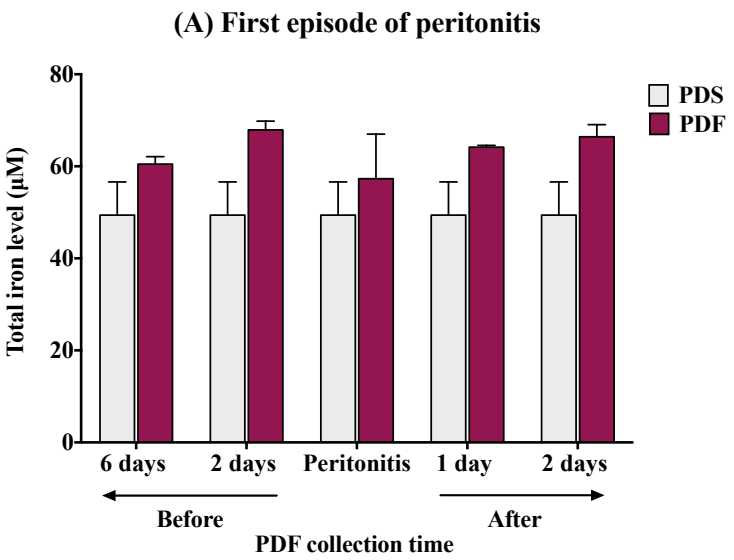


Figure 5.17 Measurements of catecholamine stress hormones concentrations in PDF of Patient 6 before, during, and after the third peritonitis and exit-site infection.
Noradrenaline, adrenaline and dopamine levels were determined using the tri cat ELISA kit (Section 2.2.1.5). Labelling at the bottom of each graph indicates the collection time of the PDF.

5.3.7 Investigation of iron levels in non-infected normal PDF and peritonitis PDF

Iron is a principal nutrient for pathogenic bacteria and as a protective measure against their acquisition of it, the immune system has evolved components such as transferrin that sequester host-iron and limit its accessibility to bacteria (Gomme *et al.*, 2005; Afzali & Goldsmith, 2004). Transferrin was detected at the highest levels during the three episodes of peritonitis (Figures 5.14 and 5.15). Therefore, it was useful to investigate changes that may exist in iron levels before, during and after each episode of peritonitis. The iron levels were measured using an iron assay kit (Section 2.2.1.4).

In the first instance, the total iron level (Fe^{+3} + Fe^{+2}) was determined in 7.5% icodextrin PDS. As described in Section 5.1.3, Patient 6 used two different types of the PDS: 2.27% glucose and 7.5% icodextrin. During the first and third peritonitis as well as during the exit-site infection, the patient had used 2.27% glucose PDS, whereas during the second peritonitis she had used 7.5% icodextrin. The total iron level of 2.27% glucose PDS was at 49.39 ± 14.41 μM (Figure 3.11). The total iron level was also measured in 7.5% icodextrin at 42.13 ± 5.93 μM. The total iron levels in the PDFs before, during and after the three episodes of peritonitis and the level in the exit-site infection were generally higher than in the corresponding PD solutions (Figures 5.18 and 5.19). The level of total iron was not, however, markedly different in the non-infected normal PDFs and the peritonitis PDFs. In addition, no major differences in total iron levels were detected in the PDFs drawn during the three episodes of peritonitis.



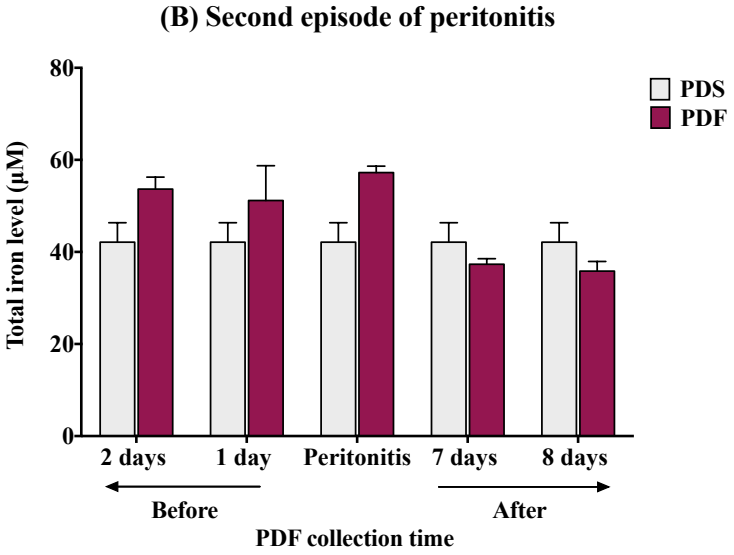


Figure 5.18 Measurements of the total iron level (Fe^{+3} + Fe^{+2}) in the PDFs before, during and after the first and second peritonitis, and in the corresponding PDS.
Patient 6 used 2.27% PDS glucose during the first peritonitis, whereas during the second peritonitis 7.5% icodextrin PDS was used. The total iron level was determined using the iron assay kit (Section 2.2.1.4).

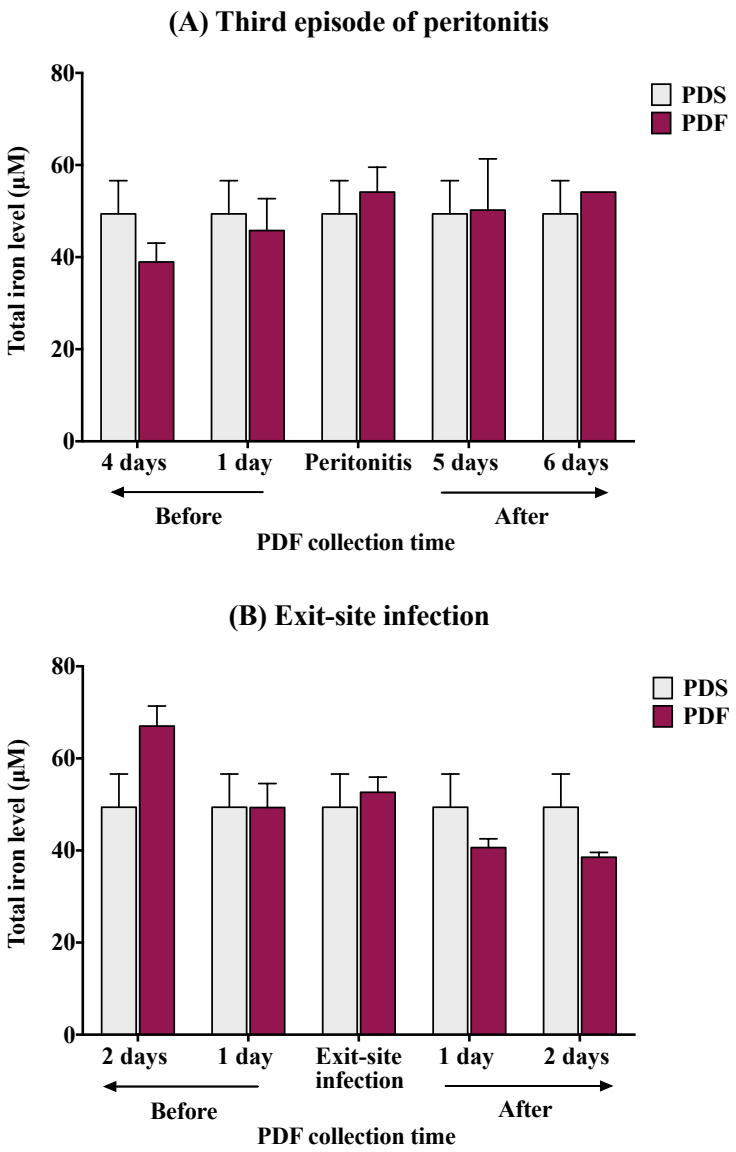


Figure 5.19 Measurements of the total iron level (Fe^{+3} + Fe^{+2}) in the PDFs before, during and after the third peritonitis, at the exit-site infection, and in the corresponding PDS.
Patient 6 used 2.27% PDS glucose during the third peritonitis as well as during the exit-site infection. The total iron level was determined using the iron assay kit (Section 2.2.1.4).

An analysis of the levels of Fe^{+3} (which binds with molecules such as transferrin) and Fe^{+2} (non-transferrin bound iron; also known as free iron) was also performed. The Fe^{+3} and Fe^{+2} levels of PDF taken during the first and second peritonitis, and in PDFs obtained before peritonitis (as non-infected normal PDF) were assessed. As shown in Figure 5.20, Fe^{+3} and Fe^{+2} were detected in the peritonitis PDFs and in the non-infected normal PDFs. The level of Fe^{+3} was higher than the level of Fe^{+2} in the non-infected normal PDF samples. During the second peritonitis, the level of Fe^{+2} was higher than the level of Fe^{+3}.

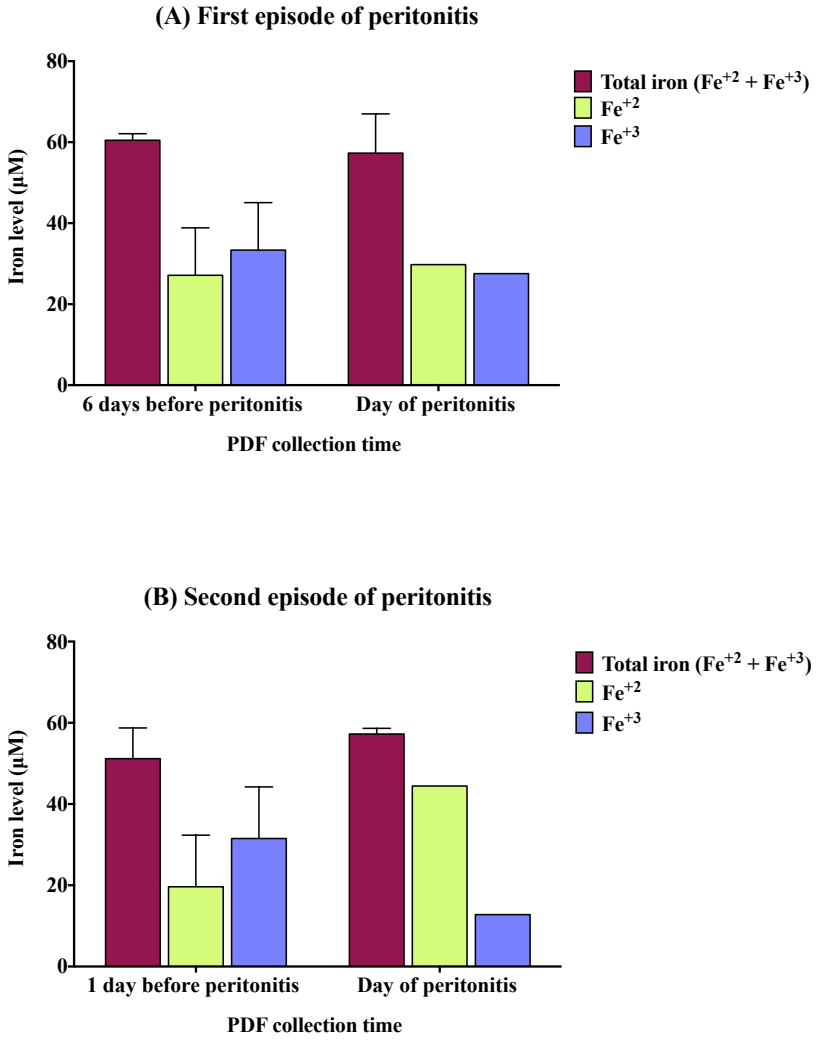


Figure 5.20 Total iron, Fe^{+2} and Fe^{+3} levels in non-infected normal PDF and peritonitis PDF.
The iron levels were determined using the iron assay kit (Section 2.2.1.4).

5.3.8 A comparison of staphylococci growth profiles in non-infected normal PDF and peritonitis PDF

It was shown that protein, transferrin and catecholamine stress hormones (in particular NE) levels were markedly higher in peritonitis PDF than non-infected normal PDF. Furthermore, total iron (Fe^{+3} + Fe^{+2}) was quantifiable in both the non-infected normal PDF and peritonitis PDF. Therefore, it was worthwhile to analyse PD pathogens (*S.aureus* and *S.epidermidis*) growth profiles in the non-infected normal PDFs (obtained before and after peritonitis) and in the peritonitis PDFs (accessed on the days of the first and second peritonitis). *S.aureus* or *S.epidermidis* was inoculated into the non-infected normal PDF and peritonitis PDF. Bacterial growth was then monitored by measuring OD at 600 nm over 24 hours (Section 2.2.1.1).

In general, the peritonitis PDFs were by far the most supportive fluids for *S.aureus* growth (Figures 5.21 A and 5.22 A), whereas the non-infected normal PDFs (collected before onset of peritonitis) had the greatest stimulatory effect on *S.epidermidis* growth (Figures 5.21 B and 5.22 B). As demonstrated in Figures 5.5, 5.14 and 5.16, the protein, transferrin and NE levels were greater in the peritonitis PDFs than in the non-infected normal PDFs. Consequently, a direct association seems to exist between *S.aureus* growth and the level of these three biochemical parameters. On the other hand, the most *S.epidermidis* growth was recognised in the non-infected normal PDFs rather than peritonitis PDFs. A possible explanation of these results that *S.epidermidis* growth may influenced by antimicrobial agents that were produced by the patient's innate immune system in response to the peritoneal infection. Because the patient was given a course of antibiotics to treat the first and second peritonitis (Section 5.1.1), *S.aureus* and *S.epidermidis* growth patterns had reached a plateau in the PDFs that were obtained after the onset of peritonitis. In summary, the peritonitis PDFs had a more positive impact on *S.aureus* growth than *S.epidermidis* growth. Moreover, a direct association may exist between the level of protein, transferrin and NE and *S.aureus* growth in the PDF.

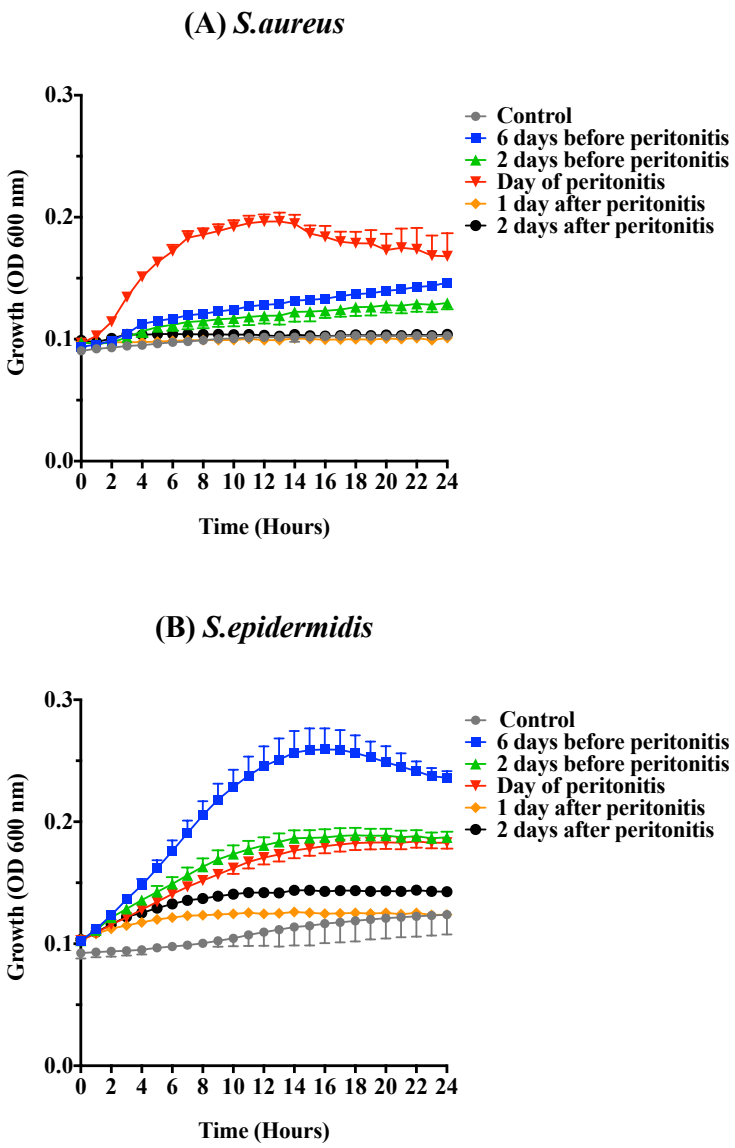


Figure 5.21 Time course of *S.aureus* and *S.epidermidis* growth in PDF of Patient 6 before, during and after the first peritonitis over 24 hours.
'Control' refers to the PDS used (2.27% glucose PDS). Labels beside the graphs refer to the PDF collection time. Approximately 10^7 CFU/ml inoculum of *S.aureus* or *S.epidermidis* culture was inoculated into the PDS or PDF and incubated at 37°C for 24 hours. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.1.1); n=3.

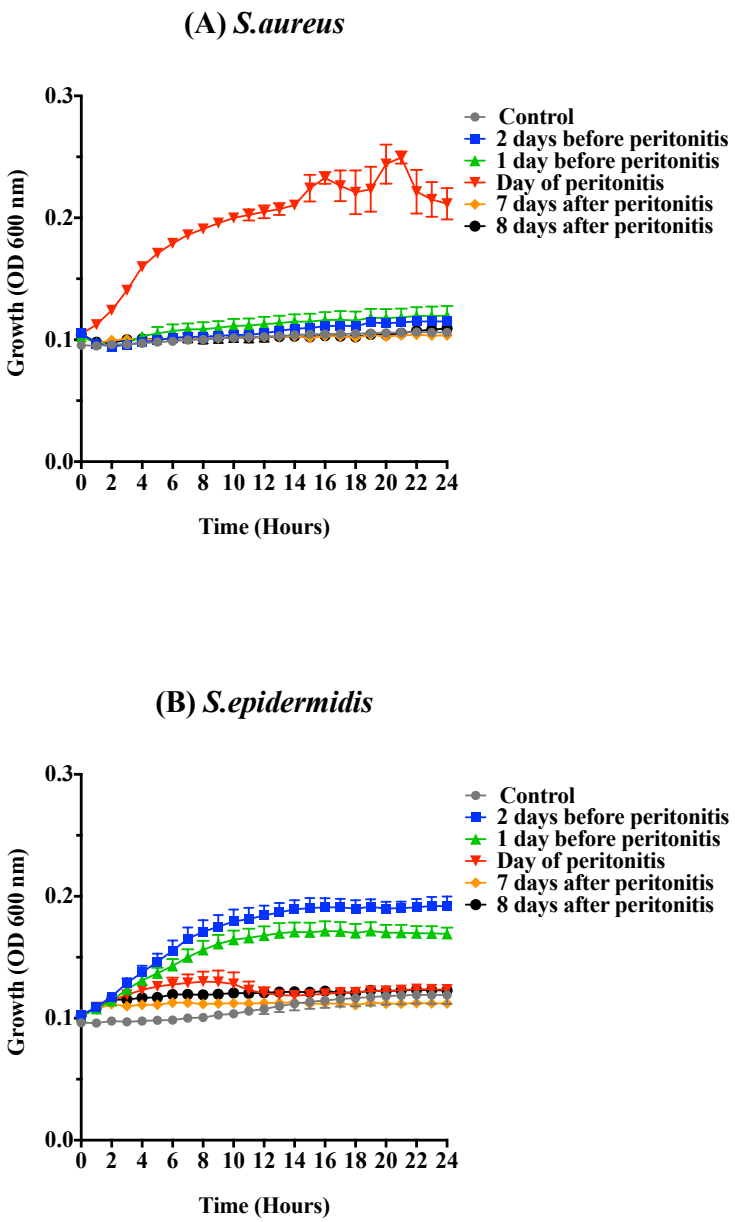


Figure 5.22 Time course of *S.aureus* and *S.epidermidis* growth in PDF of Patient 6 before, during and after the second peritonitis over 24 hours.
'Control' refers to the PDS used (7.5% icodextrin PDS). Labels beside the graphs refer to the PDF collection time. Approximately 10^7 CFU/ml inoculum of *S.aureus* or *S.epidermidis* culture was inoculated into the PDS or PDF and incubated at 37°C for 24 hours. Staphylococcal growth was monitored by measuring OD at 600 nm using the Multiscan Go Spectrophotometer (Section 2.2.1.1); n=3.

5.3.9 Uptake of transferrin complexed iron in non-infected normal PDF and peritonitis PDF

Marked differences were observed in transferrin (Figure 5.14) and NE levels (Figure 5.16) between non-infected normal PDF and peritonitis PDF. This is important because Sandrini *et al.* (2010) and Freestone *et al.* (2000) found that, in a medium containing transferrin, NE and other catecholamine stress hormones can bind iron sequestered by transferrin and provide it to bacteria. Therefore, it was necessary to examine if there were differences in the iron uptake by staphylococci between non-infected normal PDF and peritonitis PDF.

The 2.27% glucose PDS was selected because it was the dialysis solution most often used by Patient 6. Non-infected normal PDF (obtained after the patient had been on dialysis for seven months) and peritonitis PDF (accessed on the day of the second peritonitis) were chosen to represent normal and infection PDF state. The level of NE was lower in the non-infected normal PDF (0.33 ± 0.001 ng/ml) than in the peritonitis PDF (0.70 ± 0.12 ng/ml). The iron uptake investigations were performed using the protocol described by Freestone *et al.* (2000). Normalised cultures of *S.aureus* and *S.epidermidis* (OD 2 at 600 nm) were prepared and mixed with 4 ml of the PDS or 4 ml of the non-infected normal PDF or 4 ml of the peritonitis PDF. Next, 1 μg/ml of ^{55}Fe-transferrin (1 x 10^5 cpm) was pipetted into the PDS or PDF containing *S.aureus* or *S.epidermidis*. The levels of iron uptake by staphylococci were measured over the course of 4 hours of incubation using scintillation counting (Section 2.2.2.3).

On the whole, the trend of the iron uptake (in the form of ^{55}Fe) from the ^{55}Fe-transferrin in the PDS, non-infected normal PDF and peritonitis PDF was similar for both *S.aureus* and *S.epidermidis* (Figure 5.23). However, *S.aureus* had the ability to acquire considerably more Fe from the ^{55}Fe-transferrin than *S.epidermidis*. This is because *S.aureus* is a more aggressive pathogen than *S.epidermidis* and it expresses a greater array of host-iron uptake systems (Skaar & Schneewind, 2004). For both *S.aureus* and *S.epidermidis*, the uptake of iron in the 2.27% glucose PDS was greater than the uptake of iron in the non-infected normal PDF and peritonitis PDF. Although NE level was lower in the non-infected normal PDF than in the peritonitis PDF, the uptake of iron by *S.aureus* was markedly higher in the non-infected normal PDF than in the peritonitis PDF.

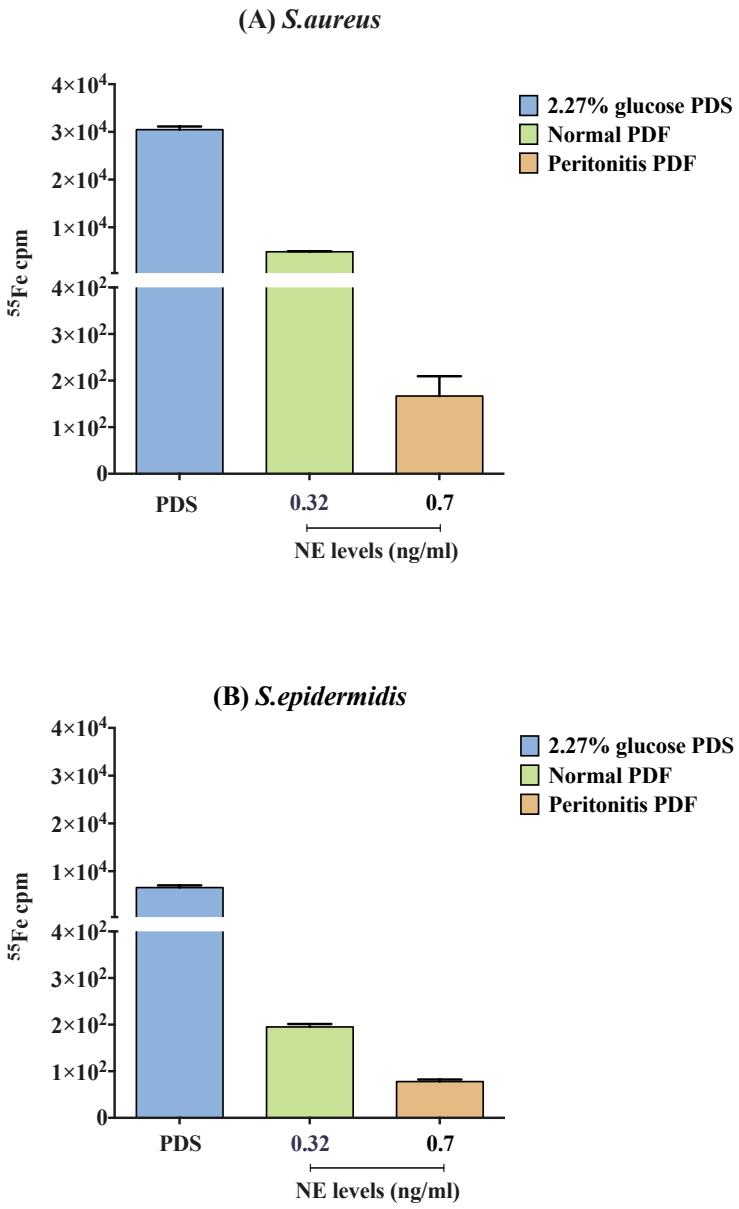


Figure 5.23 Uptake of ^{55}Fe from ^{55}Fe-labelled transferrin in PDS and PDF of Patient 6.
The bar charts demonstrate the uptake of iron by *S.aureus* and *S.epidermidis* incubated in 2.27% glucose PDS or in Patient 6's non-infected normal PDF (obtained after seven months of dialysis) or in peritonitis PDF (collected on the day of the second peritonitis) (Section 2.2.2.3); n=3.

Because the peritonitis PDF was found to have the lowest iron uptake by *S.aureus*, further iron uptake analysis was performed using a combination of PDF infected and non-infected normal PDF samples. The first combination was 2.27% glucose PDS mixed with peritonitis PDF (obtained on the day of the second peritonitis). The second combination was non-infected normal PDF (obtained after seven months on APD course) and peritonitis PDF. Afterwards, normalised cultures of *S.aureus* were incubated in each combination and ^{55}Fe uptake assays were performed as described in Section 2.2.2.3. Interestingly, reduction in the uptake of iron by *S.aureus* was observed in the peritonitis

PDF and when the peritonitis PDF was combined with either glucose PDS or non-infected normal PDF (Figure 5.24). This suggests that factor(s) that can block the uptake of iron by *S.aureus* was/were present in the peritonitis PDF.

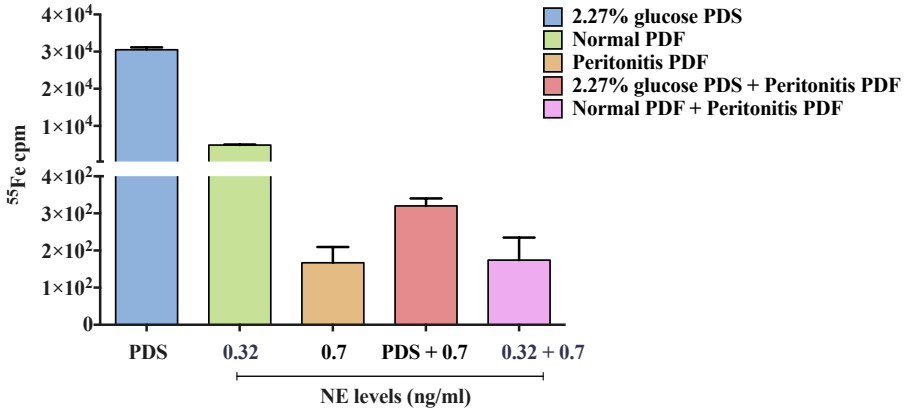


Figure 5.24 Uptake of ^{55}Fe from ^{55}Fe-labelled transferrin by *S.aureus* in combined samples from Patient 6.
S.aureus culture was incubated in single samples (2.27% glucose PDS or non-infected normal PDF or peritonitis PDF) and in combined samples (2.27% glucose PDS and peritonitis PDF or non-infected normal PDF and peritonitis PDF) (Section 2.2.2.3); n=3.

5.3.10 The association between biochemical factor levels and staphylococcal growth in non-infected normal PDF and peritonitis PDF

It was demonstrated in Chapter 3 that protein levels and the presence of transferrin, catecholamine stress hormones and iron independently and significantly predict the AUC for *S.aureus* growth in non-infected normal PDF. It was then necessary to examine whether the same associations existed between *S.aureus* or *S.epidermidis* growth and if an involvement existed for these biochemical factors in Patient 6, who experienced three episodes of peritonitis. The present chapter described the differences between staphylococcal growth patterns in both non-infected normal PDF and peritonitis PDF. The levels of protein, transferrin and catecholamine stress hormones were markedly higher in the peritonitis PDF than non-infected normal PD dialysate. Univariable linear regression analysis was therefore performed to explore possible associations between *S.aureus* or *S.epidermidis* growth (as a dependent variable) and one independent variable at a time (i.e. levels of protein, transferrin, catecholamines hormones or iron). Table 5.6 provides information about the identity of the type PDF used in the univariable linear regression analysis.

Table 5.6 Identity of the type of PDF used in the univariable linear regression analysis.

PDF identity	PDF collection time
Non-infected normal PDF	2 months before first peritonitis
Non-infected normal PDF	6 days before first peritonitis
Non-infected normal PDF	2 days before first peritonitis
Peritonitis PDF	First peritonitis
Non-infected normal PDF	2 days before second peritonitis
Non-infected normal PDF	1 day before second peritonitis
Peritonitis PDF	Second peritonitis
Non-infected normal PDF	1 month after second peritonitis

After analysis no association was detected between the AUC for *S.epidermidis* growth and protein, transferrin, catecholamines or iron levels in non-infected normal PDF and peritonitis PDF ($P > 0.05$). In sharp contrast, a significant and direct association was detected between the AUC for *S.aureus* growth and protein levels in non-infected PDF and peritonitis PDF ($P < 0.05$). Levels of transferrin and catecholamine stress hormones also were associated positively and significantly with the AUC for *S.aureus* growth in both non-infected normal PDF and peritonitis PDF ($P < 0.05$). Thus, the higher the levels of protein, transferrin and catecholamine stress hormones, the greater the *S.aureus* growth in PDF. No association was observed, however, between the AUC for *S.aureus* growth and iron levels in either non-infected normal PDF or peritonitis PDF ($P > 0.05$). Figures 5.25, 2.26, 5.27 and 5.28 provide profiles of the levels of protein, transferrin, catecholamine stress hormones and iron associated with *S.aureus* or *S.epidermidis* growth as revealed by univariable linear regression analysis.

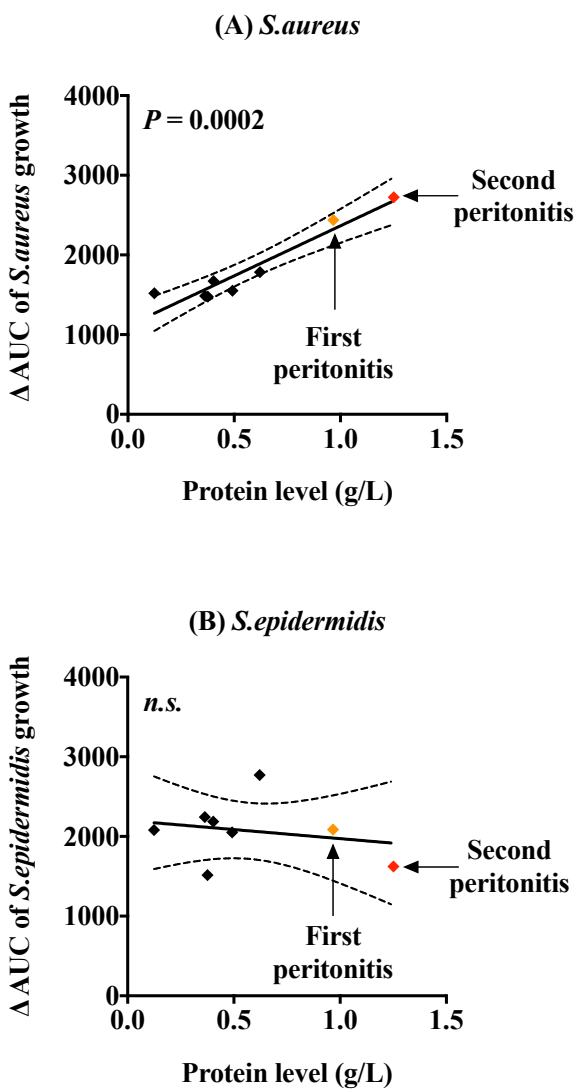


Figure 5.25 Linear regression analysis of the association between protein levels and *S.aureus* or *S.epidermidis* growth in non-infected normal PDF and peritonitis PDF.
Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of Δ AUC was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Arrows indicate peritonitis PDF; black symbols designate non-infected normal PDF samples.

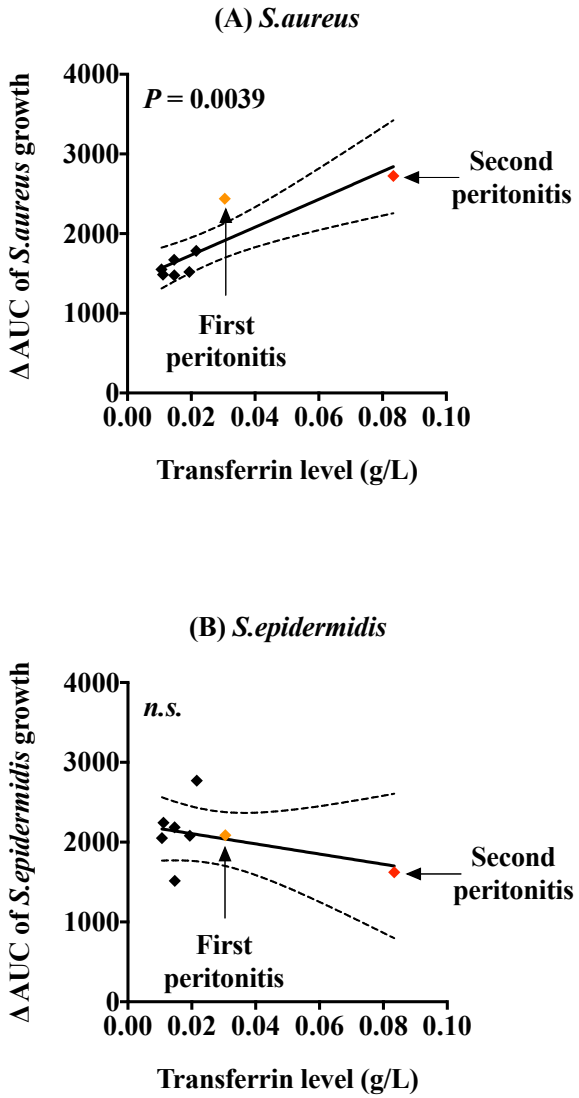


Figure 5.26 Linear regression analysis of the association between transferrin levels and *S.aureus* or *S.epidermidis* growth in non-infected normal PDF and peritonitis PDF.
Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of Δ AUC was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Arrows indicate peritonitis PDF; black symbols designate non-infected normal PDF samples.

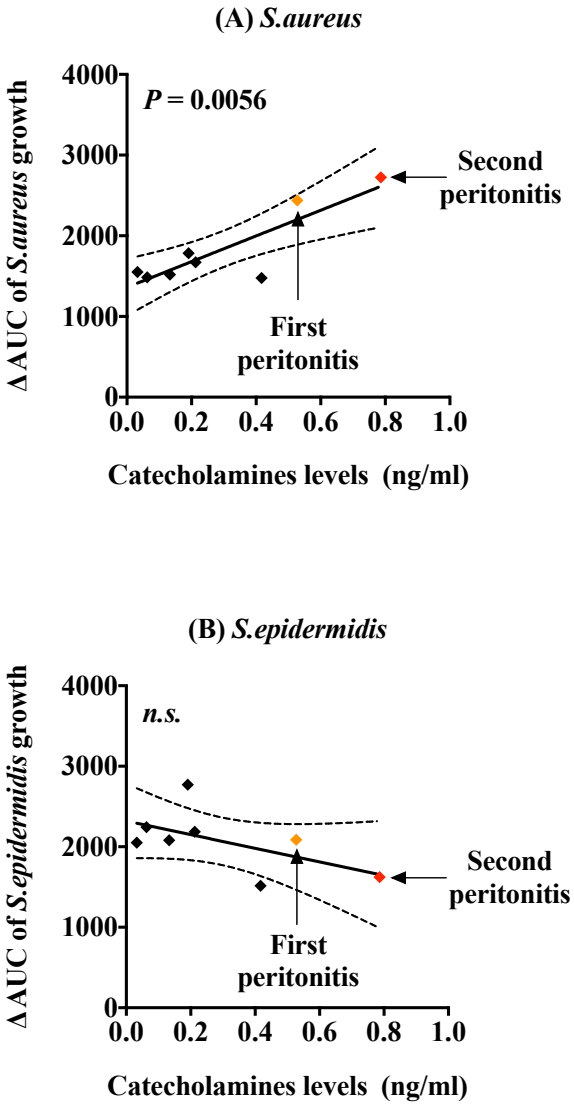


Figure 5.27 Linear regression analysis of the association between catecholamine stress hormones levels (NE + Adr + Dop) and *S.aureus* or *S.epidermidis* growth in non-infected normal PDF and peritonitis PDF.

The sum of NE, Adr and Dop was calculated for each PDF sample, then used in the analysis. Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of Δ AUC was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Arrows indicate peritonitis PDF; black symbols designate non-infected normal PDF samples.

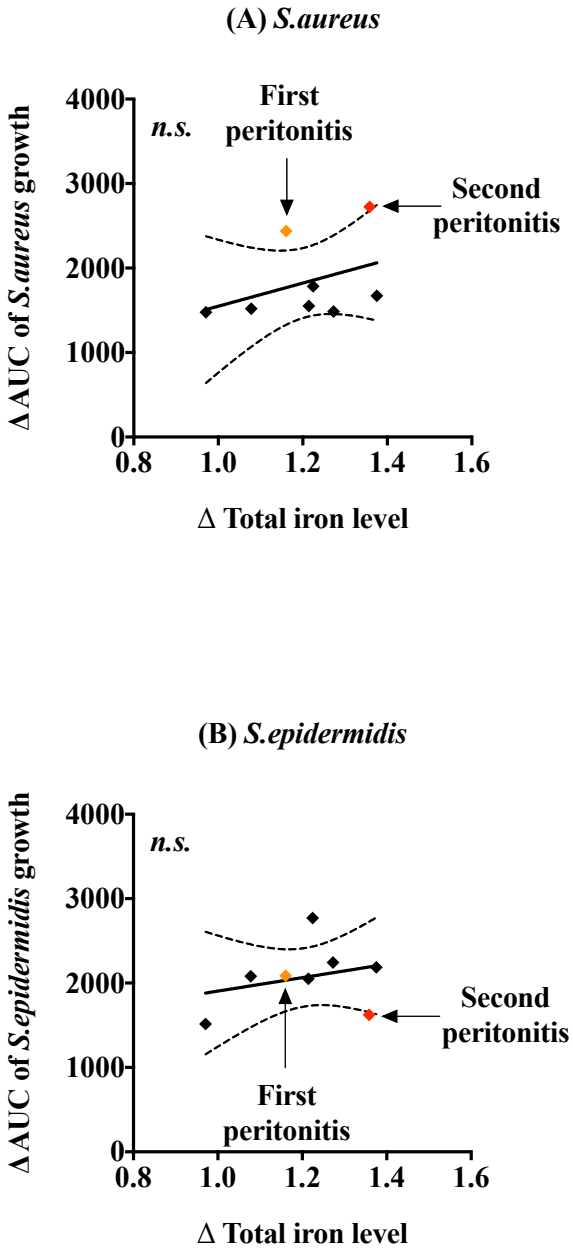


Figure 5.28 Linear regression analysis of the association between total iron levels (Fe^{+2} + Fe^{+3}) and *S.aureus* or *S.epidermidis* growth in non-infected normal PDF and peritonitis PDF.
Key: AUC: area under the curve; *n.s.* means non-significant association ($P > 0.05$). The fold difference of each variable (Δ total iron or Δ AUC) was calculated by dividing the value of the PD dialysate by the value of the corresponding PD solution. Arrows indicate peritonitis PDF; black symbols designate non-infected normal PDF samples.

5.4 Discussion

PD-associated peritonitis is a clinically significant condition considered to be a leading complication of the PD technique (Ellam & Wilkie, 2011). It initiates peritoneal membrane failure, thereby necessitating PD termination and a switch to haemodialysis (Ellam & Wilkie, 2011; Zarrinkalam *et al.*, 2001; Kerschbaum *et al.*, 2012; Li, 2001). In a review by Kerschbaum *et al.* (2012), the rate of peritonitis was documented at approximately 0.35 episodes per patient year. The occurrence of three confirmed cases of infectious peritonitis within nine months from the start of the APD course in the present prospective study is unusual. The peritoneal infections were found to be polymicrobial in nature, as Gram staining and microbial culture demonstrated the presence of both Gram-positive and Gram-negative bacteria. Later investigations revealed the infections were of gut origin; the PD catheter had nicked the colon and released a diverse microbial population, resulting in an instant infection. The central purpose of this chapter was therefore to report the longitudinal changes in the biochemical characteristics of PD dialysate during health and infection over a period of time spent by Patient 6 on APD (14 months).

The results of the present study showed that protein level in PDF peaked during each episode of peritonitis compared to levels before and after peritonitis (Figure 5.5). From a clinical point of view, peritonitis is an inflammation of the peritoneum resulting in acute damage and increased vascular permeability (Ellam & Wilkie, 2011; Davies *et al.*, 1996). The innate immune system plays an important role in fighting episodes of peritonitis by deploying a number of effective immune defence mechanisms, including release into dialysate of peritoneal WBCs (mostly macrophages), antibodies, complement factors, opsonins and defensins, which collectively increase the amount of protein lost into PDF (Zarrinkalam *et al.*, 2001). These innate immune mechanisms are activated by the release of soluble proinflammatory factors, including TNFα, leukotrienes (including IL-8), and an influx of leukocytes (mainly neutrophils) from the blood (Zarrinkalam *et al.*, 2001; Luo *et al.*, 2000). The data presented in this chapter showed a clear increase in the protein level in the PDF over the three peritonitis episodes. Such findings are in agreement with Van Diepen *et al.* (2015), who reported that the first episode of peritonitis is responsible for altering the permeability characteristics of the peritoneum. Peritonitis-associated damage is also responsible for long-lasting changes in peritoneal membrane features, which gradually render the peritoneum less effective at solute exchange (Davies *et al.*,

1996). Comparative proteomic studies were performed on non-infected normal PDF and peritonitis PDF to identify endogenous biomarker(s) that could predict a peritoneal infection. The following six proteins were found to be present at lower levels in the first and second peritonitis PDF compared to non-infected normal PDF: apolipoprotein B-100, fibronectin, alpha-2-macroglobulin, ceruloplasmin, C3 and fibrinogen gamma chain. It may be that these proteins were released by the patient's immune system during her response to polymicrobial infection (Peterson *et al.*, 2008; Menzies, 2003; Rabelink *et al.*, 1998; Hellman & Gitlin, 2002; Sahu & Lambris, 2001; Mosesson, 2005). In addition to the aforementioned proteins, four novel proteins appeared in the first and second peritonitis PDF. The first three were immunoglobulin lambda chain C regions, S100-A9 and S100-A8, which play a defensive role against infection (Gebhardt *et al.*, 2006; Vidarsson *et al.*, 2014). The fourth protein was haemoglobin; its appearance may have been due to the haemolytic activity of gut bacteria to RBCs, resulting in haemoglobin release (Han *et al.*, 2010). The levels of these 10 proteins differed between the peritonitis PDF and non-infected normal PDF; however, they were not by their activity associated with infection risk. Literature on the protein profile of non-infected normal PDF and peritonitis PDF is scarce, which makes comparing these results with those of other studies difficult. In study by Lin *et al.*, (2008), who performed 2D gel electrophoresis and SELDI-TOf MS on PDF derived from CAPD patients with or without peritonitis and reported that beta-2-microglobulin protein was present in higher levels in peritonitis PDF than in non-infected normal PDF. The beta-2-microglobulin protein was suggested to be a biological marker for CAPD peritonitis (Lin *et al.*, 2008). However, Lin *et al.* (2008) have stated that many more prospective studies are needed to establish predictive endogenous biomarkers that could anticipate PD peritonitis, which is (fortunately for patients) still a rare event. Therefore, this is an objective of the current study.

Physiologically speaking, hypoferraemia—a low level of serum iron—is a condition associated with changing levels of iron-binding proteins, including transferrin, in response to infection (Northrop-Clewes, 2008). Hypoferraemia is a protective mechanism in which iron is withheld from Fe-hungry infectious agents (Northrop-Clewes, 2008). Levels of transferrin, a negative acute phase protein, typically decrease approximately 30% in response to inflammation and infection (Northrop-Clewes, 2008). In the current study, however, the levels of transferrin in PDF did not follow this expected concentration pattern. PDF transferrin levels were greatest on the days of peritoneal infection, as shown in Figure 5.14. Further, the increase in transferrin levels in PDF during infection

paralleled the increase in catecholamine stress hormones levels, and noradrenaline in particular (Figure 5.16). It may be that transferrin and catecholamine stress hormones levels in PDF were the highest during peritonitis because the inflammation of the peritoneum results in acute damage and increased vascular permeability (Davies *et al.*, 1996; Ellam & Wilkie, 2011), a process that may promote leakage of both transferrin and catecholamine stress hormones during a dialysis exchange. It was hypothesised in this study that the levels of transferrin and catecholamine stress hormones could stimulate bacterial growth in PDF. Freestone *et al.* (2000), Freestone *et al.* (2008) and Sandrini *et al.* (2010) have shown that catecholamine stress hormones can mediate Fe^{+3} removal from transferrin. Mechanistically, the catechol part of catecholamine stress hormones can complex with the Fe^{+3} sequestered by transferrin, thereby making it accessible to bacteria (Freestone *et al.*, 2008; Freestone *et al.*, 2000; Sandrini *et al.*, 2010). It can therefore be argued that the presence of transferrin and catecholamine stress hormones in PDF during both health and infection is in agreement with the study hypothesis.

It has been reported in the literature that staphylococcal growth in body fluids, cells or tissues is induced by the presence of excess free iron (Weinberg, 2009; Sunder-Plassmann *et al.*, 1999). In the present study, Fe^{+2} (free ferrous iron), as well as Fe^{+3} (ferric iron) which exists in combination with molecules such as transferrin (Gomme *et al.*, 2005; Modun *et al.*, 1994; Parkkinen *et al.*, 2002), were detected in healthy and infected PD dialysates. In fact, the data indicated the level of total iron in non-infected normal PDF was not markedly different from that in peritonitis PDF (Figure 5.18). Typically, serum transferrin is partially saturated with Fe^{+3} (25% to 35%), maintaining the level of soluble iron at around 10^{-18} M, which is too low to enable microbial growth (Gomme *et al.*, 2005). In comparison to these normal conditions, the mean Fe^{+2} level in the non-infected normal PDF samples (obtained before the first and second peritonitis) was 2.34×10^{-5} M, which is higher than the normal free iron level in the circulation. It was also greater than the minimum iron level (4×10^{-7} to 4×10^{-6} M) required by pathogenic bacteria to grow (Park *et al.*, 2005).

PDF-transferrin was shown in this study to be a bacterial iron source. *S.aureus* and *S.epidermidis* uptake of ^{55}Fe from ^{55}Fe-transferrin was much higher in the glucose PDS than in any of the non-infected normal or peritonitis PD dialysates analysed. This shows there are some mechanisms present in PDF that reduce bacterial uptake of transferrin-iron. More interestingly, ^{55}Fe uptake from transferrin by *S.aureus* was reduced in peritonitis PDF, even when the peritonitis PDF was diluted when combined with either

glucose PDS or non-infected normal PD dialysate. One possible explanation is that the transferrin level already present in the peritonitis PDF (0.083 g/L) was higher than in the non-infected normal PDF (0.015 g/L). The bacteria in the peritonitis PDF thus were exposed to two readily accessible sources of transferrin. The first source was the high level of transferrin already present in the PDF. The second source was ^{55}Fe-transferrin, which was prepared and added to the bacterial culture in the peritonitis PDF. Competition may have occurred between the two sources of transferrin and resulted in comparatively lower bacterial uptake of ^{55}Fe in the peritonitis PDF. The non-infected normal PDF had less transferrin than did peritonitis PDF, and therefore ^{55}Fe uptake by the bacteria was higher. It may be argued from these results that the peritonitis PDF had more iron-saturated transferrin than did non-infected normal PDF. Because the glucose PDS had only one source of transferrin (^{55}Fe-transferrin), ^{55}Fe uptake by the bacteria there was then the greatest. In terms of inhibition of transferrin-iron uptake in peritonitis PDF, this needs more investigation as potentially, because iron availability is so important to growth in PDF, it could suggest a new antimicrobial.

Univariable linear regression analysis identified three parameters as significant, independent risk factors for *S.aureus* growth in PDF samples obtained from Patient 6 during health and infected states: total protein, transferrin and catecholamine stress hormones. The higher the levels of total protein, transferrin and catecholamines, the greater the degree of *S.aureus* growth in PDF. The levels of these biochemical factors continued to affect *S.aureus* growth in PDF over time. In sharp contrast, *S.epidermidis* growth showed a statistically insignificant association with the aforementioned biochemical factors. *S.aureus* is a more aggressive pathogen than *S.epidermidis* and contains a greater array of host iron-uptake systems (Skaar & Schneewind, 2004), which could explain differences between *S.aureus* and *S.epidermidis* growth responses to the identified biochemical parameters in PDF.

The literature on the association of protein loss into PDF and risk of peritonitis is limited. Dong *et al.* (2013) have shown that protein leakage into PDF is an independent predictor for the risk of peritonitis in PD patients, a finding in agreement with that of the current study. However, whether the types of protein lost are more critical than the quantity of protein lost is still unknown (Dong *et al.*, 2013). In other words, either the protein load of the PDF or some specific proteins released into the PDF act to influence bacterial growth and virulence, and so infection risk. It has been shown by Modun *et al.*, (1994) and Williams *et al.* (1995) that bacteria do not grow well in PDS, however, after the PD

exchange, the PDF is rich in serum proteins—including the iron-holding protein transferrin—which have been shown to support bacterial growth (Modun *et al.*, 1994; Williams *et al.*, 1995). My findings further support the report of those researchers, as a significant and direct association was detected between transferrin level and *S.aureus* growth in PDF. To my knowledge, no studies have looked at the association between catecholamine stress hormones and *S.aureus* growth in PDF. The data presented in this study suggest that besides total protein and transferrin, the presence of catecholamines could independently predict risk of *S.aureus* infection in PD patients. These findings confirm previous work (Freestone *et al.*, 2000; Sandrini *et al.*, 2010), which showed that transferrin becomes a bacterial iron source in the presence of catecholamine stress hormones and can induce bacterial infection.

Based on the experiments discussed in this chapter, I conclude that PDF is not always bacteriostatic in nature, because it accommodates growth-stimulating factors for *S.aureus*. Total protein, transferrin and catecholamine stress hormones levels could indeed independently predict *S.aureus* peritoneal infection in PD patients.

CHAPTER 6 General discussion

6.1 Introduction

A glomerular filtration rate value of less than 15 ml/min/1.73 m^2 is considered to be a critical biomarker for end stage kidney disease (ESKD) (Levey & Coresh, 2012). ESKD is fatal unless it is managed by renal replacement therapy–either kidney transplantation or kidney dialysis (Chaudhary *et al.*, 2011). One form of kidney dialysis is peritoneal dialysis (PD). PD is the simplest and most economical therapeutic technique for ESKD patients and is also a home-based therapy that provides more independence and flexibility in terms of the patient's lifestyle than the more complex machine-based haemodialysis (Chaudhary *et al.*, 2011). Although there are significant advantages of PD, the percentage of PD patients in the UK has decreased since 2000 (MacNeill *et al.*, 2016). The most likely reason for the underutilisation of PD is its potential complications, particularly the loss of proteins into the peritoneal dialysis fluid (PDF) and peritonitis (Ellam & Wilkie, 2011).

PD-associated peritonitis is an inflammation of the peritoneal membrane, which is most often caused by an infectious agent. Repeated peritonitis typically results in peritoneal membrane failure and necessitates PD termination and a switch to haemodialysis (Li, 2001). There are a considerable number of patient risk factors for PD-associated peritonitis have been reported in the literature, including having an insufficient educational level, having a low socioeconomic status, using the connect PD system and using CAPD versus APD (Kerschbaum *et al.*, 2012). Despite the identifying of the risk factors for the PD-associated peritonitis, its occurrence is still related with significant morbidity and mortality rates. It is still unclear why some PD patients are more susceptible to infection than others. This means there are undiscovered prognostic factors, possibly endogenous host factors, which may play a central role in the establishment of a PD-related peritonitis. The central aim of the present study was therefore to widen our knowledge and understanding of the host-associated components present within the PDF, which may play a key role in stimulating bacterial growth and virulence, and so explain differences between rates of patient infections. This will allow investigating the study hypothesis that the levels of catecholamine stress hormones and iron-binding proteins (such as transferrin) in PDF may increase patient susceptibility to peritoneal infection by stimulating bacterial growth via iron-providing.

6.2 Characterisation studies of PDF

The data presented in Chapter 3 involved a comprehensive characterisation of the PDF derived from nine PD patients who were new to PD therapy. The PDF samples were collected at the start of their dialysis and at different times during the PD time-course. As a result, inter- and intra-patient variations in the protein levels in the PDF were observed. Proteomic profiling showed that the PD patients generally had the same protein profile in their PD dialysates as seen by protein patterns on 8% and 12% SDS-PAGE gels. In Table 3.5, 18 proteins were identified in the patient PDF samples that were of plasma origin. The identified proteins can be classified into seven categories according to their biological functions: (1) acute phase proteins, such as albumin and transferrin; (2) complement factors, including C3; (3) apolipoproteins, such as apolipoprotein A-I; (4) coagulation proteins, including fibrinogen; (5) extracellular matrix proteins, such as fibronectin; (6) vitamin-binding proteins, such as retinol-binding protein; and (7) enzymes, such as tRNA-dihydrouridine synthase. The majority of the identified proteins were basically involved in innate immune defence, which might because they are constantly lost into dialysate be linked to a systemic deficiency of the immune defence proteins, which could then increase the patient's susceptibility to infection. Apart from tRNA-dihydrouridine synthase presence, which is unique to this study, the proteins identified in the PDF in Table 3.5 were previously observed by other investigators (Wen *et al.*, 2013; Wang *et al.*, 2010; Oliveira *et al.*, 2014; Cuccurullo *et al.*, 2011).

In contrast to the PD solution (the solution that is infused into the peritoneal cavity and contains glucose and electrolytes), the PDF (the fluid collected after the PD exchange and has been modified with host-proteins, hormones and other factors), as expected was more supportive for staphylococcal growth. Such findings are in agreement with other studies, which have shown that PD solution converts from non-supportive medium (before dialysis exchange) to supportive medium (after dialysis exchange) for staphylococcal growth (Verbrugh *et al.*, 1984; Williams *et al.*, 1995; McDonald *et al.*, 1986; Sheth *et al.*, 1986). The staphylococcal growth behaviour was found in Chapter 3 to be dissimilar in PDF samples obtained from different patients as well as in PDF samples derived from the same patient over time, indicating that there were inter- and intra-patient differences in the biochemical characteristics of the PDF. This study examined biochemical factors in the PDF of PD patients toward a better understanding how differences affect risk of peritonitis.

Fe^{+2} (non-transferrin bound iron, also termed free iron), as well as Fe^{+3} (ferric iron), which exists in combination with molecules such as transferrin (Gomme *et al.*, 2005; Modun *et al.*, 1994; Parkkinen *et al.*, 2002), were detected at variable levels in the range of PDF samples. Under normal conditions, serum transferrin is usually partially saturated with Fe^{+3} (25% to 35%) to maintain the level of Fe^{+2} at around 10^{-18} M, which is too low to enable microbial growth (Gomme *et al.*, 2005). In comparison to normal conditions, the mean Fe^{+2} level in the PDF samples was detected at 2.9×10^{-5} M, which is higher than the Fe^{+2} level in the circulation. It is also more than the minimum iron level required by pathogenic bacteria to grow (4×10^{-7} to 4×10^{-6} M) (Park *et al.*, 2005). Some investigators have reported that staphylococcal growth in body fluids, cells or tissues is induced by the presence of excessive Fe^{+2} (Weinberg, 2009; Sunder-Plassmann *et al.*, 1999). Thus, in the iron context, unlike body tissue fluids PDF is not a bacteriostatic medium, which means that it might increase a patient's susceptibility to peritoneal infection.

Transferrin is an iron-binding protein which has a major presence in plasma (Afzali & Goldsmith, 2004). It is a key part of the innate immunity as it makes extracellular body fluids bacteriostatic due to its ability to bind Fe^{+2} and thus inhibit the growth of bacteria (Afzali & Goldsmith, 2004). However, it has been shown by Freestone *et al.* (2000) and Sandrini *et al.* (2010) that in the presence of catecholamine stress hormones (NE, Adr and Dop), the transferrin becomes a bacterial iron source and can promote bacterial infection induction. Catecholamine stress hormones are a group of tyrosine-derived effectors, which are chemically characterised by having a catechol and an opposing amine side chain (Freestone *et al.*, 2008). Catecholamine stress hormones are released in response to stress either physiologically or psychologically (Collins & Bercik, 2009). The available data in Chapter 3 indicated inter- and intra- patient variances in transferrin as well as catecholamine stress hormones levels in the PDF. The most abundant catecholamine stress hormone in the PDF samples was NE, which was also detected across all PDF samples. Clinically speaking, NE is the principal neurotransmitter released by the sympathetic nerves and it serves as an index for sympathetic outflow (Aneman *et al.*, 1996), which could explain why its level is the greatest in PDF samples in Chapter 3. Various researchers (Sandrini *et al.*, 2010; Anderson & Armstrong, 2008; Freestone *et al.*, 2000; Freestone *et al.*, 2002; Freestone *et al.*, 2007; Freestone *et al.*, 2008; Neal *et al.*, 2001) have shown that the bacterial growth is significantly stimulated in the presence of transferrin by catecholamine-iron provision from the transferrin. The catechol part of the NE was found by Sandrini *et al.* (2010) to complex with the Fe^{+3} sequestered by

transferrin and reduce it to Fe^{+2}. The resultant NE-Fe^{+2} compound then dissociates and is internalised by bacteria (Sandrini *et al.*, 2010). Furthermore, urea-PAGE gel studies of the iron-binding status of PDF transferrin (showed in Chapter 3) revealed a significant presence of differic transferrin (iron-saturated transferrin) in all of the dialysates of the nine PD patients. This means that the transferrin, which normally works as a major innate immune defence, inhibiting microbial growth by limiting iron accessibility in blood, is actually iron-saturated in PDF.

Given all of the above considerations, the simultaneous presence of iron-saturated transferrin and the catecholamine NE in the PDF could lead to NE-Fe^{+2} complex formation, which subsequently affects the iron binding capacity of transferrin. It is, therefore, possible to argue that the co-localisation of the catecholamines and iron-saturated transferrin in PDF weakens the bacteriostatic nature of PDF and results in production of what could be regarded as a nutritious bacterial growth medium. This means good PD hygiene is absolutely essential to minimise infection and development of peritonitis.

6.3 Investigation of PDF exposure on the virulence of *S.aureus* and *S.epidermidis*

Another objective of the present study was to investigate how PDF may affect *S.aureus* and *S.epidermidis* virulence, as both are major causes of peritoneal infections in PD patients. Based on the experiments analysed in Chapter 4, I demonstrated that the most important aspect of *S.aureus* and *S.epidermidis* virulence, biofilm production, is enhanced by the presence of host-factors in PDF. The release of transferrin, catecholamines and fibrinogen into PDF could enhance staphylococcal attachment to the PD catheter and thereby participate in biofilm development. Neither species haemolytic or proteolytic activities were greatly stimulated by PDF, but the virulence factor activities were still being expressed when the bacteria were incubated in the dialysate, suggesting that haemolytic and proteolytic activities could be involved in the progress of a PD peritonitis.

6.4 Profiling changes in PDF during health and infection

In Chapter 5, longitudinal changes in the biochemical characteristics of PD dialysate during health and infection over a period of time spent by Patient 6 on automated

peritoneal dialysis (14 months) were studied. Under physiological conditions, hypoferraemia is a protective mechanism in which iron is withheld from iron-hungry bacteria (Northrop-Clewes, 2008). Levels of transferrin, typically decrease ~30% in response to infection (Northrop-Clewes, 2008). In Chapter 5, however, the levels of transferrin in PDF did not follow this expected concentration pattern. PDF transferrin levels were greatest on the days of peritoneal infection. Further, the increase in transferrin levels in PDF during infection paralleled the increase in catecholamine stress hormones levels, and NE in particular. It may be that transferrin and NE levels in PDF were the highest during infection because the inflammation of the peritoneum results in acute damage and increased vascular permeability (Davies *et al.*, 1996; Ellam & Wilkie, 2011), a process that may promote leakage of both transferrin and catecholamines during a dialysis exchange. It was hypothesised in this study that the levels of transferrin and catecholamine stress hormones could stimulate bacterial growth in PDF. Freestone *et al.* (2000; 2008) and Sandrini *et al.* (2010) have shown that catecholamine stress hormones can mediate Fe^{+3} removal from transferrin. Mechanistically, the catechol part of catecholamine stress hormones can complex with the Fe^{+3} sequestered by transferrin, thereby making it accessible to bacteria (Freestone *et al.*, 2008; Freestone *et al.*, 2000; Sandrini *et al.*, 2010). It can therefore be argued that the presence of transferrin and catecholamine stress hormones in PDF during both health and infection is in agreement with the study hypothesis.

In this study, PDF-transferrin was shown to be a source of iron for PD-infection causing bacteria. *S.aureus* and *S.epidermidis* uptake of ^{55}Fe from ^{55}Fe-transferrin was much higher in the glucose PDS than in any of the non-infected normal or peritonitis PD dialysates analysed. This finding shows a mechanism or mechanisms are present in PDF that reduce staphylococcal uptake of transferrin-iron. More interestingly, ^{55}Fe uptake from transferrin by *S.aureus* was reduced in peritonitis PDF, even when the peritonitis PDF was diluted by combining it with either glucose PDS or non-infected normal PD dialysate, suggesting there are factors present in the peritonitis PDF that can block host iron uptake by *S.aureus*. Unfortunately, due to time limitations, it was not possible to investigate these factors; thus further study is a priority. Because iron availability is vital for bacterial growth in PDF and indeed all patient tissues, identifying molecules that can block iron uptake could provide a novel approach for antimicrobial protective therapy development.

6.5 Identifying PDF risk factors

Two parameters, transferrin and catecholamine stress hormones (NE, Adr and Dop), were identified as significant ($P < 0.05$) independent risk factors for *S.aureus* growth in the non-infected normal PD dialysates obtained at the beginning of dialysis from nine PD patients. These two parameters also were identified as significant, independent risk factors for *S.aureus* growth in PD dialysates obtained from Patient 6 during health and infected states ($P < 0.05$). Together, these findings suggest that the higher the levels transferrin and catecholamines, the greater the degree of *S.aureus* growth in PDF.

6.6 Summary

Based on the findings discussed in this chapter, I have to conclude that PDF is not always bacteriostatic in nature, as PDF accommodates growth-stimulating factors for pathogens such as *S.aureus*. Specifically, transferrin and catecholamines levels appear to be stimulatory factors for *S.aureus* multiplication in PDF. This finding could lead to the development of a simple dipstick prognostic test to identify patients with high catecholamine and transferrin levels and who thus may be more susceptible to infection. Use of such a test would have the potential to reduce a great deal of PD-patient suffering, and even save lives.

6.7 Future work

PD-associated peritonitis can lead to PD termination and a transfer to haemodialysis, or even loss of life. Identification of risk factors predisposing certain patients to develop PD-associated peritonitis is therefore necessary. Understanding the underlying mechanisms of PD-associated peritonitis will help determine management strategies to reduce its risk. More importantly, risk minimisation will help maximise patients' time with the PD technique, as for some, PD is the *only* convenient option to replace the functioning of their damaged kidneys. Thus, it will improve the odds for patients' survival as well as improve their quality of life. One basic improvement to the further study of host-related risk factors for peritonitis is to increase sample size by recruiting many more PD patients and collecting PD dialysates during their PD time-course. The results of this study indicated the presence of factors that can block bacterial uptake of iron in peritonitis PDF. Identification of the factors with this inhibitory capacity are a future priority, given the importance of iron availability for bacterial growth in PDF. Identifying human produced

iron uptake blocking factors could provide a novel approach for antimicrobial development. Biochemical profiling such as heat and protease treatment, molecular weight determination, would identify if the novel factor was a protein. Also mass spectroscopy analysis can be performed to create molecular profiles of non-infected and infected PDF and identify low molecular weight components produced by PD patients. Levels of transferrin and catecholamines in PDF were shown in the current study to associate independently, positively and significantly with PD pathogen growth such as *S.aureus*. It is therefore important to explore whether the same associations exist between transferrin and catecholamines in PDF and the growth of normal gut flora (Gram-negative bacteria) such as *Escherichia coli* and *Klebsiella pneumoniae*, which are also responsible for severe infectious peritonitis in PD patients. Determining these relationships will reveal whether the presence of transferrin and catecholamines reliably predict the growth of peritonitis-causing bacteria and whether they could be targeted for therapy.

CHAPTER 7 Appendices

Appendix I

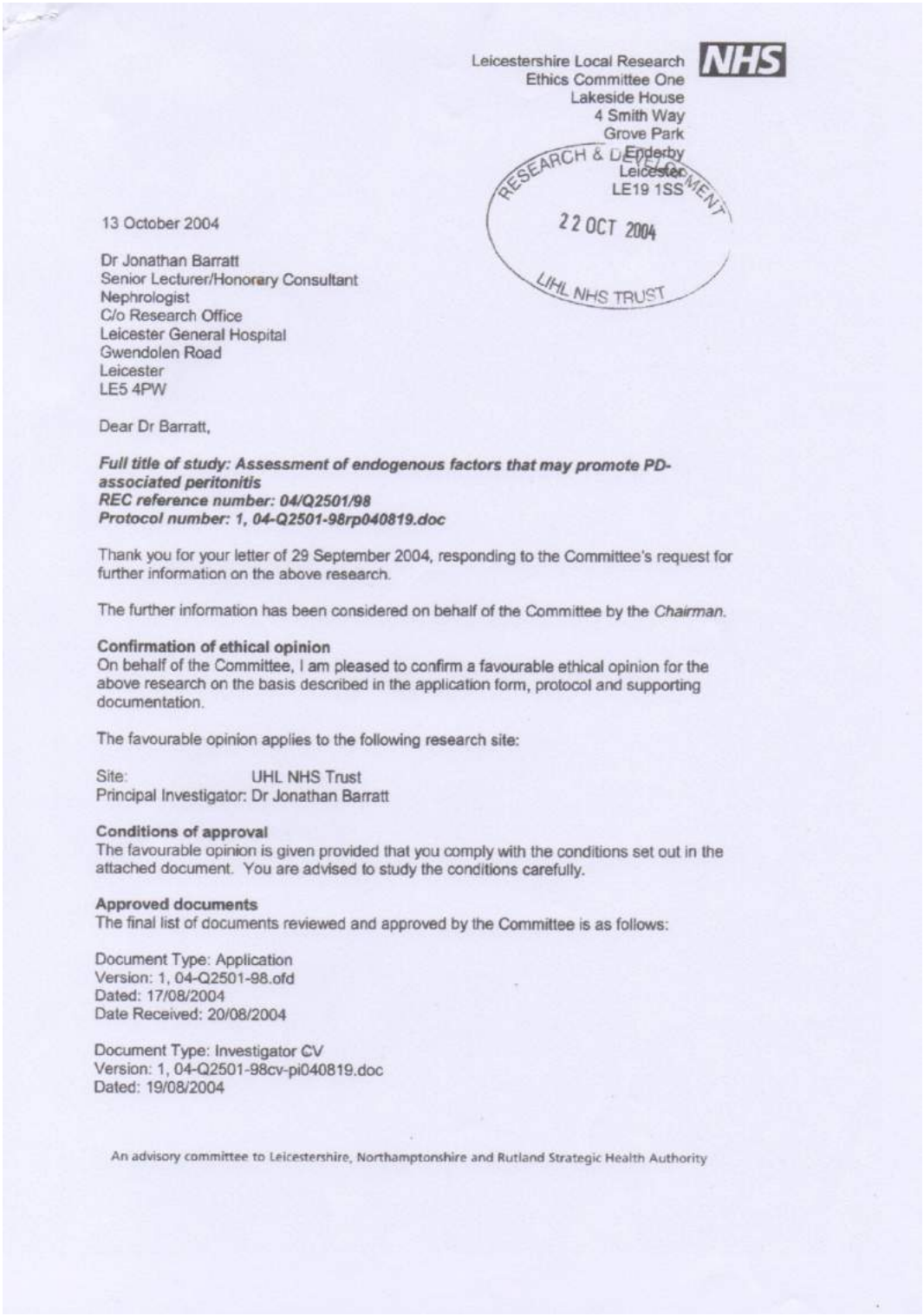
Publications completed during the course of this dissertation:

Sandrini, S., Aldriwesh, M., Alruways, M., Freestone, P., 2015. Microbial endocrinology: host-bacteria communication within the gut microbiome. *The Journal of Endocrinology.* **225,** R21-34.

Appendix II

A copy of the ethical approval for the present study from the Leicestershire Local Research Ethics Committee One in Leicester, UK (Section 2.1.2).

Page 1:



Leicestershire Local Research
Ethics Committee One
Lakeside House
4 Smith Way
Grove Park
Enderby
Leicester
LE19 1SS

NHS

RESEARCH & DEVELOPMENT
22 OCT 2004
UHL NHS TRUST

13 October 2004

Dr Jonathan Barratt
Senior Lecturer/Honorary Consultant
Nephrologist
C/o Research Office
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Dear Dr Barratt,

Full title of study: Assessment of endogenous factors that may promote PD-associated peritonitis
REC reference number: 04/Q2501/98
Protocol number: 1, 04-Q2501-98rp040819.doc

Thank you for your letter of 29 September 2004, responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the *Chairman*.

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

The favourable opinion applies to the following research site:

Site: UHL NHS Trust
Principal Investigator: Dr Jonathan Barratt

Conditions of approval
The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

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

Document Type: Application
Version: 1, 04-Q2501-98.ofd
Dated: 17/08/2004
Date Received: 20/08/2004

Document Type: Investigator CV
Version: 1, 04-Q2501-98cv-pi040819.doc
Dated: 19/08/2004

An advisory committee to Leicestershire, Northamptonshire and Rutland Strategic Health Authority

Page 2:

Date Received: 20/08/2004

Document Type: Protocol
Version: 1, 04-Q2501-98rp040819.doc
Dated: 19/08/2004
Date Received: 20/08/2004

Document Type: Participant Information Sheet
Version: 2, 04-Q2501-98is-p040929.doc
Dated: 29/09/2004
Date Received: 29/09/2004

Document Type: Participant Consent Form
Version: 2, 04-Q2501-98cf-p040929.doc
Dated: 29/09/2004
Date Received: 29/09/2004

Document Type: Response to Request for Further Information
Version:
Dated: 29/09/2004
Date Received: 29/09/2004

Management approval
The study may not commence until final management approval has been confirmed by the organisation hosting the research.

All researchers and research collaborators who will be participating in the research must obtain management approval from the relevant host organisation before commencing any research procedures. Where a substantive contract is not held with the host organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Notification of other bodies
We shall notify the research sponsor, UHL NHS Trust and the Medicines and Health-Care Products Regulatory Agency that the study has a favourable ethical opinion.

Statement of compliance *(from 1 May 2004)*
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC reference number: 04/Q2501/98 **Please quote this number on all correspondence**

Yours sincerely,

Dr Carl Edwards
Chairman

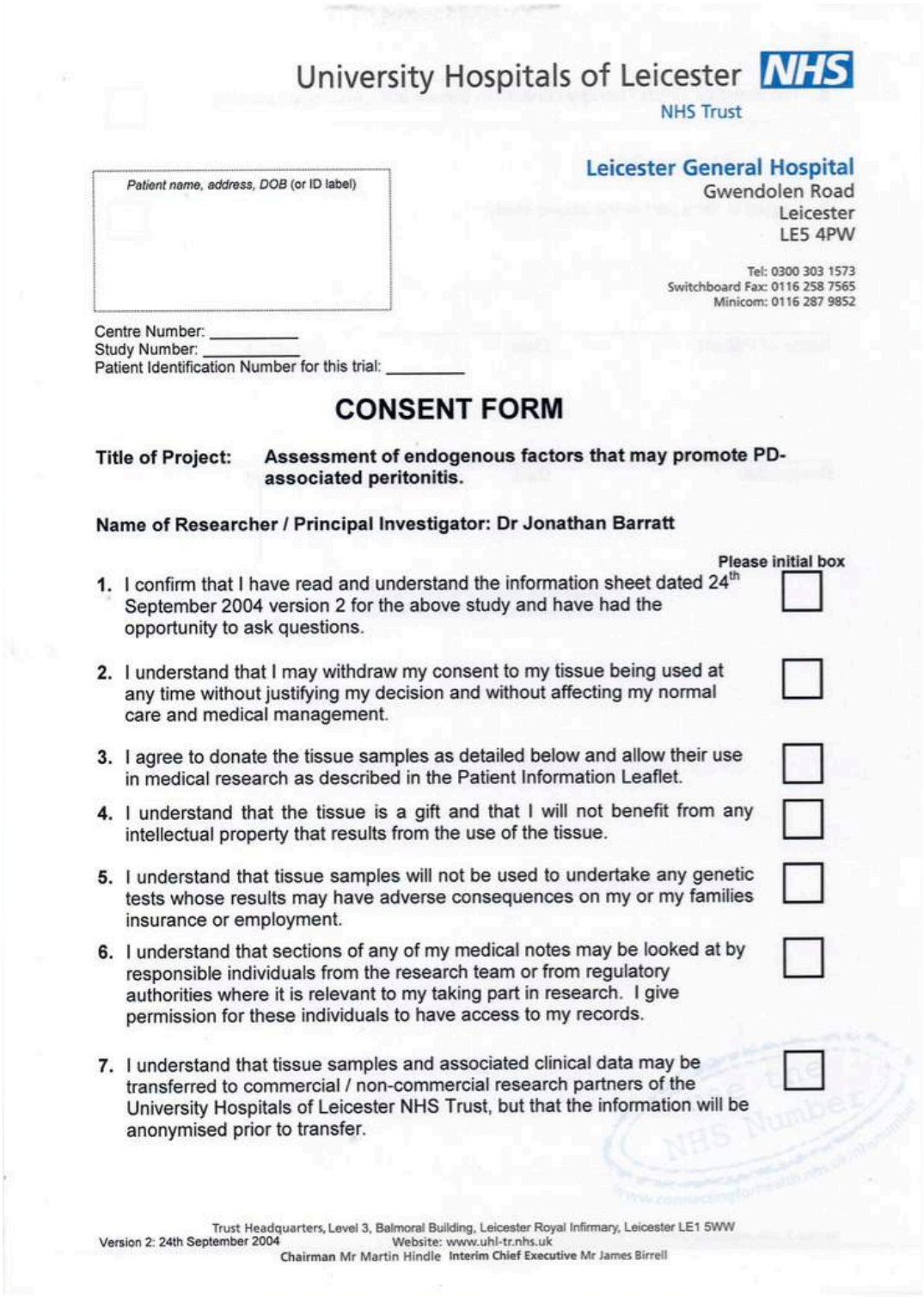
Enclosures *Standard approval conditions [SL-AC1 or SL-AC2]*

An advisory committee to Leicestershire, Northamptonshire and Rutland Strategic Health Authority

Appendix III

A copy of the consent form, which each peritoneal dialysis patient read and signed when they enrolled in this study, before the peritoneal dialysis fluids were analysed (Section 2.1.2).

Page 1:



University Hospitals of Leicester NHS
NHS Trust

Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Tel: 0300 303 1573
Switchboard Fax: 0116 258 7565
Minicom: 0116 287 9852

Patient name, address, DOB (or ID label)

Centre Number: _____
Study Number: _____
Patient Identification Number for this trial: _____

CONSENT FORM

Title of Project: **Assessment of endogenous factors that may promote PD-associated peritonitis.**

Name of Researcher / Principal Investigator: Dr Jonathan Barratt

Please initial box

1. I confirm that I have read and understand the information sheet dated 24th September 2004 version 2 for the above study and have had the opportunity to ask questions. ☐
2. I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management. ☐
3. I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet. ☐
4. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐
5. I understand that tissue samples will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment. ☐
6. I understand that sections of any of my medical notes may be looked at by responsible individuals from the research team or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
7. I understand that tissue samples and associated clinical data may be transferred to commercial / non-commercial research partners of the University Hospitals of Leicester NHS Trust, but that the information will be anonymised prior to transfer. ☐

Trust Headquarters, Level 3, Balmoral Building, Leicester Royal Infirmary, Leicester LE1 5WW
Version 2: 24th September 2004 Website: www.uhl-tr.nhs.uk
Chairman Mr Martin Hindle Interim Chief Executive Mr James Birrell

Page 2:

8. The samples which I hereby consent to donate are: **(please type if possible)** ☐

……PD effluent fluid

9. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Researcher	Date	Signature

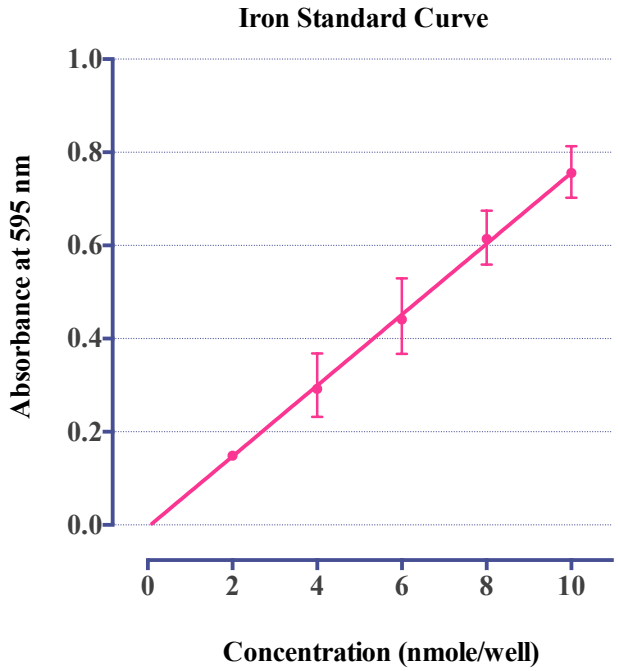
Original for researcher/site file/CRF
copy for patient, copy for hospital notes

Version 2: 24th September 2004

Appendix IV

Representative iron standard curve

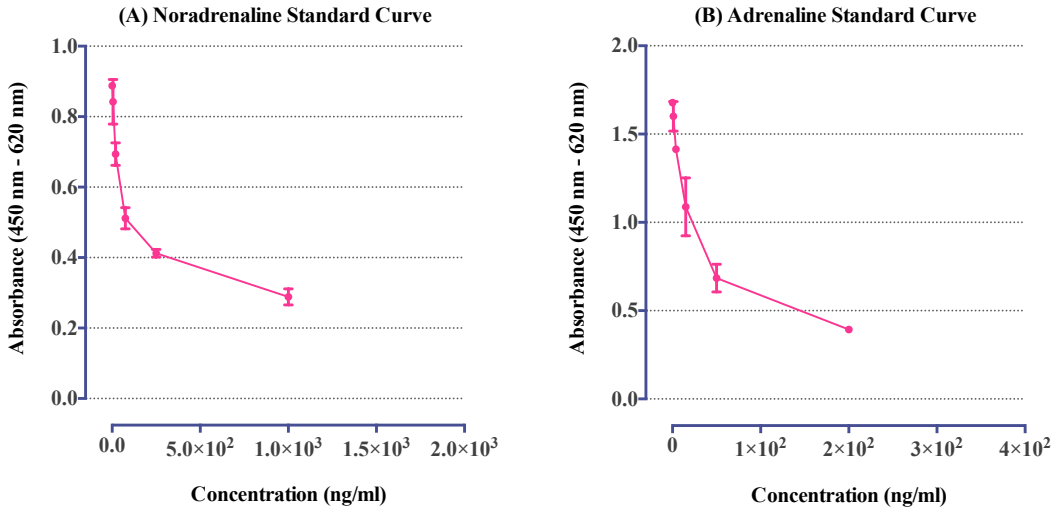
The iron standard curve was produced each time the assay was conducted. The mean values were calculated for the triplicate readings for each standard and PDS or PDF. The standard curves were produced by plotting the mean absorbance reading at 595 nm of the standards (linear, y-axis) versus the corresponding standard concentrations (logarithmic, x-axis). The best-fit line was then determined by linear regression analysis. The amount of iron (nmole) in the PDS/PDF was determined by comparing it with the standard curve, and then used in the equation mentioned in the materials and methods chapter (Section 2.2.1.4) to determine the final iron concentration.

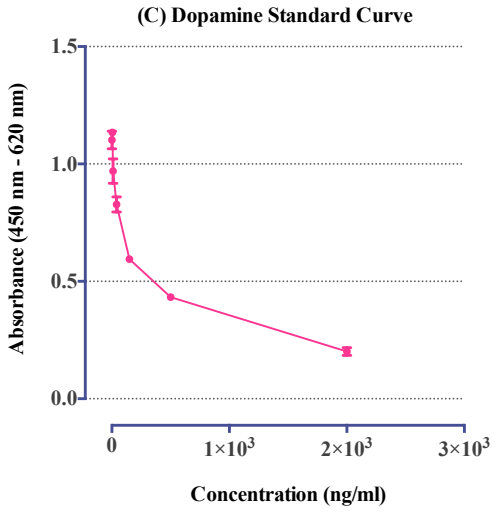


Appendix V

Typical calibration curves for noradrenaline, adrenaline and dopamine

The catecholamine stress hormone standard curves were created utilising the tri cat ELISA kit, as described in the materials and methods chapter (Section 2.2.1.5), and were produced each time the assay was conducted. The calibration curves were produced by plotting the absorbance reading of the standards (linear, y-axis) versus the corresponding standard concentrations (logarithmic, x-axis). The absorbance values were read at 450 nm and at a reference wavelength of 620 nm. The 450 nm values were then subtracted from the 620 nm data to yield final absorbance readings. The best-fit line was then determined by non-linear regression analysis.

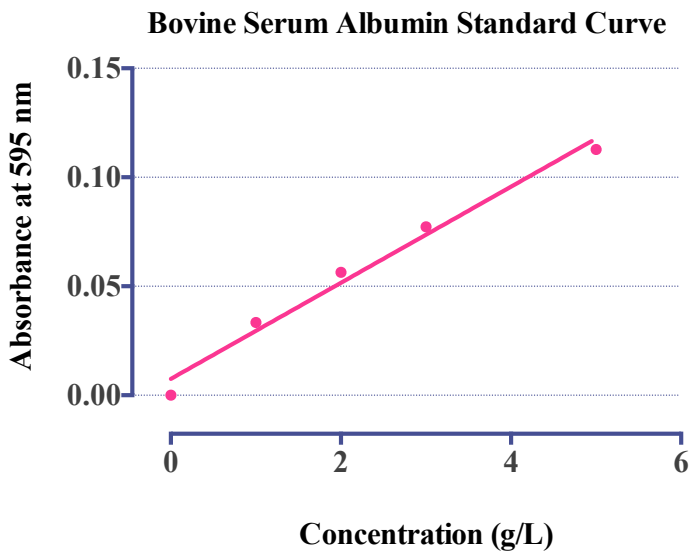




Appendix VI

Typical standard curve for the Bio-Rad protein assay using bovine serum albumin (BSA) as the standard protein

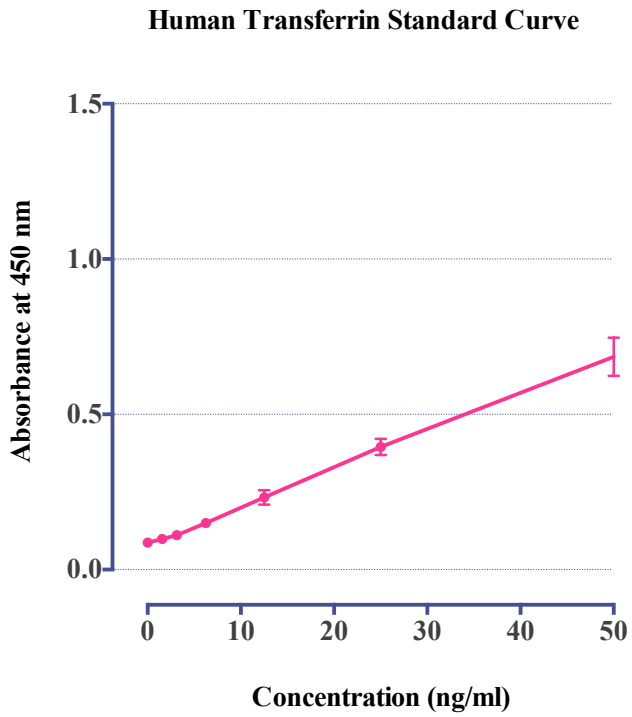
The BSA standard curve was created each time the assay was performed. The absorbance values were exported into a Prism file and the data were used to generate a protein standard curve. The protein concentration of unknown PDF samples was determined by comparison with the standard curve, and then multiplied by the dilution factor (Section 2.2.1.6).



Appendix VII

Representative human transferrin standard curve

The human transferrin standard curve was generated each time the assay was performed. The mean values were calculated for the triplicate readings for each standard and unknown PDF sample. The graph was plotted using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance readings on the y-axis. The best-fit line was then determined by regression analysis using a log-log or semi-log curve fit. The unknown PDF transferrin concentration was determined from the standard curve, and the value was multiplied by the dilution factor (Section 2.2.1.10).

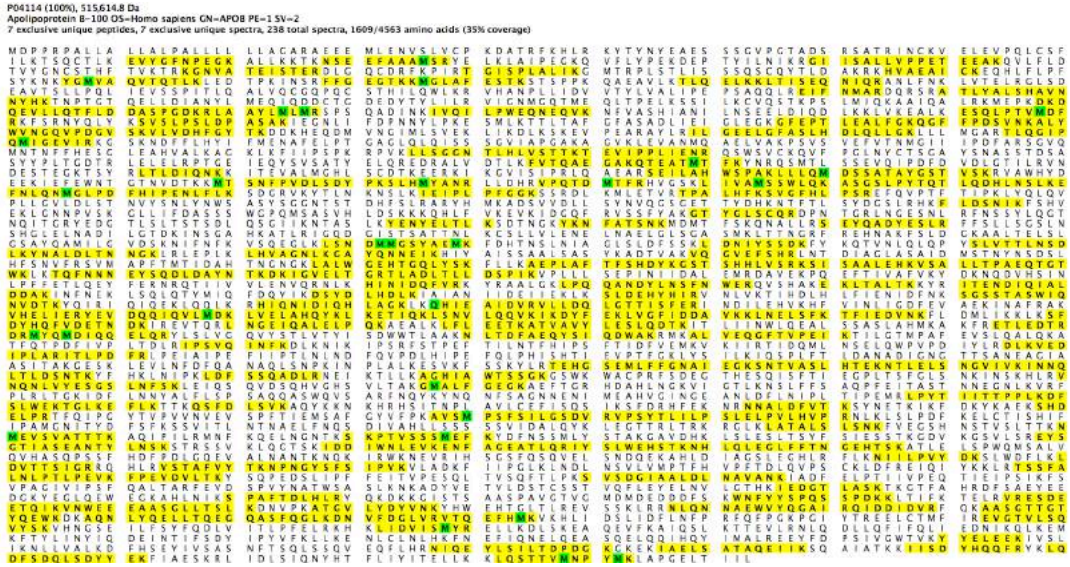


Appendix VIII

Protein sequence using liquid chromatography-mass spectrometry technique (LC-MS)

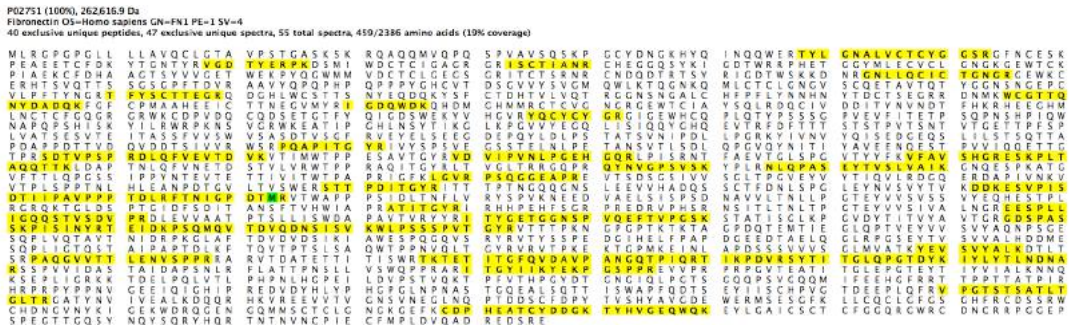
Protein 1

Apolipoprotein B-100



Protein 2

Fibronectin

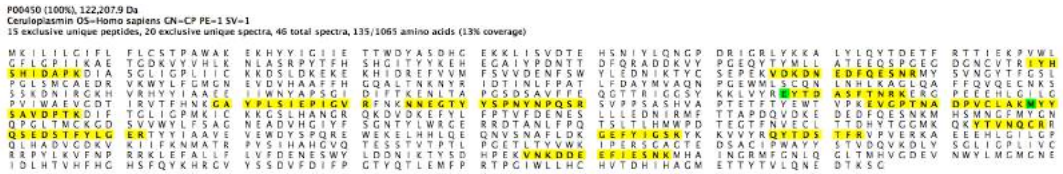


Protein 3

Alpha-2-macroglobuline

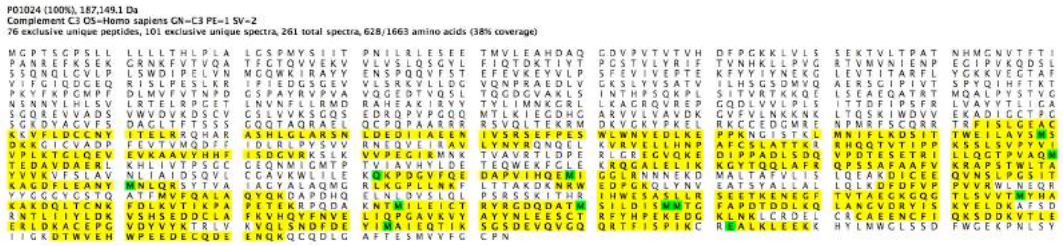
Protein 4

Ceruloplasmin



Protein 5

Complement component 3

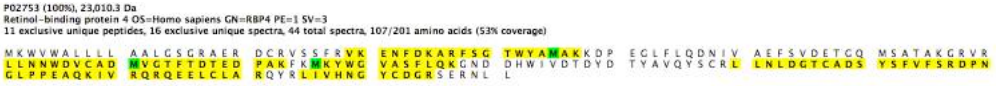


Protein 6

Fibrinogen gamma chain

Protein 15

Retinol-binding protein

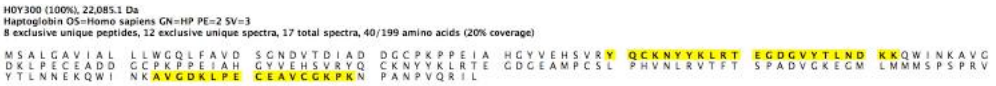


Protein 16

Beta-2-microglobulin

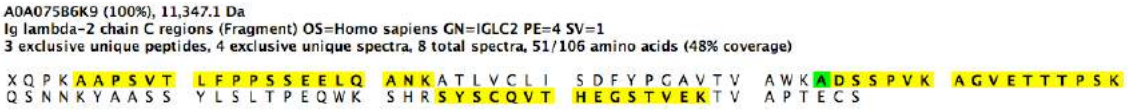
Protein 18

Haptoglobin



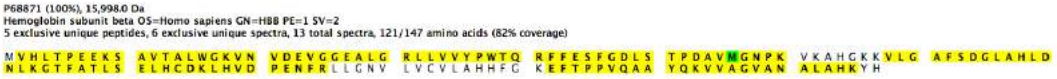
Protein 19

Ig lambda-2 chain C regions



Protein 22

Haemoglobin subunit beta



Protein sequence using Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

Protein 7

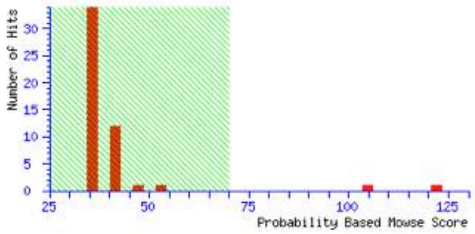
Serotransferrin

University of Leicester **Mascot Search Results**

```
User            : Mascot Wizard
Email           : sya2@le.ac.uk
Search title    : MA_01_0001.dat - Sample Info, G:\160309\MA_01_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2014_11 (547085 sequences; 194742747 residues)
Timestamp       : 11 Mar 2016 at 12:35:18 GMT
Top Score       : 122 for P02787, Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 70 are significant (p<0.05).



Protein 8

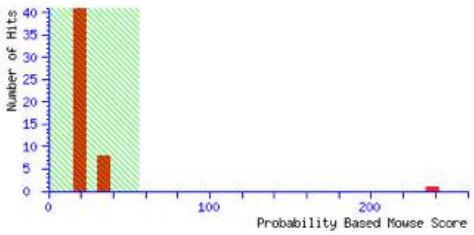
Serum albumin

University of Leicester **Mascot Search Results**

```
User            :
Email           :
Search title    : MA_02_0001.dat - Sample Info, G:\160309\MA_02_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2014_11 (547085 sequences; 194742747 residues)
Taxonomy        : Homo sapiens (human) (20194 sequences)
Timestamp       : 14 Sep 2016 at 13:04:21 GMT
Top Score       : 238 for P02768, Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 56 are significant (p<0.05).



Protein 9

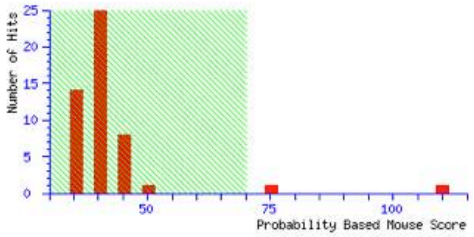
Alpha-1-antitrypsin

University of Leicester **Mascot Search Results**

```
User            : Mascot Wizard
Email           : sya2@le.ac.uk
Search title    : MA_03_0001.dat - Sample Info, G:\160309\MA_03_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2014_11 (547085 sequences; 194742747 residues)
Timestamp       : 11 Mar 2016 at 12:35:53 GMT
Top Score       : 110 for P01009, Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 70 are significant (p<0.05).



Protein 10

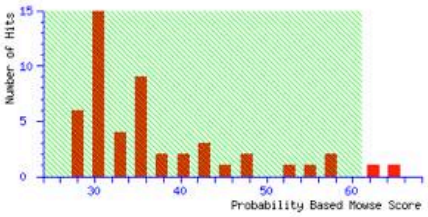
tRNA-dihydrouridine (20) synthase [NAD (P) +] – like (Fragment)

University of Leicester **Mascot Search Results**

```
User            :
Email           :
Search title    : MA_04_0001.dat - Sample Info, G:\160309\MA_04_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniprotHuman 2015_02 (67914 sequences; 22756652 residues)
Timestamp       : 11 Mar 2016 at 12:42:16 GMT
Top Score       : 65 for J3QLD5, tRNA-dihydrouridine(20) synthase [NAD(P)+]-like (Fragment) OS=Homo sapiens GN=DUS2 PE=4 SV=1
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 61 are significant (p<0.05).



Protein 11

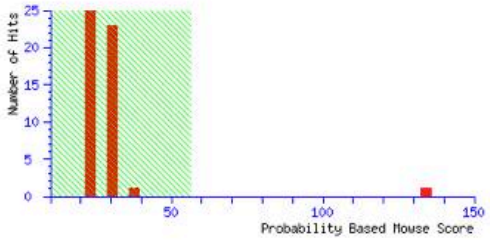
Apolipoprotein A-IV

University of Leicester **Mascot Search Results**

```
User            :
Email           :
Search title    : AM_02_0001.dat - Sample Info, F:\141027\AM_02_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2014_11 (547085 sequences; 194742747 residues)
Taxonomy        : Homo sapiens (human) (20194 sequences)
Timestamp       : 14 Sep 2016 at 13:20:52 GMT
Top Score       : 134 for P06727, Apolipoprotein A-IV OS=Homo sapiens GN=APOA4 PE=1 SV=3
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 56 are significant (p<0.05).



Protein 12

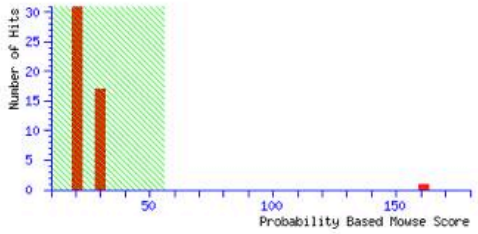
Fibrinogen beta chain

University of Leicester **Mascot Search Results**

```
User            :
Email           :
Search title    : AM_01_0001.dat - Sample Info, F:\141027\AM_01_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2014_11 (547085 sequences; 194742747 residues)
Taxonomy        : Homo sapiens (human) (20194 sequences)
Timestamp       : 14 Sep 2016 at 13:19:22 GMT
Top Score       : 161 for P02675, Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 56 are significant (p<0.05).



Protein 13

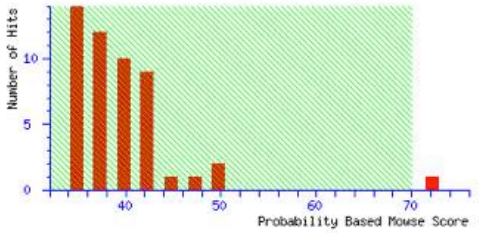
Ig kappa chain C region

University of Leicester **Mascot Search Results**

```
User            : Mascot Wizard
Email           : sya2@le.ac.uk
Search title    : MA_05_0001.dat - Sample Info, G:\160309\MA_05_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2014_11 (547085 sequences; 194742747 residues)
Timestamp       : 11 Mar 2016 at 12:39:26 GMT
Top Score       : 72 for P01834, Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 70 are significant (p<0.05).



Protein 14

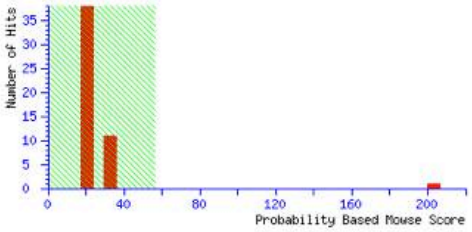
Apolipoprotein A-I

University of Leicester **Mascot Search Results**

```
User            :
Email           :
Search title    : MA_06_0001.dat - Sample Info, G:\160309\MA_06_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2014_11 (547085 sequences; 194742747 residues)
Taxonomy        : Homo sapiens (human) (20194 sequences)
Timestamp       : 14 Sep 2016 at 13:15:32 GMT
Top Score       : 203 for P02647, Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 56 are significant (p<0.05).



Protein 17

Transthyretin

University of Leicester **Mascot Search Results**

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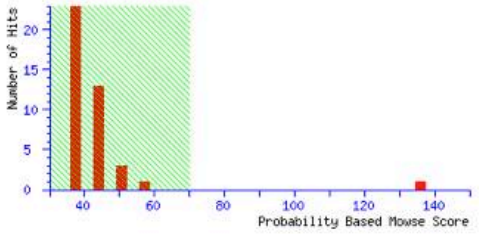
User            : Mascot Wizard
Email           : sya2@le.ac.uk
Search title    : AM_04_0001.dat - Sample Info, F:\141024\AM_04_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2014_07 (546000 sequences; 194259968 residues)
Timestamp       : 27 Oct 2014 at 15:11:47 GMT
Top Score       : 136 for P02766, Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 70 are significant (p<0.05).



Protein 20

Protein S100-A9

University of Leicester **Mascot Search Results**

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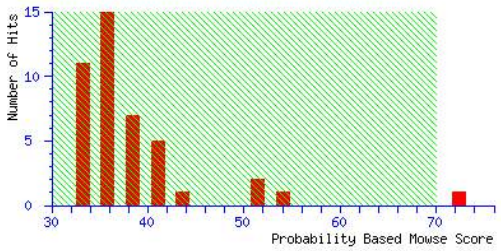
User            : Mascot Wizard
Email           : sya2@le.ac.uk
Search title    : MA_03_0001.dat - Sample Info, F:\140516\MA_03_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2012_08 (537505 sequences; 190795142 residues)
Timestamp       : 16 May 2014 at 13:25:32 GMT
Top Score       : 72 for P06702, Protein S100-A9 OS=Homo sapiens GN=S100A9 PE=1 SV=1
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 70 are significant (p<0.05).



Protein 21

Protein S100-A8

University of Leicester **Mascot Search Results**

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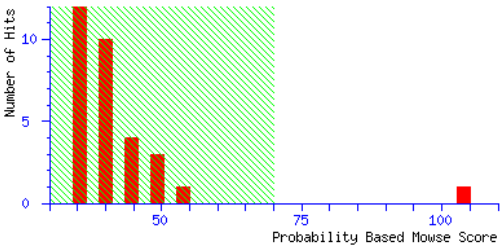
User            : Mascot Wizard
Email           : sya2@le.ac.uk
Search title    : MA_11_0001.dat - Sample Info, F:\140516\MA_11_0001.dat submitted from Mascot Wizard on HT-4575
Database        : UniProtKB-SwissProt 2012_08 (537505 sequences; 190795142 residues)
Timestamp       : 16 May 2014 at 13:31:45 GMT
Top Score       : 104 for P05109, Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 70 are significant (p<0.05).



CHAPTER 8 References

Afzali, B. & Goldsmith, D.J., 2004. Intravenous iron therapy in renal failure: Friend and foe? *Journal of Nephrology.* **17,** 487-495.

Ahmetagic, A., Numanovic, F., Ahmetagic, S., Rakovac-Tupkovic, L., Porobic-Jahic, H., 2013. Etiology of peritonitis. *Medical Archives.* **67,** 278.

Akoh, J.A. & Hakim, N.S., 2001. *Dialysis access: current practice.* World Scientific.

Alayash, A.I., 2011. Haptoglobin: old protein with new functions. *Clinica Chimica Acta.* **412,** 493-498.

Anderson, M.T. & Armstrong, S.K., 2008. Norepinephrine mediates acquisition of transferrin-iron in Bordetella bronchiseptica. *Journal of Bacteriology.* **190,** 3940-3947.

Aneman, A., Eisenhofer, G., Olbe, L., Dalenback, J., Nitescu, P., Fandriks, L., Friberg, P., 1996. Sympathetic discharge to mesenteric organs and the liver. Evidence for substantial mesenteric organ norepinephrine spillover. *The Journal of Clinical Investigation.* **97,** 1640-1646.

Ash, S.R. & Janle, E.M., 1993. T-fluted peritoneal dialysis catheter. *Advances in Peritoneal Dialysis.* **9,** 223-223.

Austin, P.D., Hand, K.S., Elia, M., 2012. Factors that influence *Staphylococcus epidermidis* growth in parenteral nutrition with and without lipid emulsion: A study framework to inform maximum duration of infusion policy decisions. *Clinical Nutrition.* **31,** 974-980.

Baker, S., Griffiths, C. and Nicklin, J., 2011. *Microbiology.* Fourth Edition ed. New York NY 10016, USA: Garland Science, Taylor & Francis Group, LLC.

Barnett, N. & Mamode, N., 2011. Kidney transplantation. *Surgery (Oxford).* **29,** 330-335.

Barratt, J., Topham, P. and Harris, K.P., 2008. *Oxford Desk Reference: Nephrology.* Oxford University Press, USA.

Barretti, P., Montelli, A.C., Batalha, J.E., Caramori, J.C., Maria de Lourdes, R., 2009. The role of virulence factors in the outcome of staphylococcal peritonitis in CAPD patients. *BMC Infectious Diseases.* **9,** 1.

Barretti, P., Moraes, T.M., Camargo, C.H., Caramori, J.C., Mondelli, A.L., Montelli, A.C., Maria de Lourdes, R., 2012. Peritoneal dialysis-related peritonitis due to *Staphylococcus aureus*: a single-center experience over 15 years. *PloS One.* **7,** e31780.

Barry, A.L., Lachica, R.V., Atchison, F.W., 1973. Identification of *Staphylococcus aureus* by simultaneous use of tube coagulase and thermonuclease tests. *Applied Microbiology.* **25,** 496-497.

Bazzi, C., Rizza, V., Olivieri, G., Casellato, D., D'Amico, G., 2015. Tubular reabsorption of high, middle and low molecular weight proteins according to the tubulo-interstitial damage marker N-acetyl-β-d-glucosaminidase in glomerulonephritis. *Journal of Nephrology.* **28,** 541-548.

Beasley, F.C. & Heinrichs, D.E., 2010. Siderophore-mediated iron acquisition in the staphylococci. *Journal of Inorganic Biochemistry.* **104,** 282-288.

Bersenas, A.M., 2011. A clinical review of peritoneal dialysis. *Journal of Veterinary Emergency and Critical Care.* **21,** 605-617.

Bhakdi, S. & Tranum-Jensen, J., 1991. Alpha-toxin of *Staphylococcus aureus*. *Microbiological Reviews.* **55,** 733-751.

Blumenkrantz, M.J., Gahl, G.M., Kopple, J.D., Kamdar, A.V., Jones, M.R., Kessel, M., Coburn, J.W., 1981. Protein losses during peritoneal dialysis. *Kidney International.* **19,** 593-602.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* **72,** 248-254.

Brantly, M., Nukiwa, T., Crystal, R.G., 1988. Molecular basis of alpha-1-antitrypsin deficiency. *The American Journal of Medicine.* **84,** 13-31.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria. *Science (New York, N.Y.).* **303,** 1532-1535.

Burger, D. & Dayer, J., 2002. High-density lipoprotein-associated apolipoprotein AI: the missing link between infection and chronic inflammation? *Autoimmunity Reviews.* **1,** 111-117.

Burkart, J.M., Jordan, J.R., Durnell, T.A., Case, L.D., 1992. Comparison of exit-site infections in disconnect versus nondisconnect systems for peritoneal dialysis. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **12,** 317-320.

Cameron, J.S., 1995. Host defences in continuous ambulatory peritoneal dialysis and the genesis of peritonitis. *Pediatric Nephrology.* **9,** 647-662.

Canadian, C., 1989. Peritonitis in continuous ambulatory peritoneal dialysis (CAPD): a multi-centre randomized clinical trial comparing the Y-connector disinfectant system to standard systems. *Perit Dial Int.* **9,** 159-164.

Carozzi, S., Nasini, M.G., Schelotto, C., Caviglia, P.M., Canepa, M., Zanin, T., Cantaluppi, A., Salit, M., 1990. Bacterial peritonitis and beta-2 microglobulin (B2M) production by peritoneal macrophages (PM0) in CAPD patients'. *Adv Perit Dial.* **6,** 106-109.

Castrale, C., Evans, D., Verger, C., Fabre, E., Aguilera, D., Ryckelynck, J.P., Lobbedez, T., 2010. Peritoneal dialysis in elderly patients: report from the French Peritoneal Dialysis Registry (RDPLF). *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **25,** 255-262.

Chan, T.M., Leung, J.K., Sun, Y., Lai, K.N., Tsang, R.C., Yung, S., 2003. Different effects of amino acid-based and glucose-based dialysate from peritoneal dialysis patients on mesothelial cell ultrastructure and function. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **18,** 1086-1094.

Chaudhary, K., Sangha, H., Khanna, R., 2011. Peritoneal dialysis first: rationale. *Clinical Journal of the American Society of Nephrology : CJASN.* **6,** 447-456.

Chen, T.W., Li, S.Y., Chen, J.Y., Yang, W.C., 2008. Training of peritoneal dialysis patients--Taiwan's experiences. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **28 Suppl 3,** S72-5.

Chern, Y.B., Ho, P.S., Kuo, L.C., Chen, J.B., 2013. Lower education level is a major risk factor for peritonitis incidence in chronic peritoneal dialysis patients: a retrospective cohort study with 12-year follow-up. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **33,** 552-558.

Cho, Y.M., Youn, B.S., Lee, H., Lee, N., Min, S.S., Kwak, S.H., Lee, H.K., Park, K.S., 2006. Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. *Diabetes Care.* **29,** 2457-2461.

Chow, K.M., Szeto, C.C., Leung, C.B., Law, M.C., Li, P.K., 2005. Impact of social factors on patients on peritoneal dialysis. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **20,** 2504-2510.

Clarkson, M.R., Brenner, B.M. and Magee, C., 2010. *Pocket Companion to Brenner and Rector's The Kidney.* Elsevier Health Sciences.

Cockayne, A., Hill, P.J., Powell, N.B., Bishop, K., Sims, C., Williams, P., 1998. Molecular cloning of a 32-kilodalton lipoprotein component of a novel iron-regulated *Staphylococcus epidermidis* ABC transporter. *Infection and Immunity.* **66,** 3767-3774.

Cohen, A., Martin, M., Mizanin, J., Konkle, D.F., Schwartz, E., 1990. Vision and hearing during deferoxamine therapy. *The Journal of Pediatrics.* **117,** 326-330.

Collins, S.M. & Bercik, P., 2009. The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. *Gastroenterology.* **136,** 2003-2014.

Cotton, J.L., Tao, J., Balibar, C.J., 2009. Identification and Characterization of the *Staphylococcus aureus* Gene Cluster Coding for Staphyloferrin A. *Biochemistry.* **48,** 1025-1035.

Couchoud, C., Bolignano, D., Nistor, I., Jager, K.J., Heaf, J., Heimburger, O., Van Biesen, W., European Renal Best Practice (ERBP) Diabetes Guideline Development Group, 2015. Dialysis modality choice in diabetic patients with end-stage kidney disease: a systematic review of the available evidence. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **30,** 310-320.

Cuccurullo, M., Evangelista, C., Vilasi, A., Simeoni, M., Avella, F., Riccio, E., Memoli, B., Malorni, A., Capasso, G., 2011. Proteomic analysis of peritoneal fluid of patients treated by peritoneal dialysis: effect of glucose concentration. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **26,** 1990-1999.

Daly, C.D., Campbell, M.K., MacLeod, A.M., Cody, D.J., Vale, L.D., Grant, A.M., Donaldson, C., Wallace, S.A., Lawrence, P.D., Khan, I.H., 2001. Do the Y-set and double-bag systems reduce the incidence of CAPD peritonitis? A systematic review of randomized controlled trials. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **16,** 341-347.

Dasgupta, M., Ulan, R., Bettcher, K., Burns, V., Lam, K., Dossetor, J., Costerton, J., 1986. Effect of exit site infection and peritonitis on the distribution of biofilm encased adherent bacterial microcolonies (BABM) on Tenckhoff (T) catheters in patients undergoing continuous ambulatory peritoneal dialysis (CAPD). *Adv Perit Dial.* **2,** 102-109.

Dasgupta, M.K., 2002. Biofilms and infection in dialysis patients, *Seminars in dialysis,* 2002, Wiley Online Library pp338-346.

Davenport, A., 2009. Peritonitis remains the major clinical complication of peritoneal dialysis: the London, UK, peritonitis audit 2002-2003. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **29,** 297-302.

Davies, S.J., Bryan, J., Phillips, L., Russell, G.I., 1996. Longitudinal changes in peritoneal kinetics: the effects of peritoneal dialysis and peritonitis. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **11,** 498-506.

Dey, S. & Rosen, B.P., 1995. Dual mode of energy coupling by the oxyanion-translocating ArsB protein. *Journal of Bacteriology.* **177,** 385-389.

Dinges, M.M., Orwin, P.M., Schlievert, P.M., 2000. Exotoxins of *Staphylococcus aureus*. *Clinical Microbiology Reviews.* **13,** 16-34.

Don, B.R. and Kaysen, G., 2004. Poor nutritional status and inflammation: serum albumin: relationship to inflammation and nutrition, *Seminars in dialysis,* 2004, Wiley Online Library pp432-437.

Dong, J., Chen, Y., Luo, S., Xu, R., Xu, Y., 2013. Peritoneal protein leakage, systemic inflammation, and peritonitis risk in patients on peritoneal dialysis. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **33,** 273-279.

Drapeau, G.R., 1978. Role of metalloprotease in activation of the precursor of staphylococcal protease. *Journal of Bacteriology.* **136,** 607-613.

Dubin, G., Chmiel, D., Mak, P., Rakwalska, M., Rzychon, M., Dubin, A., 2001. Molecular cloning and biochemical characterisation of proteases from *Staphylococcus epidermidis*. *Biological Chemistry.* **382,** 1575-1582.

Dudek, R.W., 2007. *High - Yield Kidney.* Philadelphia, Pa.; London: Lippincott Williams & Wilkins.

Dulaney, J.T. & Hatch, F.E., 1984. Peritoneal dialysis and loss of proteins: a review. *Kidney International.* **26,** 253-262.

Duwe, A.K., Vas, S.I., Weatherhead, J.W., 1981. Effects of the composition of peritoneal dialysis fluid on chemiluminescence, phagocytosis, and bactericidal activity in vitro. *Infection and Immunity.* **33,** 130-135.

Eisenberg, E.S., Ambalu, M., Szylagi, G., Aning, V., Soeiro, R., 1987. Colonization of skin and development of peritonitis due to coagulase-negative staphylococci in patients undergoing peritoneal dialysis. *The Journal of Infectious Diseases.* **156,** 478-482.

Eknoyan, G., Lameire, N., Eckardt, K., Kasiske, B., Wheeler, D., Levin, A., Stevens, P., Bilous, R., Lamb, E., Coresh, J., 2013. KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. *Kidney Int.* **3,** 5-14.

Ellam, T. & Wilkie, M., 2011. Peritoneal dialysis. *Medicine.* **39,** 434-437.

Elliott, D.A., 2000. Hemodialysis. *Clinical Techniques in Small Animal Practice.* **15,** 136-148.

Episkopou, V., Maeda, S., Nishiguchi, S., Shimada, K., Gaitanaris, G.A., Gottesman, M.E., Robertson, E.J., 1993. Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. *Proceedings of the National Academy of Sciences of the United States of America.* **90,** 2375-2379.

Evans, R.W. & Williams, J., 1980. The electrophoresis of transferrins in urea/polyacrylamide gels. *The Biochemical Journal.* **189,** 541-546.

Fang, W., Qian, J., Lin, A., Rowaie, F., Ni, Z., Yao, Q., Bargman, J.M., Oreopoulos, D.G., 2008. Comparison of peritoneal dialysis practice patterns and outcomes between a Canadian and a Chinese centre. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **23,** 4021-4028.

Farias, M.G., Soucie, J.M., McClellan, W., Mitch, W.E., 1994. Race and the risk of peritonitis: an analysis of factors associated with the initial episode. *Kidney International.* **46,** 1392-1396.

Filipek, R., Rzychon, M., Oleksy, A., Gruca, M., Dubin, A., Potempa, J., Bochtler, M., 2003. The Staphostatin-staphopain complex: a forward binding inhibitor in complex with its target cysteine protease. *The Journal of Biological Chemistry.* **278,** 40959-40966.

Fishbane, S., 1999. Review of issues relating to iron and infection. *American Journal of Kidney Diseases.* **34,** s47-s52.

Flessner, M.F., 1991. Peritoneal transport physiology: insights from basic research. *Journal of the American Society of Nephrology : JASN.* **2,** 122-135.

Fontan, M.P., Cambre, H.D., Rodriguez-Carmona, A., Muniz, A.L., Falcon, T.G., 2009. Treatment of peritoneal dialysis-related peritonitis with ciprofloxacin monotherapy: clinical outcomes and bacterial susceptibility over two decades. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **29,** 310-318.

Freestone, P.P., Williams, P.H., Haigh, R.D., Maggs, A.F., Neal, C.P., Lyte, M., 2002. Growth stimulation of intestinal commensal Escherichia coli by catecholamines: a possible contributory factor in trauma-induced sepsis. *Shock.* **18,** 465-470.

Freestone, P.P., Haigh, R.D., Williams, P.H., Lyte, M., 1999. Stimulation of bacterial growth by heat-stable, norepinephrine-induced autoinducers. *FEMS Microbiology Letters.* **172,** 53-60.

Freestone, P.P., Sandrini, S.M., Haigh, R.D., Lyte, M., 2008. Microbial endocrinology: how stress influences susceptibility to infection. *Trends in Microbiology.* **16,** 55-64.

Freestone, P.P., Haigh, R.D., Lyte, M., 2008. Catecholamine inotrope resuscitation of antibiotic-damaged staphylococci and its blockade by specific receptor antagonists. *The Journal of Infectious Diseases.* **197,** 1044-1052.

Freestone, P.P., Haigh, R.D., Lyte, M., 2007. Specificity of catecholamine-induced growth in Escherichia coli O157:H7, Salmonella enterica and Yersinia enterocolitica. *FEMS Microbiology Letters.* **269,** 221-228.

Freestone, P.P., Haigh, R.D., Williams, P.H., Lyte, M., 2003. Involvement of enterobactin in norepinephrine-mediated iron supply from transferrin to enterohaemorrhagic Escherichia coli. *FEMS Microbiology Letters.* **222,** 39-43.

Freestone, P.P., Lyte, M., Neal, C.P., Maggs, A.F., Haigh, R.D., Williams, P.H., 2000. The mammalian neuroendocrine hormone norepinephrine supplies iron for bacterial growth in the presence of transferrin or lactoferrin. *Journal of Bacteriology.* **182,** 6091-6098.

Furkert, J., Zeier, M., Schwenger, V., 2008. Effects of peritoneal dialysis solutions low in GDPs on peritonitis and exit-site infection rates. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **28,** 637-640.

Gebhardt, C., Németh, J., Angel, P., Hess, J., 2006. S100A8 and S100A9 in inflammation and cancer. *Biochemical Pharmacology.* **72,** 1622-1631.

Giangrande, A., Limido, A., Cantù, P., Allaria, P., 1980. SDS-Polyacrylamide electrophoresis of protein loss during continuous ambulatory peritoneal dialysis. *International Journal of Clinical & Laboratory Research.* **10,** 117-120.

Gokal, R., Khanna, R., Krediet, R.T. and Nolph, K.D., 2013. *Textbook of peritoneal dialysis.* Springer Science & Business Media.

Goldstein, M., Carrillo, M., Ghai, S., 2013. Continuous ambulatory peritoneal dialysis—a guide to imaging appearances and complications. *Insights into Imaging.* **4,** 85-92.

Gomme, P.T., McCann, K.B., Bertolini, J., 2005. Transferrin: structure, function and potential therapeutic actions. *Drug Discovery Today.* **10,** 267-273.

Gorman, S., Adair, C., Mawhinney, W., 1994. Incidence and nature of peritoneal catheter biofilm determined by electron and confocal laser scanning microscopy. *Epidemiology and Infection.* **112,** 551-559.

Govindarajulu, S., Hawley, C.M., McDonald, S.P., Brown, F.G., Rosman, J.B., Wiggins, K.J., Bannister, K.M., Johnson, D.W., 2010. *Staphylococcus aureus* peritonitis in Australian peritoneal dialysis patients: predictors, treatment, and outcomes in 503 cases. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **30,** 311-319.

Grodstein, G.P., Blumenkrantz, M.J., Kopple, J.D., Moran, J.K., Coburn, J.W., 1981. Glucose absorption during continuous ambulatory peritoneal dialysis. *Kidney International.* **19,** 564-567.

Gruys, E., Toussaint, M.J., Niewold, T.A., Koopmans, S.J., 2005. Acute phase reaction and acute phase proteins. *Journal of Zhejiang University.Science.B.* **6,** 1045-1056.

Guyton, A. & Hall, J., 2006. Textbook of medical physiology, 11th Edition.

Gyurcsik, B. & Nagy, L., 2000. Carbohydrates as ligands: coordination equilibria and structure of the metal complexes. *Coordination Chemistry Reviews.* **203,** 81-149.

Hain, H. & Kessel, M., 1987. Aspects of new solutions for peritoneal dialysis. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **2,** 67-72.

Hall, J.E., 2015. *Guyton and Hall textbook of medical physiology.* Elsevier Health Sciences.

Hammer, N.D. & Skaar, E.P., 2012. The impact of metal sequestration on *Staphylococcus aureus* metabolism. *Current Opinion in Microbiology.* **15,** 10-14.

Han, J.S., Jang, I., Ryu, H., Kim, W., 2010. Hemolysin Gene Expression in the hns Knockout Mutant of Klebsiella pneumoniae UN Strain. *Journal of the Korean Society for Applied Biological Chemistry.* **53,** 401-406.

Han, S.H., Lee, J.E., Kim, D.K., Moon, S.J., Kim, H.W., Chang, J.H., Kim, B.S., Kang, S.W., Choi, K.H., Lee, H.Y., Han, D.S., 2008. Long-term clinical outcomes of peritoneal dialysis patients: single center experience from Korea. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **28 Suppl 3,** S21-6.

Hellman, N.E. & Gitlin, J.D., 2002. Ceruloplasmin metabolism and function. *Annual Review of Nutrition.* **22,** 439-458.

Ishibashi, F., 1994. Glomerular clearance and tubular reabsorption of transferrin in microtransferrinuric patients with non-insulin-dependent diabetes. *Diabetes Research and Clinical Practice.* **25,** 169-175.

Jaeger, K.E., Ransac, S., Dijkstra, B.W., Colson, C., van Heuvel, M., Misset, O., 1994. Bacterial lipases. *FEMS Microbiology Reviews.* **15,** 29-63.

Jarvis, E.M., Hawley, C.M., McDonald, S.P., Brown, F.G., Rosman, J.B., Wiggins, K.J., Bannister, K.M., Johnson, D.W., 2010. Predictors, treatment, and outcomes of non-Pseudomonas Gram-negative peritonitis. *Kidney International.* **78,** 408-414.

Kanekar, P., Nilegaonkar, S., Sarnaik, S., Kelkar, A., 2002. Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. *Bioresource Technology.* **85,** 87-93.

Kaplan, R.A., Alon, V., Hellerstein, S., Warady, B.A., 1993. Unusual causes of peritonitis in three children receiving peritoneal dialysis. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **13,** 60-63.

Kasprzak, J.M., Czerwoniec, A., Bujnicki, J.M., 2012. Molecular evolution of dihydrouridine synthases. *BMC Bioinformatics.* **13,** 1.

Katirtzoglou, A., Oreopoulos, D., Husdan, H., Leung, M., Ogilvie, R., Dombros, N., 1980. Reappraisal of protein losses in patients undergoing continuous ambulatory peritoneal dialysis. *Nephron.* **26,** 230-233.

Kerro Dego, O., van Dijk, J.E., Nederbragt, H., 2002. Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. A review. *The Veterinary Quarterly.* **24,** 181-198.

Kerschbaum, J., Konig, P., Rudnicki, M., 2012. Risk factors associated with peritoneal-dialysis-related peritonitis. *International Journal of Nephrology.* **2012,** 483250.

Khovidhunkit, W., Duchateau, P.N., Medzihradszky, K.F., Moser, A.H., Naya-Vigne, J., Shigenaga, J.K., Kane, J.P., Grunfeld, C., Feingold, K.R., 2004. Apolipoproteins A-IV and AV are acute-phase proteins in mouse HDL. *Atherosclerosis.* **176,** 37-44.

Kim, J.E., Park, S.J., Oh, J.Y., Kim, J.H., Lee, J.S., Kim, P.K., Shin, J.I., 2015. Noninfectious Complications of Peritoneal Dialysis in Korean Children: A 26-Year Single-Center Study. *Yonsei Medical Journal.* **56,** 1359-1364.

Kitsati, N., Liakos, D., Ermeidi, E., Mantzaris, M.D., Vasakos, S., Kyratzopoulou, E., Eliadis, P., Andrikos, E., Kokkolou, E., Sferopoulos, G., Mamalaki, A., Siamopoulos, K., Galaris, D., 2015. Rapid elevation of transferrin saturation and serum hepcidin concentration in hemodialysis patients after intravenous iron infusion. *Haematologica.* **100,** e80-3.

Kluytmans, J., van Belkum, A., Verbrugh, H., 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clinical Microbiology Reviews.* **10,** 505-520.

Koeppen, B.M. & Stanton, B.A., 2012. *Renal Physiology: Mosby Physiology Monograph Series.* Elsevier Health Sciences.

Kofteridis, D.P., Valachis, A., Perakis, K., Maraki, S., Daphnis, E., Samonis, G., 2010. Peritoneal dialysis-associated peritonitis: clinical features and predictors of outcome. *International Journal of Infectious Diseases.* **14,** e489-e493.

Kopriva-Altfahrt, G., Konig, P., Mundle, M., Prischl, F., Roob, J.M., Wiesholzer, M., Vychytil, A., Austrian Study Group for Prevention of Peritoneal Catheter-Associated Infections, Arneitz, K., Karner, A., Artes, R., Wolf, E., Auinger, M., Pawlak, A., Fraberger, J., Hofbauer, S., Galvan, G., Salmhofer, H., Pichler, B., Wazel, M., Gruber, M., Thonhofer, A., Hager, A., Malajner, S., Heiss, S., Braunsteiner, T., Zweiffler, M., Konig, P., Rudnicki, M., Kogler, R., Kohlhauser, D., Wiesinger, T., Kopriva-Altfahrt, G., Moser, E., Kotanko, P., Loibner, H., Nitz, H., Miska, H.J., Wenzel, R., Wolfer, M., Mundle, M., Breuss, H., Holzl, B., Prischi, F., Schmekal, B., Riener, E.M., Roob, J.M., Wonisch, W., Vikydal, R., Vychytil, A., Frank, B., Wieser, C., Wiesholzer, M., Pokorny, K., 2009. Exit-site care in Austrian peritoneal dialysis centers -- a nationwide survey. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **29,** 330-339.

Kotsanas, D., Polkinghorne, K.R., Korman, T.M., Atkins, R.C., Brown, F., 2007. Risk factors for peritoneal dialysis-related peritonitis: Can we reduce the incidence and improve patient selection? *Nephrology.* **12,** 239-245.

Krediet, R., ed, 2011. *Progress in peritoneal dialysis.* Rijeka, Croatia: Janeza Trdine 9, 51000.

Lacey, J.M., Bergen, H.R., Magera, M.J., Naylor, S., O'Brien, J.F., 2001. Rapid determination of transferrin isoforms by immunoaffinity liquid chromatography and electrospray mass spectrometry. *Clinical Chemistry.* **47,** 513-518.

Lai, K.N., 2009. *A Practical Manual of Renal Medicine: Nephrology, Dialysis, and Transplantation.* World Scientific.

Leblanc, M., Ouimet, D. and Pichette, V., 2001. Dialysate leaks in peritoneal dialysis, *Seminars in dialysis,* 2001, Wiley Online Library pp50-54.

Levey, A.S. & Coresh, J., 2012. Chronic kidney disease. *The Lancet.* **379,** 165-180.

Levy, J., Brown, E. and Lawrence, A., 2015. *Oxford handbook of dialysis.* Oxford University Press.

Levy, M., Dromer, F., Brion, N., Leturdu, F., Carbon, C., 1988. Community-acquired pneumonia: importance of initial noninvasive bacteriologic and radiographic investigations. *CHEST Journal.* **93,** 43-48.

Levy, J., Brown, E. and Daley, C., 2009. *Oxford Handbook of Dialysis.* [e-book]. Oxford, GB: Oxford University Press. Available from : http://site.ebrary.com/lib/leicester/docDetail.action?docID=10581418&ppg=1.

Li, P.K., 2001. Peritoneal dialysis related infections: challenges ahead. *Hong Kong Journal of Nephrology.* **3,** 1-2.

Li, P.K., Szeto, C.C., Piraino, B., Bernardini, J., Figueiredo, A.E., Gupta, A., Johnson, D.W., Kuijper, E.J., Lye, W.C., Salzer, W., Schaefer, F., Struijk, D.G., International Society for Peritoneal Dialysis, 2010. Peritoneal dialysis-related infections recommendations: 2010 update. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **30,** 393-423.

Liabeuf, S., Lenglet, A., Desjardins, L., Neirynck, N., Glorieux, G., Lemke, H., Vanholder, R., Diouf, M., Choukroun, G., Massy, Z.A., 2012. Plasma beta-2 microglobulin is associated with cardiovascular disease in uremic patients. *Kidney International.* **82,** 1297-1303.

Lin, W., Tsai, C., Chen, C., Lee, W., Su, C., Wu, Y., 2008. Proteomic analysis of peritoneal dialysate fluid in patients with dialysis-related peritonitis. *Renal Failure.* **30,** 772-777.

Lindsay, J., Riley, T., Mee, B., 1994. Production of siderophore by coagulase-negative staphylococci and its relation to virulence. *European Journal of Clinical Microbiology and Infectious Diseases.* **13,** 1063-1066.

Lowy, F.D., 1998. *Staphylococcus aureus* infections. *New England Journal of Medicine.* **339,** 520-532.

Luo, Q., Cheung, A.K., Kamerath, C.D., Reimer, L.G., Leypoldt, J.K., 2000. Increased protein loss during peritonitis associated with peritoneal dialysis is neutrophil dependent. *Kidney International.* **57,** 1736-1742.

Lyte, M. & Freestone, P., 2009. Microbial endocrinology comes of age. *Microbe.* **4,** 169-175.

Lyte, M., Freestone, P.P., Neal, C.P., Olson, B.A., Haigh, R.D., Bayston, R., Williams, P.H., 2003. Stimulation of *Staphylococcus epidermidis* growth and biofilm formation by catecholamine inotropes. *The Lancet.* **361,** 130-135.

MacNeill, S.J., Casula, A., Shaw, C., Castledine, C., 2016. UK Renal Registry 18th Annual Report: Chapter 2 UK Renal Replacement Therapy Prevalence in 2014: National and Centre-specific Analyses. *Nephron.* **132 Suppl 1,** 41-68.

Matos, J., Harmon, R., Langlois, B., 1995. Lecithinase reaction of *Staphylococcus aureus* strains of different origin on Baird-Parker medium. *Letters in Applied Microbiology.* **21,** 334-335.

McDonald, W.A., Watts, J., Bowmer, M.I., 1986. Factors affecting *Staphylococcus epidermidis* growth in peritoneal dialysis solutions. *Journal of Clinical Microbiology.* **24,** 104-107.

Menzies, B.E., 2003. The role of fibronectin binding proteins in the pathogenesis of *Staphylococcus aureus* infections. *Current Opinion in Infectious Diseases.* **16,** 225-230.

Milačič, R. & Benedik, M., 1999. Determination of trace elements in a large series of spent peritoneal dialysis fluids by atomic absorption spectrometry. *Journal of Pharmaceutical and Biomedical Analysis.* **18,** 1029-1035.

Miller, S.J. & Morgan, B.W., 2014. Deferoxamine. In Wexler, P., ed, *Encyclopedia of Toxicology (Third Edition).* Oxford: Academic Press. 1154-1156.

Modun, B., Kendall, D., Williams, P., 1994. Staphylococci express a receptor for human transferrin: identification of a 42-kilodalton cell wall transferrin-binding protein. *Infection and Immunity.* **62,** 3850-3858.

Molnar, C., Hevessy, Z., Rozgonyi, F., Gemmell, C.G., 1994. Pathogenicity and virulence of coagulase negative staphylococci in relation to adherence, hydrophobicity, and toxin production in vitro. *Journal of Clinical Pathology.* **47,** 743-748.

Montenegro, J., Saracho, R., Gallardo, I., Martinez, I., Munoz, R., Quintanilla, N., 2007. Use of pure bicarbonate-buffered peritoneal dialysis fluid reduces the incidence of CAPD peritonitis. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **22,** 1703-1708.

Moraes, T.P., Pecoits-Filho, R., Ribeiro, S.C., Rigo, M., Silva, M.M., Teixeira, P.S., Pasqual, D.D., Fuerbringer, R., Riella, M.C., 2009. Peritoneal dialysis in Brazil: twenty-five years of experience in a single center. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **29,** 492-498.

Morris, L.S., Evans, J., Marchesi, J.R., 2012. A robust plate assay for detection of extracellular microbial protease activity in metagenomic screens and pure cultures. *Journal of Microbiological Methods.* **91,** 144-146.

Mosesson, M., 2005. Fibrinogen and fibrin structure and functions. *Journal of Thrombosis and Haemostasis.* **3,** 1894-1904.

Munib, S., 2004. Continuous Ambulatory Peritoneal Dialysis (CAPD). *Gomal Journal of Medical Sciences.* **4,** .

Nakamoto, H., Kawaguchi, Y., Suzuki, H., 2006. Is technique survival on peritoneal dialysis better in Japan? *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **26,** 136-143.

Neal, C.P., Freestone, P.P., Maggs, A.F., Haigh, R.D., Williams, P.H., Lyte, M., 2001. Catecholamine inotropes as growth factors for *Staphylococcus epidermidis* and other coagulase-negative staphylococci. *FEMS Microbiology Letters.* **194,** 163-169.

NICE clinical guideline, 2011. Chronic kidney disease (stage 5): peritoneal dialysis. **125.**

Northrop-Clewes, C.A., 2008. Interpreting indicators of iron status during an acute phase response--lessons from malaria and human immunodeficiency virus. *Annals of Clinical Biochemistry.* **45,** 18-32.

Oasgupta, M.K., Bettcher, K.B., Ulan, R.A., Burns, V., Lam, K., Oossetor, J.B., Costerton, J.W., 1987. Relationship of adherent bacterial biofilms to peritonitis in chronic ambulatory peritoneal dial ysis. *Peritoneal Dialysis International.* **7,** 168-173.

Oliveira, E., Araújo, J.E., Gómez-Meire, S., Lodeiro, C., Perez-Melon, C., Iglesias-Lamas, E., Otero-Glez, A., Capelo, J.L., Santos, H.M., 2014. Proteomics analysis of the peritoneal dialysate effluent reveals the presence of calcium-regulation proteins and acute inflammatory response. *Clinical Proteomics.* **11,** 1.

Otto, M., 2009. *Staphylococcus epidermidis*—the'accidental'pathogen. *Nature Reviews Microbiology.* **7,** 555-567.

Otto, M., 2008. Staphylococcal biofilms. *Bacterial biofilms.* Springer. 207-228.

Owen, J.E., Walker, R.G., Lemon, J., Brett, L., Mitrou, D., Becker, G.J., 1992. Randomized study of peritonitis with conventional versus O-set techniques in continuous ambulatory peritoneal dialysis. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **12,** 216-220.

Oygar, D., Yalin, A., Altiparmak, M., Ataman, R., Serdengecti, K., 2011. Obligatory referral among other factors associated with peritonitis in peritoneal dialysis patients. *Nefrologia.* **31,** 435-440.

Pajek, J., Guček, A., Škoberne, A., Pintar, T., 2011. Severe peritonitis in patients treated with peritoneal dialysis: a case series study. *Therapeutic Apheresis and Dialysis.* **15,** 250-256.

Park, R.Y., Sun, H.Y., Choi, M.H., Bae, Y.H., Shin, S.H., 2005. Growth of *Staphylococcus aureus* with defective siderophore production in human peritoneal dialysate solution. *Journal of Microbiology (Seoul, Korea).* **43,** 54-61.

Parkkinen, J., Von Bonsdorff, L., Ebeling, F., Sahlstedt, L., 2002. Function and therapeutic development of apotransferrin. *Vox Sanguinis.* **83,** 321-326.

Pattinson, H., Koninckx, P., Brosens, I., Vermylen, J., 1981. Clotting and fibrinolytic activities in peritoneal fluid. *BJOG: An International Journal of Obstetrics & Gynaecology.* **88,** 160-166.

Peterson, M.M., Mack, J.L., Hall, P.R., Alsup, A.A., Alexander, S.M., Sully, E.K., Sawires, Y.S., Cheung, A.L., Otto, M., Gresham, H.D., 2008. Apolipoprotein B is an innate barrier against invasive *Staphylococcus aureus* infection. *Cell Host & Microbe.* **4,** 555-566.

Pihl, M., Davies, J.R., Johansson, A.C., Svensater, G., 2013. Bacteria on catheters in patients undergoing peritoneal dialysis. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **33,** 51-59.

Piraino, B., Bernardini, J., Brown, E., Figueiredo, A., Johnson, D.W., Lye, W.C., Price, V., Ramalakshmi, S., Szeto, C.C., 2011. ISPD position statement on reducing the risks of peritoneal dialysis-related infections. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **31,** 614-630.

Popovich, R.P., Moncrief, J.W., Nolph, K.D., GHODS, A.J., TWARDOWSKI, Z.J., Pyle, W., 1978. Continuous ambulatory peritoneal dialysis. *Annals of Internal Medicine.* **88,** 449-456.

Potempa, J. & Travis, J., 2000. Proteinases as virulence factors in bacterial diseases and as potential targets for therapeutic intervention with proteinase inhibitors. *Proteases as targets for therapy.* Springer. 159-188.

Qamar, M., Sheth, H., Bender, F.H., Piraino, B., 2009. Clinical outcomes in peritoneal dialysis: impact of continuous quality provement initiatives. *Advances in Peritoneal Dialysis.Conference on Peritoneal Dialysis.* **25,** 76-79.

Raaijmakers, R., Pluk, W., Schroder, C.H., Gloerich, J., Cornelissen, E.A., Wessels, H.J., Willems, J.L., Monnens, L.A., van den Heuvel, L.P., 2008. Proteomic profiling and identification in peritoneal fluid of children treated by peritoneal dialysis. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **23,** 2402-2405.

Rabelink, T.J., Reijngoud, D., Gadellaa, M.M., Voorbij, H.A., Stellaard, F., Kaysen, G.A., 1998. Plasma &agr; 2 macroglobulin is increased in nephrotic patients as a result of increased synthesis alone. *Kidney International.* **54,** 530-535.

Reed, S.B., Wesson, C.A., Liou, L.E., Trumble, W.R., Schlievert, P.M., Bohach, G.A., Bayles, K.W., 2001. Molecular characterization of a novel *Staphylococcus aureus* serine protease operon. *Infection and Immunity.* **69,** 1521-1527.

Rodrigues, A., Matos, C., Silva, F., Fonseca, I., Nogueira, C., Santos, J., Silva, A., Cabrita, A., 2006. Long-term peritoneal dialysis experience in Portugal. *International Journal of Artificial Organs.* **29,** 1109.

Rodriguez-Carmona, A., Perez Fontan, M., Garcia Falcon, T., Fernandez Rivera, C., Valdes, F., 1999. A comparative analysis on the incidence of peritonitis and exit-site infection in CAPD and automated peritoneal dialysis. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **19,** 253-258.

Ruger, W., van Ittersum, F.J., Comazzetto, L.F., Hoeks, S.E., ter Wee, P.M., 2011. Similar peritonitis outcome in CAPD and APD patients with dialysis modality continuation during peritonitis. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **31,** 39-47.

Sahu, A. & Lambris, J.D., 2001. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunological Reviews.* **180,** 35-48.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. *Molecular cloning.* Cold spring harbor laboratory press New York.

Sandrini, S., Alghofaili, F., Freestone, P., Yesilkaya, H., 2014. Host stress hormone norepinephrine stimulates pneumococcal growth, biofilm formation and virulence gene expression. *BMC Microbiology.* **14,** 1.

Sandrini, S., Aldriwesh, M., Alruways, M., Freestone, P., 2015. Microbial endocrinology: host-bacteria communication within the gut microbiome. *The Journal of Endocrinology.* **225,** R21-34.

Sandrini, S.M., Shergill, R., Woodward, J., Muralikuttan, R., Haigh, R.D., Lyte, M., Freestone, P.P., 2010. Elucidation of the mechanism by which catecholamine stress hormones liberate iron from the innate immune defense proteins transferrin and lactoferrin. *Journal of Bacteriology.* **192,** 587-594.

Saran, R., Li, Y., Robinson, B., Abbott, K.C., Agodoa, L.Y., Ayanian, J., Bragg-Gresham, J., Balkrishnan, R., Chen, J.L., Cope, E., Eggers, P.W., Gillen, D., Gipson, D., Hailpern, S.M., Hall, Y.N., He, K., Herman, W., Heung, M., Hirth, R.A., Hutton, D., Jacobsen, S.J., Kalantar-Zadeh, K., Kovesdy, C.P., Lu, Y., Molnar, M.Z., Morgenstern, H., Nallamothu, B., Nguyen, D.V., O'Hare, A.M., Plattner, B., Pisoni, R., Port, F.K., Rao, P., Rhee, C.M., Sakhuja, A., Schaubel, D.E., Selewski, D.T., Shahinian, V., Sim, J.J., Song, P., Streja, E., Kurella Tamura, M., Tentori, F., White, S., Woodside, K., Hirth, R.A., 2016. US Renal Data System 2015 Annual Data Report: Epidemiology of Kidney Disease in the United States. *American Journal of Kidney Diseases : The Official Journal of the National Kidney Foundation.* **67,** A7-8.

Ščančar, J., Milačič, R., Benedik, M., Križaj, I., 2003. Total metal concentrations in serum of dialysis patients and fractionation of Cu, Rb, Al, Fe and Zn in spent continuous ambulatory peritoneal dialysis fluids. *Talanta.* **59,** 355-364.

Schwartz, G.J. & Work, D.F., 2009. Measurement and estimation of GFR in children and adolescents. *Clinical Journal of the American Society of Nephrology : CJASN.* **4,** 1832-1843.

Sheth, N.K., Bartell, C.A., Roth, D.A., 1986. In vitro study of bacterial growth in continuous ambulatory peritoneal dialysis fluids. *Journal of Clinical Microbiology.* **23,** 1096-1098.

Shigidi, M.M., Fituri, O.M., Chandy, S.K., Asim, M., Al Malki, H.A., Rashed, A.H., 2010. Microbial spectrum and outcome of peritoneal dialysis related peritonitis in Qatar. *Saudi Journal of Kidney Diseases and Transplantation : An Official Publication of the Saudi Center for Organ Transplantation, Saudi Arabia.* **21,** 168-173.

Simons, J.F., van Kampen, M.D., Riel, S., Götz, F., Egmond, M.R., Verheij, H.M., 1998. Cloning, purification and characterisation of the lipase from *Staphylococcus epidermidis*. *European Journal of Biochemistry.* **253,** 675-683.

Sitter, T. & Sauter, M., 2005. Impact of glucose in peritoneal dialysis: saint or sinner? *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **25,** 415-425.

Skaar, E.P. & Schneewind, O., 2004. Iron-regulated surface determinants (Isd) of *Staphylococcus aureus*: stealing iron from heme. *Microbes and Infection.* **6,** 390-397.

Smith, D.G., Wilcox, M.H., Williams, P., Finch, R.G., Denyer, S.P., 1991. Characterization of cell envelope proteins of *Staphylococcus epidermidis* cultured in human peritoneal dialysate. *Infection and Immunity.* **59,** 617-624.

Sritippayawan, S., Chiangjong, W., Semangoen, T., Aiyasanon, N., Jaetanawanitch, P., Sinchaikul, S., Chen, S., Vasuvattakul, S., Thongboonkerd, V., 2007. Proteomic analysis of peritoneal dialysate fluid in patients with different types of peritoneal membranes. *Journal of Proteome Research.* **6,** 4356-4362.

Stepanović, S., Vuković, D., Dakić, I., Savić, B., Švabić-Vlahović, M., 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods.* **40,** 175-179.

Storm, T., Zeitz, C., Cases, O., Amsellem, S., Verroust, P.J., Madsen, M., Benoist, J.F., Passemard, S., Lebon, S., Jonsson, I.M., Emma, F., Koldso, H., Hertz, J.M., Nielsen, R., Christensen, E.I., Kozyraki, R., 2013. Detailed investigations of proximal tubular function in Imerslund-Grasbeck syndrome. *BMC Medical Genetics.* **14,** 111-2350-14-111.

Sunder-Plassmann, G., Patruta, S.I., Hörl, W.H., 1999. Pathobiology of the role of iron in infection. *American Journal of Kidney Diseases.* **34,** s25-s29.

Sweny, P., Rubin, R.H. and Tolkoff-Rubin, N., 2003. *The infectious complications of renal disease.* Oxford University Press.

Tendolkar, P.M., Baghdayan, A.S., Gilmore, M.S., Shankar, N., 2004. Enterococcal surface protein, Esp, enhances biofilm formation by Enterococcus faecalis. *Infection and Immunity.* **72,** 6032-6039.

Teufel, P. & Gotz, F., 1993. Characterization of an extracellular metalloprotease with elastase activity from *Staphylococcus epidermidis*. *Journal of Bacteriology.* **175,** 4218-4224.

Thirugnanasambathan, T., Hawley, C.M., Badve, S.V., McDonald, S.P., Brown, F.G., Boudville, N., Wiggins, K.J., Bannister, K.M., Clayton, P., Johnson, D.W., 2012. Repeated Peritoneal Dialysis–Associated Peritonitis: A Multicenter Registry Study. *American Journal of Kidney Diseases.* **59,** 84-91.

Thodis, E., Passadakis, P., Lyrantzopooulos, N., Panagoutsos, S., Vargemezis, V., Oreopoulos, D., 2005. Peritoneal catheters and related infections. *International Urology and Nephrology.* **37,** 379-393.

Tjiong, H.L., Zijlstra, F.J., Rietveld, T., Wattimena, J.L., Huijmans, J.G., Swart, G.R., Fieren, M.W., 2007. Peritoneal protein losses and cytokine generation in automated peritoneal dialysis with combined amino acids and glucose solutions. *Mediators of Inflammation.* **2007,** 97272.

Tobudic, S., Kratzer, C., Poeppl, W., Vychytil, A., Burgmann, H., 2011. Impact of various peritoneal dialysis solutions on the growth of common bacterial and yeast pathogens. *Peritoneal Dialysis International.* **31,** 688-692.

Todar, K., 2006. *Todar's online textbook of bacteriology.* University of Wisconsin-Madison Department of Bacteriology Madison, Wis, USA.

Twardowski, Z., Ksiazek, A., Majdan, M., Janicka, L., Bochenska-Nowacka, E., Sokolowska, G., Gutka, A., Zbikowska, A., 1981. Kinetics of continuous ambulatory peritoneal dialysis (CAPD) with four exchanges per day. *Clinical Nephrology.* **15,** 119-130.

Tyan, Y., Su, S., Ting, S., Wang, H., Liao, P., 2013. A comparative proteomics analysis of peritoneal dialysate before and after the occurrence of peritonitis episode by mass spectrometry. *Clinica Chimica Acta.* **420,** 34-44.

Van Asbeck, B., Marcelis, J., Marx, J., Struyvenberg, A., Van Kats, J., Verhoef, J., 1983. Inhibition of bacterial multiplication by the iron chelator deferoxamine: potentiating effect of ascorbic acid. *European Journal of Clinical Microbiology.* **2,** 426-431.

Van Diepen, A.T., van Esch, S., Struijk, D.G., Krediet, R.T., 2015. The first peritonitis episode alters the natural course of peritoneal membrane characteristics in peritoneal dialysis patients. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **35,** 324-332.

Vathsala, A., 2005. Preventing renal transplant failure. *Ann Acad Med Singapore.* **34,** 36-43.

Verbrugh, H.A., Keane, W.F., Conroy, W.E., Peterson, P.K., 1984. Bacterial growth and killing in chronic ambulatory peritoneal dialysis fluids. *Journal of Clinical Microbiology.* **20,** 199-203.

Vidarsson, G., Dekkers, G., Rispens, T., 2014. IgG subclasses and allotypes: from structure to effector functions. *Frontiers in Immunology.* **5,** 520.

Vikrant, S., Guleria, R.C., Kanga, A., Verma, B.S., Singh, D., Dheer, S.K., 2013. Microbiological aspects of peritonitis in patients on continuous ambulatory peritoneal dialysis. *Indian Journal of Nephrology.* **23,** 12-17.

Von Bonsdorff, L., Lindeberg, E., Sahlstedt, L., Lehto, J., Parkkinen, J., 2002. Bleomycin-detectable iron assay for non-transferrin-bound iron in hematologic malignancies. *Clinical Chemistry.* **48,** 307-314.

Von Bonsdorff, L., Sahlstedt, L., Ebeling, F., Ruutu, T., Parkkinen, J., 2003. Apotransferrin administration prevents growth of *Staphylococcus epidermidis* in serum of stem cell transplant patients by binding of free iron. *FEMS Immunology and Medical Microbiology.* **37,** 45-51.

Vuong, C. & Otto, M., 2002. *Staphylococcus epidermidis* infections. *Microbes and Infection.* **4,** 481-489.

Wang, H.Y., Tian, Y.F., Chien, C.C., Kan, W.C., Liao, P.C., Wu, H.Y., Su, S.B., Lin, C.Y., 2010. Differential proteomic characterization between normal peritoneal fluid and diabetic peritoneal dialysate. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association.* **25,** 1955-1963.

Weinberg, E.D., 2009. Iron availability and infection. *Biochimica Et Biophysica Acta (BBA)-General Subjects.* **1790,** 600-605.

Wen, Q., Zhang, L., Mao, H., Tang, X., Rong, R., Fan, J., Yu, X., 2013. Proteomic analysis in peritoneal dialysis patients with different peritoneal transport characteristics. *Biochemical and Biophysical Research Communications.* **438,** 473-478.

Westra, W.M., Kopple, J.D., Krediet, R.T., Appell, M., Mehrotra, R., 2007. Dietary protein requirements and dialysate protein losses in chronic peritoneal dialysis patients. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **27,** 192-195.

Wilcox, M.H., Smith, D.G., Evans, J.A., Denyer, S.P., Finch, R.G., Williams, P., 1990. Influence of carbon dioxide on growth and antibiotic susceptibility of coagulase-negative staphylococci cultured in human peritoneal dialysate. *Journal of Clinical Microbiology.* **28,** 2183-2186.

Williams, P., Swift, S., Modun, B., 1995. Continuous ambulatory peritoneal dialysis-associated peritonitis as a model device-related infection: phenotypic adaptation, the staphylococcal cell envelope and infection. *Journal of Hospital Infection.* **30,** 35-43.

Williams, J. & Moreton, K., 1980. The distribution of iron between the metal-binding sites of transferrin human serum. *The Biochemical Journal.* **185,** 483-488.

Witowski, J., Jörres, A., Korybalska, K., Ksiazek, K., Wisniewska-Elnur, J., Bender, T.O., Passlick-Deetjen, J., Breborowicz, A., 2003. Glucose degradation products in peritoneal dialysis fluids: do they harm? *Kidney International.* **63,** S148-S151.

Yam, L., Li, C.Y., Crosby, W., 1971. Cytochemical identification of monocytes and granulocytes. *American Journal of Clinical Pathology.* **55,** 283-290.

Yao, Y., Sturdevant, D.E., Otto, M., 2005. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. *The Journal of Infectious Diseases.* **191,** 289-298.

Yoo, H., Ku, S., Kim, S., Bae, J., 2015. Early diagnosis of sepsis using serum hemoglobin subunit Beta. *Inflammation.* **38,** 394-399.

Zarrinkalam, K.H., Leavesley, D.I., Stanley, J.M., Atkins, G.J., Faull, R.J., 2001. Expression of defensin antimicrobial peptides in the peritoneal cavity of patients on peritoneal dialysis. *Peritoneal Dialysis International : Journal of the International Society for Peritoneal Dialysis.* **21,** 501-508.

Zorzanello, M.M., 2004. Peritoneal dialysis and hemodialysis: similarities and differences. *Nephrology Nursing Journal.* **31,** 588.