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Local microRNAs in Peritoneal Dialysis-Related Peritonitis

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DECLARATION

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Acknowledgements

Summary

Scientific background

Infection remains a major cause of morbidity, mortality and technique failure in PD patients. Identification of peritonitis episodes and the causative organism is slow and unreliable. The immune system has evolved to be specific for different pathogens, suggesting a pathogen-specific "immune fingerprint" may be able to distinguish distinct pathogens to guide more accurate treatment decisions. microRNAs are post-transcriptional regulators of most human genes and have roles in the majority of biological processes and pathways. They are stable, accurate and specific biomarkers in biological fluids. My work aimed to combine the immune fingerprint model with specific microRNAs in PD effluent to identify peritonitis episodes and ascertain the role of extracellular microRNAs in the acute immune response.

Approach

The microRNA profile of PD effluent was analysed in peritonitis patients with different infectious organisms to identify candidate biomarkers. The cellular source of these microRNAs was identified, and the functional release of one microRNA (miR-223) into the extracellular space was analysed for functional stabilisation in extracellular vesicles.

Results

microRNA profiles are altered in infected compared to uninfected PD effluent, which may have diagnostic value in acute peritonitis. Four microRNAs were identified as candidate biomarkers (miR-223, miR-27a, miR-21 and miR-31), with distinct cell-specific expression patterns. Potential mRNA targets of these microRNAs were identified. miR-223 was found to be functionally stabilised in PD effluent from peritonitis patients, with a proportion likely to be incorporated into neutrophil-derived exosomes.

Conclusions

My studies prove that microRNAs are useful biomarkers of infection in PD-related peritonitis and have the potential to contribute to a pathogen-specific immune fingerprint. Exosome-encapsulated microRNAs may have a functional role in intercellular signalling between immune cells responding to the infection and the local tissue, to help clear the infection and resolve the inflammation.

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List of abbreviations

AAD	Allergic airway disease
AGE	Advanced glycation end-product
AGO	Argonaute
AKI	Acute kidney injury
ANOVA	Analysis of variance
APC	Antigen presenting cell
APD	Automated peritoneal dialysis
ARMCX1/ALEX1	Armadillo repeat containing X-linked 1/ Arm protein lost in epithelial cancers chromosome X protein 1
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
AU	Adenylate-uridylate
BCR	B cell receptor
C. elegans	*Caenorhabditis elegans*
CAPD	Continuous ambulatory peritoneal dialysis
CCL18	Chemokine (C-C motif) ligand 18
CCL2/MCP-1	C-C Motif Chemokine Ligand 2/monocyte chemoattractant protein-1
CCNC	Cyclin C
CCPD	Continuous cyclical peritoneal dialysis
CCR4-NOT	Carbon catabolite repressor 4- negative on TATA complex
CD	Cluster of differentiation
CDK8	Cyclin dependent kinase 8
cDNA	Complementary DNA
CDS	Coding sequence
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CNS	Coagulase negative *Staphylococcus* species
CoM	Conditioned media
CRP	C-reactive protein
CT	Computed tomography
Ct	Cycle threshold

CVD	Cardiovascular disease
CXCL8	Chemokine (C-X-C motif) ligand 8
DC	Dendritic cell
DCP2	Decapping protein 2
DGCR8	DiGeorge syndrome Critical Region 8
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
dsRBP	Double stranded RNA binding protein
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eIF4A	Translation initiation factor
ELISA	Enzyme-linked immunosorbent assay
EMT/MMT	Epithelial/mesothelial-to-mesenchymal transition
EPS	Encapsulating peritoneal sclerosis
ERK	Extracellular-signal-regulated kinase
ESRD	End stage renal disease
EV	Extracellular vesicles
EXP5	Exportin 5
FACS	Fluorescently activated cell sorting
FasL	Fas ligand
FBXW7	F-box and WD repeat domain containing 7
FCS	Foetal calf serum
FSC-A/FSC-H	Forward scatter area/high
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
GDP	Glucose degradation product
GFR	Glomerular filtration rate
GTP	Guanosine tri-phosphate
GW182	Trinucleotide repeat containing adaptor 6A
GWAS	Genome-wide association studies
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma

HCV	Hepatitis C virus
HD	Haemodialysis
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HPFB	Human peritoneal fibroblasts
HPMC	Human peritoneal mesothelial cells
HTA	Human Tissue Authority
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL6ST	Interleukin 6 signal transducer
IP or i.p.	Intraperitoneal
IPD	Intermittent peritoneal dialysis
ISPD	International Society for Peritoneal Dialysis
IV	Intravenous
KDIGO	Kidney Disease Improving Global Outcomes
KO	Knock out
LAR II	Luciferase Assay Reagent II
LB	Luria broth
LBH	Limb bud and heart development
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
M. leprae	*Mycobacterium leprae*
M. tuberculosis	*Mycobacterium tuberculosis*
MAC	Membrane attack complex
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
miR-21$^{-/-}$	miR-21 knockout
miR-C	Control microRNA

miRNA*	microRNA passenger strand
miRNA/miR	microRNA
MMD	Monocyte to macrophage differentiation associated
MMP	Matrix metalloprotease
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Multiple sclerosis
NCR	Natural cytotoxicity receptors
ncRNA	Non-coding RNA
NEAA	Non-essential amino acids
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa B
NIPD	Nightly intermittent peritoneal dialysis
NK	Natural killer cell
NLR	Nucleotide-binding oligomerisation domain-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
OA	Osteoarthritis
OD$_{600}$	Optical density at 600 nm
PAM3	Pam(3) CSK(4)
PAMP	Pathogen-associated molecular pattern
PAN2-PAN3	Poly(A) specific ribonuclease subunit complex
PAX9	Paired box 9
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered-saline
PCR	Polymerase chain reaction
PD	Peritoneal dialysis
pDC	Plasmacytoid dendritic cell
PDCD4	Programmed cell death protein 4
PKCε/PRKCE	Protein kinase C epsilon
PMN	Polymorphonuclear
Pol II	RNA polymerase II
PRDM1	PR/SET domain 1
PRR	Pathogen-associated molecular pattern recognition receptor

qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RAN	Nuclear GTP-binding protein
RASA1	Ras p21 protein activator 1
RBC	Red blood cells
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
RhoB	Ras homolog gene family member B
RISC	RNA-induced silencing complex
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RQ	Relative quantification
RRF	Residual renal function
RRT	Renal replacement therapy
RSV	Respiratory syncytial virus
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SEM	Standard error of the mean
siRNA	Small interfering RNA
SIRS	Systemic Inflammatory Response Syndrome
SLC16A10	Solute carrier family 16 member 10
sno-135	Small nucleolar RNA 135
SNP	Single nucleotide polymorphism
snRNA U6	Small nuclear RNA U6
SPRY2	Sprouty RTK signalling antagonist 2
sRNA	Small bacterial RNA
SSC-A/FSC-A	Side scatter and forward scatter area/high
stRNA	Small temporal RNA molecule
TCIM/TC-1/C8orf4	Transcriptional and immune response regulator/ thyroid cancer-1/chromosome 8 open reading frame 4
TCR	T cell receptor
TGF-β	Transforming growth factor beta

Th	T helper subset
TLDA	TaqMan Low Density Array
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TPD	Tidal peritoneal dialysis
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRBP	TAR RNA-binding protein
Treg	Regulatory T cell
TRF	Time-resolved fluorometry
UHW	University hospital Wales
UTI	Urinary tract infection
UTR	Untranslated region
v-miRNA	Viral microRNA
VAV3	Vav guanine nucleotide exchange factor 3
VEGF	Vascular endothelial growth factor
VRE	Vancomycin-resistant *Enterococcus*
WT	Wildtype
XRN1	Exoribonuclease 1
ZSWIM6	Zinc finger SWIM-type containing 6

1   Introduction

1.1   Chronic Kidney Disease

The kidneys are essential organs located at the back of the abdomen, which are approximately the size of a fist in healthy adults. These bean-shaped organs are involved in regulatory functions in the body, including maintaining the composition of the blood. They remove excess organic molecules and electrolytes, and are involved in the maintenance of acid-base balance and blood pressure (via salt and water balance), as well as production of hormones (e.g. erythropoietin) and enzymes (e.g. renin). Kidneys filter out excess waste products and water from the blood and produce urine to secrete these out. The blood is filtered in the glomeruli where it undergoes selective secretion and reabsorption of different molecules and water, before urine is produced in the downstream nephron segments.[1]

1.1.1   Kidney disease

If the kidneys suffer an injury then it is called kidney disease, with the length of time defining whether it is an acute kidney injury (AKI) or chronic kidney disease (CKD). If the renal function declines for less than 3 months, this is termed AKI, and the patient often recovers to full renal function. However, it is possible the patient may progress to CKD, if the injury and reduction in renal function persists for longer than 3 months. AKI and CKD are separate but related disorders, with AKI giving an increased chance of the occurrence of CKD, whilst the opposite is true as CKD is an independent risk factor for AKI. This progression to CKD is more likely if the AKI is severe or the patient has multiple episodes of AKI, regardless of the underlying pathology.[2]

Renal replacement therapies (RRT) are required for patients with end stage renal disease (ESRD), to replace the normal filtration function of the kidneys. The severity of renal failure is assessed by the glomerular filtration rate (GFR), with a GFR of 90 to 120 ml/min/1.73 m^2 considered normal. A GFR of below 60 ml/min/1.73 m^2 for 3 or more months is a sign of CKD, and a GFR lower than 15 ml/min/1.73 m^2 is a sign of kidney failure. The most common cause of ESRD in the UK is diabetic nephropathy (27% of patients in 2014), with glomerulonephritis (13%) and polycystic kidney disease (7%) the next most common diagnoses (of those with known aetiology).[2]

1.1.1.1 Diagnosis

The clinical presentation of both types of kidney diseases is marked by a range of non-specific signs including high blood pressure, nausea and generalised itching. The diagnosis is therefore reliant on biochemical markers such as the estimated GFR, through measurement of the creatinine concentration in plasma and use of an equation established in the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI). The GFR is an estimate of the amount of blood passing through the glomeruli per minute. The combination of the GFR and albuminuria levels (levels of protein or albumin in the urine) are used to diagnose AKI or CKD, and also the stage of disease, and to specify treatment options to be considered, as shown in Table 1.1. The latter stages of CKD are termed ESRD, or kidney failure. The specific guidelines on diagnosis are set out by the Kidney Disease Improving Global Outcomes (KDIGO) CKD Work Group, and these include a diagnosis of the underlying pathology, if possible, to help characterise the stage, and likely prognosis and treatment options.[3–6]

1.1.1.2 Causes

The most common causes of kidney disease are diabetes and hypertension, which are often chronic, lifestyle-related disorders (especially type 2 diabetes) and are becoming more common, meaning the prevalence of kidney disease is increasing worldwide. Elderly populations in developed countries have the highest rates of CKD, although rates are now also increasing in developing countries. In developing countries, the causes of CKD may be more widespread, including infections with nephrotoxic effects such as glomerulonephritis, human immunodeficiency virus (HIV) (and associated anti-retroviral therapies) and enteric infections that cause haemolytic-uraemic syndrome. Other risk factors include treatments with nephrotoxic effects, for example herbal treatments and their interactions with conventional therapies. Sometimes the cause of CKD is unknown, especially in Asia and sub-Saharan Africa.[2,5]

1.1.1.3 Complications

Both AKI and CKD can have many complications, most notably cardiovascular disease (CVD) and mortality. This is likely due to the commonly associated comorbidities of diabetes and hypertension, although CKD is an independent risk factor for both these complications. Other complications that require clinical interventions include volume overload, metabolic

GFR category	GFR (ml/min/1.73m^2)	Terms
G1	>90	Normal or high
G2	60-89	Mildly decreased
G3a	45-59	Mildly to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased
G5	<15	Kidney failure

Albuminuria category	AER (mg/24hr)	ACR (mg/mmol)	ACR (mg/g)	Terms
A1	<30	<3	<30	Normal to mildly increased
A2	30-300	3-30	30-300	Moderately increased
A3	>300	>30	>300	Severely increased

GFR = Glomerular filtration rate

AER = albumin excretion rate

ACR = albumin-to-creatinine ratio

Table 1.1: GFR and albuminuria categories used to classify CKD stage, adapted from[6].

complications (e.g. acidosis), hypertension, anaemia, mineral and bone disorders, uraemia, dyslipidaemia and infection.[4,5]

The biggest problem with AKI or CKD is the build-up of toxic waste products and water, as the kidneys cannot remove them from the blood. If the renal function declines to such an extent that the kidneys can no longer function to a sufficient level, then a renal replacement therapy must be initiated to keep the patient alive.[4,5]

1.1.2 Renal replacement therapies

The most effective treatment for ESRD patients is a kidney transplant, however, due to the lack of organs available for transplantation, dialysis is normally initiated whilst the patient waits for a suitable donor. After 90 days on RRT, only 10% of patients have received a transplant. There are two types of dialysis which patients can undergo; haemodialysis (HD) and peritoneal dialysis (PD). HD is initiated in 70% of patients as a primary treatment, whereas PD is only started in 20% of patients.[2] Internationally, 269,000 patients received PD in 2013, compared to 2.25 million who used HD and 675,000 with a renal transplant.[7]

1.1.2.1 Kidney transplant

Kidney transplant is the best RRT due to the improved quality of life and increased survival, compared to dialysis. The first successful kidney transplant is thought to have happened in 1954 between a pair of identical twins in the US. Through advances in immunosuppression, organ matching and preservation, and chemoprophylaxis of opportunistic infections, patient survival is now greater than 95%, and graft survival over 90%. This RRT is also cheaper than long-term dialysis, however the demand for organs greatly exceeds the supply in all parts of the world. Paired living donor schemes have attempted to bridge this gap, but there is still a shortfall in suitable donor organs.[2,8,9]

1.1.2.2 Dialysis

Dialysis is defined as the diffusion of molecules in solution across a semipermeable membrane along an electrochemical concentration gradient, and is used to replace the function of the kidneys in patients with ESRD. There are 2 main modalities of dialysis that patients can choose to initiate when they have kidney failure; HD and PD. Both methods

are effective at restoring the intracellular and extracellular fluid environment that is characteristic of normal kidney function.[10]

Toxins and waste products, such as urea, have to be transported out of the blood, with the concentration and size of the solutes the main determinant of the diffusion rates. Small molecules, e.g. urea, diffuse rapidly, whilst larger more complex molecules, e.g. albumin, diffuse more slowly. Solutes are also removed from the blood by ultrafiltration, which is a convective process driven by hydrostatic or osmotic pressure gradients to remove excess body water.[10]

1.1.2.2.1 Patient choice

The patient choice between HD and PD can depend on a variety of factors, such as patient preference, geographical location relative to a dialysis centre, age, employment (of patient and family), obesity and level of residual renal function (RRF).[11] Patients receiving PD are more likely to rate their care highly, compared to those undergoing HD,[12] however, the rates of take-up of PD are still low.[2] The apparent preference by patients for PD may be due to several factors including the better quality of life, flexibility, ability to conduct treatment at home and lower costs of PD, compared to HD.[12] Furthermore, complications related to infections appear more common in HD than in PD, survival is superior in the first 2-3 years of treatment and the RRF is preserved.[11,13,14]

1.1.2.2.2 Haemodialysis

In HD, the blood is removed from the body and cleaned in a dialysis machine, or dialyser. The blood is passed across synthetic semipermeable membranes, with a dialysate on the other side. This allows for diffusion of small toxins out of the blood and essential solutes into the blood, as well as ultrafiltration to remove excess water. The first human HD was performed in 1924 by Prof Georg Haas. This involved removing 400 ml of blood from an ESRD patient and filtering using collodion membrane. Although successful, research into this treatment was largely discontinued for 20 years, before the first "artificial kidney" was developed in the 1940s by Kolff et al, and used similar materials as Haas, but an automated system to clean more blood more rapidly. After this development, further optimisation showed the intermittent HD schedule was optimal. The standard current treatment regime, first described in the 1960s, consists of three sessions a week of in-centre HD, where the patient travels to a HD centre and the HD is conducted by trained professionals, with the

prescription calculated to remove about two-thirds of the total-body urea content during each treatment.[10,15–17]

In-centre HD is both expensive and disruptive for the patient, therefore there is some movement towards provision of home HD. This is where a relative or friend of the patient is trained to conduct HD in the patient's home, allowing more frequent dialysis sessions and reducing disruption to the patient's life, allowing more flexibility to continue working or travelling. However, this does require both the patient and the carer to take responsibility for the treatment and intensive training at the dialysis centre.[14,22]

1.1.2.2.2.1 Advantages

Although both HD and PD offer good dialysis and effective RRT for ESRD patients, HD is often the more favoured treatment. This is due to a number of advantages including the continual assessment of the patient by medical staff, meaning doses can be adjusted in real time and even automatically by new advances in feedback control systems. Also, the rate of complications is quite low, due to the fact that trained medical professionals are completing, and constantly monitoring, the treatment. This is a great benefit especially for older, more frail patients, as they can be monitored closely and do not have to take responsibility for the treatment themselves, which may be a challenge for some. Also, the social benefits to regularly coming to the dialysis unit may be an advantage for patients who can become isolated, especially those who are older. In the UK, more doctors are familiar with HD as a treatment, due to the higher prevalence of it as an RRT than PD, therefore it is often favoured, and the complications are so rare that they are seen as part of the treatment and can be dealt with effectively and rapidly. The development of home HD systems has also brought down the costs associated with patient transport to the unit and staff hours. Although the start-up costs may be more for this treatment, the overall benefit is significant. Home HD is also associated with an increased quality of life therefore this is an important development that can be utilised further to benefit patients and reduce costs. The cognitive function of patients with ESRD is poor, and this increases with dialysis treatment of both modalities (HD and PD).[10,18–22]

1.1.2.2.2.2 Disadvantages

There are, however, a number of disadvantages associated with HD compared to PD. The nature of in-centre HD means there are associated costs for patient transport and staff

hours. The transport and length of treatment time also massively disrupts a patient's professional and personal life, with the lack of flexibility and independence challenging, especially for those of a working age. One of the major clinical disadvantages to HD is that this therapy causes a loss of any RRF. New regimes of incremental HD are being developed to attempt to counter this, but RRF is still lost eventually. HD is also associated with a risk of intradialytic hypotension, and infection with the central venous access. The risk of infection is also present in PD, but infections into the blood stream can become more serious very rapidly. In older patients especially, another clinical complication is that vascular access can be challenging to gain and maintain. This complex treatment does have a reasonably high rate of comorbidities, although these are generally managed successfully. Some studies suggest that the quality of life of patients is decreased on HD, although this is not clear, and some may suggest an improvement in quality of life, compared to PD, especially when patients are on home HD. There has been a recent suggestion that, although cognitive function increases with dialysis, including HD, PD is associated with a greater increase over one year.[18–24]

All of this shows how HD can be both a very effective RRT and have some problems that mean patients (and the treating doctors) may choose other RRTs. Home HD is an especially important development that will be essential to advancing this treatment.[18,19] The advantages and disadvantages of PD as an RRT will be discussed in more detail below.

1.2 Peritoneal dialysis

1.2.1 General overview

Peritoneal dialysis uses the natural ability of the peritoneal membrane to act as an ultrafiltration barrier. A PD solution (dialysate) is inserted into the peritoneal cavity via a permanent abdominal catheter. During the subsequent dwell time, the peritoneal membrane acts as a dialyser to remove waste products by diffusion and ultrafiltration.[25,26] The dialysate consists of water, osmotic agents and electrolytes in a buffer. The osmotic agents make the solution hyperosmolar and allow water to be removed from the blood following an osmotic gradient. Buffers, such as bicarbonate and lactate, are used to control acidosis. Electrolytes are also added, (e.g. sodium, calcium and magnesium) to prevent the excessive removal of these essential metals from the blood.[27] These will be discussed further below.

There are three barriers between the dialysate and the capillary blood, these are the capillary wall (which is the most important for solute transport), the interstitium (offers some resistance to solute transport, mainly large solutes only) and the mesothelial cell layer (which isn't a major barrier to water or solute transport). According to the three-pore hypothesis, there are three sizes of pores in the capillary endothelium which size-restrict solute transport. The most common type of pore is the small pores, with an average radius of 40 to 50 Å, which mediate the transport of lower-molecular-weight solutes. The large pores are less numerous and have an average radius of >150 Å, they are also more variable in size. Ultrasmall pores are only 3 to 5 Å wide and are called aquaporins. These transcellular pores allow only the transport of water molecules via an osmotic gradient. Waste products are removed from the blood by a concentration gradient, via diffusion or convection (solute transport that occurs alongside water during ultrafiltration).[28–30]

1.2.2 Peritoneal Dialysis Development

1.2.2.1 First uses

PD as a technique was first successfully described in dogs in 1945 by Seligman and colleagues.[31] It was first used in humans as a treatment for kidney failure patients, primarily in response to fatal uraemia in soldiers on the battlefront. The prediction that uraemia is a reversible process led to the development of a simple alternative to restore filtration and keep ESRD patients alive. The dogs used in the pioneering experiments were able to survive 3-10 days post nephrectomy and died eventually of complications, often related to peritonitis and contamination of the catheter. This technique was seen as a success in dogs, with 40-75% of normal renal function restored, in terms of urea clearance, correct acidosis, and prevention of death from uraemia.[31,32] This then led to a desire to optimise it for human patients, and a number of case studies showed successful use as a RRT, with extension of life and elimination of uraemia, although patients often died from other complications.[32–34]

1.2.2.2 Dialysis solution development

PD solutions consist of electrolytes and an osmotic agent in a buffer. The electrolyte can include sodium, potassium, magnesium and calcium, with their role to maintain the homeostatic balance of the body and generate a concentration gradient to remove excess

electrolytes. An osmotic agent is used to make the solution hyperosmolar and generate an osmotic gradient to allow ultrafiltration of water out of blood, most commonly used is glucose but it can be amino acids or icodextrin. Although glucose is a good osmotic agent, it can also negatively impact the peritoneal membrane via glucose degradation products (GDPs, discussed further below), therefore alternatives have been used. Icodextrin is a high molecular weight glucose polymer that does not diffuse across the peritoneal membrane, it absorbs by convection much slower than glucose. This, and amino acids, as alternatives both also have their own disadvantages (discussed further below). The buffer is important to maintain the pH as biocompatible as possible, and the most commonly used is either lactate or bicarbonate.[35–37]

The three main components of PD solutions are important to allow efficient dialysis, however their impact on the peritoneal membrane has recently become important. Due to the nature of the components, they are naturally not "biocompatible", and can increase the rate of fibrosis, epithelial/mesothelial-to-mesenchymal transition (EMT/MMT) and systemic inflammation associated with PD. More biocompatible solutions have therefore been developed. As mentioned before GDPs can negatively impact the compatibility of the PD solution with the peritoneal membrane which is encounters. GDPs are generated from glucose after heat sterilisation, and are both directly and indirectly toxic to cells, as well as nephrotoxic in animal models. GDPs rapidly forms advanced glycation end-products (AGE), which are also toxic to the peritoneal membrane. To combat these toxic by-products, low-GDP solutions have been developed to help maintain RRF and peritoneal membrane integrity better. One way of reducing GDPs formed in heat sterilisation is to decrease the pH, making it acidic. However, this can also have negative impacts on the peritoneal membrane causing fibrosis and EMT/MMT, ultimately leading to technique failure. Multi-component systems allowing the development of neutral pH, low-GDP solutions have greatly advanced the biocompatibility of PD solutions. To avoid the generation of GDPs, other osmotic agents have been developed. Amino acids are useful osmotic agents and do not produce GDPs, however they are limited by the development of metabolic acidosis. Icodextrin is a viable alternative to glucose as an osmotic agent and has biocompatibility benefits. This polydispersed, high molecular weight glucose polymer does not diffuse across the peritoneal membrane, maintaining the osmotic gradient. It does not generate GDP or AGE, and has other clinical benefits including better ultrafiltration and attenuated metabolic effects that are caused by systemic glucose absorption.[35,37–39]

The use of biocompatible PD solutions can reduce the cumulative and progressive injury to the peritoneal membrane caused by traditional PD solutions. They have many benefits including the preservation of RRF, stability of peritoneal membrane characteristics, reduced incidence and severity of peritonitis episodes (due to decreased impairment of peritoneal host defences), reduced systemic inflammation and decreased inflow pain. Biocompatible PD solutions are more expensive to generate than traditional PD solutions, but they are seen as cost-effective due to the reduced costs of the negative impacts of a lack of biocompatibility (i.e. reduced technique failure due to fibrosis, fewer and less severe peritonitis episodes), meaning the overall cost to the healthcare provider is reduced.[35,37–41]

1.2.2.3 Different modalities

PD can be conducted in one of two modalities, either as manual continuous ambulatory peritoneal dialysis (CAPD) or automated peritoneal dialysis (APD)

1.2.2.3.1 Continuous ambulatory peritoneal dialysis (CAPD)

This manual method of performing PD exchanges was first used in 1978 by Popvich et al.[42] This technique uses multiple manual exchanges of PD fluid per day, to be conducted by either a carer or the patient themselves. Before CAPD, PD was only used in intensive care settings as the patients required constant care and attachment to machinery. The ability to disconnect patients from tubing and allow them to continue normal life was revolutionary. From the discovery that this was an effective RRT, this has become the most common modality of PD in many countries as it is very effective at biochemical control and improves patient quality of life. PD is the most common mode of chronic dialysis in Hong Kong, as well as in developing countries such as El Salvador and Mexico. This technique requires minimal training for the patient/carer and can be done anywhere, with no electricity or running water required, just clean conditions and the ability to conduct a sterile PD fluid exchange, with the costs of medical personnel also relatively low. CAPD was initially described with five exchanges per day but can be done with at least three to five exchanges. One of the main complications often associated more highly with CAPD than APD is the increased incidence of peritonitis, due to the manual exchanges allowing opportunity for infectious organisms to enter the sterile peritoneum. Since the development of the Y-set

and double bag system, which allows washing out of the catheter before new PD fluid is introduced, the rates of peritonitis are decreased.[42–46]

1.2.2.3.2 Automated peritoneal dialysis (APD)

This broad term refers to all PD exchanges conducted using a machine, which includes continuous cyclical PD (CCPD), intermittent PD (IPD), nightly intermittent PD (NIPD), and tidal PD (TPD).[47,48] When PD was first used in a hospital setting in the 1960s, a form of APD was used, where a peritoneal cycler conducted multiple 2 litre exchanges over 10 hours.[49] When CAPD was described in 1976, this became the method of choice for PD due to its effectiveness and flexibility.[47] The first form of APD to be developed was CCPD in 1981, where three or four exchanges are performed automatically overnight as well as an additional exchange that remains in the abdomen during the day.[47,48,50,51] IPD historically had inadequate dialysis and malnutrition but was improved in the late 1960s and early 1970s, before being adapted as TPD. IPD consists of frequent, short exchanges over 8-10 hours (often overnight, called NIPD), three-times weekly. This still is not as efficient as other methods of PD, so is reserved for patients with high solute transport rates and limited ultrafiltration. TPD is similar to IPD in that there are many short exchanges, often overnight, but these exchanges do not involve a full drain, with approximately 50% of dialysate left in the abdomen after each exchange, until the final exchange. This is also not used as widely and is often selected for patients with problems or pain either with the in-flow or drain stages. Both IPD and TPD have periods with no dialysate in the abdomen, called dry periods or phases.[47,48,51–53]

The advancement of technology has meant automatic cyclers now come with a wide range of technologies to make this treatment as straightforward as possible for the patient or carer to use. For example, these machines are often able to perform all different forms of APD and can be programmed with the patient's prescription. They have easy-to-use display screens and can record all data about previous exchanges. The exchanges are automatically monitored whilst they are being done, with troubleshooting alerts for any detected problems.[47,48]

APD is becoming the first-choice PD treatment for a wide range of patients. Children and young people often select APD due to the flexibility of adjusting fluid exchanges by small volumes as required, a dry phase during the day time (to help with body-image issues

associated with a distorted fluid-filled abdomen), and no exchanges throughout the day (causing minimal disruption to the patient's or carer's day). Elderly patients are often put onto APD due to the minimal manual dexterous activity required to set up an exchange. APD is also used when patients have low RRF and high peritoneal membrane transport characteristics, as well as those with ultrafiltration failure and those suffering burnout from CAPD. APD is not suitable for all patients due to the high start-up costs, long periods required in bed and clinical problems such as poor blood pressure control.[47,48]

1.2.2.3.3 Comparison of modalities

Both CAPD and APD have been used for a number of years, allowing observational studies to analyse the benefits and disadvantages of both. In terms of clinical outcomes, there appears to be no difference in the rates of overall survival, peritonitis, technique failure, other infectious complications, hospitalisation and blood pressure control.[43,54–56] The rates of ultrafiltration are also comparable,[57] although some limited, older studies suggest CAPD may be more effective at this.[58] The maintenance of RRF is important as this is a strong predictor of patient outcome. Some studies suggest RRF is better maintained in CAPD,[59] whilst others suggest APD is more effective.[60] More recent studies show no difference in RRF retention between the modalities.[43,54,56] APD originally offered a beneficial reduction in peritonitis rates due to fewer connections and disconnections required compared to CAPD,[43,61] however, with the introduction of the safer twin-bag system, infection rates in APD and CAPD patients are now comparable.[43,56,62,63] One benefit of APD to the clinicians, and therefore the patients too, is that the prescription is more flexible with respect to dwell times and volumes as well as other factors.[43,54,63,64] The psychosocial benefits of APD have meant the rates of take-up are increasing. These include a reduced disruption to the professional and personal life of patients, as exchanges can be performed at night.[43,54,56,64] As a result, APD is thought to offer improved quality of life,[62,64] although this may not always be the case.[43,54,64] While APD is associated with higher start-up costs compared to CAPD, for the more expensive equipment including the cycler itself, the overall cost of the two modalities is similar, with APD sometimes seen as cheaper in the long term.[65,66] Overall, the two modalities of PD are seen as equally good in many comparisons, therefore the choice of modality is generally through personal choice of the patient and clinician.

1.2.3 Technique failure

Despite its clinical advantages as a RRT for ESRD patients, PD can often not be sustained for an extended period of time. This technique failure can be the result of either infectious or non-infectious complications. Technique failure is defined as insufficient dialysis of the blood, and another type of RRT must be initiated, normally HD.[67] This is quite common with CAPD patients, as 39% of patients experience an episode of technique failure within the first 3 years of treatment (defined as a switch to HD of 30 days or more).[68] The main cause of technique failure is peritonitis, closely followed by dialysis inadequacy or ultrafiltration failure.[67] The majority of technique failure occurs during the first two years of PD treatment, with risk factors including age, CVD and loss of RRF. Early technique failure is normally due to either choice or problems with the catheter, whilst ultrafiltration failure is a major cause in more long-term PD treatments. Infectious complications are a consistent reason for technique failure across all time frames. Another reason for stopping PD (particularly early on) is the availability of a transplant.[69] The main clinical causes for technique failure can be divided into infectious and non-infectious complications.

1.2.3.1 Non-infectious complications

Long term exposure of the peritoneal membrane to non-biocompatible PD fluids can alter the membrane functionality, causing inflammation, fibrosis, neoangiogenesis and eventually ultrafiltration failure, leading to technique failure. The major cause of these events in non-infectious complications are glucose and GDPs in the non-biocompatible PD fluid. Inflammation is caused by multiple factors, including accumulation of uremic toxins such as water-soluble compounds (e.g. urea), and small lipid-soluble or protein bound compounds (e.g. asymmetric dimethylarginine and homocysteine). Modified protein accumulation, retention of cytokines and mechanical stress of the vascular wall (i.e. due to hypertension) can also disrupt the peritoneal membrane through inflammation. Co-morbidities such as advanced age and diabetes, and extra-osseous calcification also contribute to this inflammatory reaction. In this process, inflammatory factors (e.g. tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6)) are produced by mesothelial cells, and macrophages are recruited, even without an infection. Chronic inflammation of the peritoneal membrane can cause ultrafiltration failure itself, but it is often caused by a combination of inflammation with fibrosis, neoangiogenesis and EMT/MMT.[70–72]

Chronic inflammation stimulates mesothelial cells to produce transforming growth factor-beta (TGF-β), which stimulates vasodilation, increased cell wall permeability, rapid solute transport and low ultrafiltration capacity, concurrent with fibrosis. One of the primary events in fibrosis is EMT/MMT, where mesothelial cells increase synthesis of extracellular matrix components and transition to fibroblasts (which then proliferate), whilst synthesising pro-inflammatory and pro-angiogenic factors (including IL-6 and chemokine (C-C motif) ligand 18 (CCL18)). These factors then recruit macrophages to the peritoneal cavity, increasing the inflammatory reaction and fibrosis of the peritoneal membrane.[70–72]

Alongside fibrosis, angiogenesis is a major cause of ultrafiltration failure. Pro-angiogenic factors released during chronic inflammation and fibrosis increase the vascular network in the peritoneal membrane, which then decreases the glucose-driven osmotic pressure of the dialysate. Alongside this, changes in the vascular wall (such as thickening and increased permeability) cause an exacerbation of small solute transport. These events, primarily caused by mesothelial cell production of vascular endothelial growth factor (VEGF), reduce the time for exchange of waste products, therefore ultrafiltration fails. When this occurs, PD is no longer effective at filtering the blood, and another RRT must be utilised.[70–72]

1.2.3.1.1 Encapsulating Peritoneal Sclerosis

Encapsulating peritoneal sclerosis (EPS) is a severe form of peritoneal membrane fibrosis. This is a very uncommon complication of PD, with prevalence reported as between 0.4% and 8.9% of PD patients in a variety of different single-centre, multi-centre and national registry studies.[73] Diagnosis is by a combination of structural and functional features, including computed tomography (CT) scan appearance and bowel obstruction, caused by a fibrous cocoon wrapped around the bowel, differentiated from membrane fibrosis caused by long term PD. It is common for EPS to develop up to five years after conclusion of PD treatment. The mortality rate of EPS patients can be as high as 55%, with this complication being particularly serious in the first year after diagnosis. The risk of EPS is increased with both duration of PD and incidence of peritonitis, as well as ultrafiltration failure and use of less biocompatible PD solutions. EPS can be classed into four different stages: pre-EPS, inflammatory, encapsulating and chronic, with treatments differing dependent on the stage, as well as underlying cause of EPS.[73–76]

1.2.3.2 Infectious complications

Although PD patients have better survival prospects in the first 2 years than HD patients,[11] infectious complications can be common and often serious. Infections in PD patients account for approximately two thirds of all PD catheter removals and about one third of transfers to HD. Peritonitis is the most common infectious complication; it accounts for 22% of all technique failures, is the cause of 4% of all deaths in PD patients and is a contributing factor in 16% of deaths.[7,77,78]

1.2.3.2.1 Type of infectious

1.2.3.2.1.1 Exit site infection

An acute exit site infection is defined as a purulent drainage at the catheter-epidermal interface, which may be associated with redness, tenderness, increased granuloma and oedema. Exit site infections can also be chronic (where the acute infection is either not treated properly or recurs after antibiotic withdrawal) or equivocal (where the infection and associated symptoms are mild, this often progresses to an acute infection if not treated). If the infection does not respond to antibiotic treatment (either systemic or local application at the exit site, for at least two weeks), or the catheter itself shows signs of infections, then the catheter must be removed to prevent progression to tunnel infections or peritonitis. Exit site infections are more common in the first year of PD treatment than later on. Treatment of exit sites with prophylactic topical antibiotic cream (such as mupirocin) is an effective preventative measure against these infections, which are normally caused by touch contamination.[79–82]

1.2.3.2.1.2 Tunnel infection

A tunnel infection is characterised by inflammation along the tunnel of the peritoneal catheter. This can present clinically as erythema, oedema and tenderness over the subcutaneous pathway, but can be subclinical and only diagnosed by ultrasonographic identification of inflammation or collection along the catheter. Tunnel infections usually occur in the presence of an exit site infection but can occur alone, with progression to peritonitis likely if the infection is left untreated or does not respond to treatment. Tunnel infections often have more severe consequence for the patient than exit site infections, and are highly predictive of catheter loss either due to replacement or removal, and subsequent transition to an alternative RRT. Tunnel infections are treated with standard

first-line antibiotic treatments, although they often require longer courses than exit site infections (i.e. three weeks rather than two weeks).[79,81–84]

1.2.3.3 Peritonitis

Peritonitis is one of the most serious complications of PD, with considerable associated mortality and morbidity. Peritonitis is a direct contributor to approximately 20% of technique failures and 2-6% of deaths of PD patients. Peritonitis is not only a complication itself, but can contribute to peritoneal membrane fibrosis and ultrafiltration failure, and may also increase the risk of EPS.[74,75,78,85,86]

1.2.3.3.1 Diagnosis

Peritonitis is diagnosed when cloudy PD effluent is observed, along with abdominal pain and a leukocyte count of >100 cells/mm^3 with >50% polymorphonuclear (PMN) cells. Standard culture techniques are used to identify the causative organism to decide on appropriate long-term treatment, whilst an empirical treatment regime is initiated before culture results are known. It is recommended by the International Society for Peritoneal Dialysis (ISPD) that culture-negative peritonitis should not be greater than 20% of peritonitis episodes in each hospital, as identifying the causative organism can greatly aid more accurate treatment decisions, as well as identifying the source of the infection, to help prevent further contamination from the same source.[87,88]

1.2.3.3.2 Epidemiology and Microbiology

PD-related peritonitis rates range widely across different centres and countries. Reported rates globally range from 0.06-1.66 episodes per year, per patient, with rates within a single country varying as much as 0.14-1.0 per year (across the London Thames centres). These large differences are likely down to variations in patient demographics, the local spectrum of microorganisms, patient training, infection prevention, and possibly accuracy in recording.[78,89]

Peritonitis rates are generally decreasing over time, with some single-centre studies showing almost a 50% decrease in rates of total peritonitis episodes (0.57 episodes/patient-year in 1993 to 0.29 in 2005 in a centre in Korea).[90] Comparable results were also seen in single-centre studies in Brazil, Portugal and Taiwan.[91–93] This is mainly due to a reduction in Gram positive infections, while rates of Gram negative episodes

remain similar. This can be attributed to improvements in PD equipment (such as the introduction of the twin-bag connection system) leading to a reduction in contamination with skin commensal organisms during connection procedures.[78,94] Other factors that have reduced rates of infection include better identification of peritonitis risk factors and introduction of prophylactic antibiotic treatment.[78,85,95]

The most common causative organism for PD-related peritonitis are Gram positive organisms, which accounts for 56% to 71% of all peritonitis episodes, dependent on the centre. This includes coagulase negative *Staphylococcus spp.* (CNS) and *Staphylococcus aureus*, two of the most common infecting organisms. The majority of other peritonitis infections are caused by Gram negative bacteria, which make up 19% to 38% of infections, including *Escherichia coli*. The guidelines from the ISPD recommend no more than 20% of peritonitis episodes should be counted as "culture negative", i.e. have no detectable organism after microbiological culture techniques. This is true for the majority of centres studied, with the proportion of peritonitis episodes without a positive culture result varying from 16% to 23%. A small proportion of infections are caused by fungi (between 1% and 15%). Although these represent only a small proportion of peritonitis episodes, they can cause severe infections with detrimental outcomes.[96–98]

1.2.3.3.2.1 Single organism infections

Most peritonitis episodes are caused by a single bacterial organism (up to 85%), with most of those being from Gram positive organisms. The primary peritonitis-causing organism is CNS, including *S. epidermidis*. This skin commensal typically causes a relatively mild peritonitis episode, which usually responds well to first-line antimicrobial treatment and can be resolved, often without hospitalisation (although different regional practices may mean rates of hospitalisation can vary). The next most common infecting organism is *S. aureus*, followed by *Streptococcus* and *Enterococcus* species. These all cause relatively minor infections that can often be resolved quickly. Episodes caused by antimicrobial-resistant bacteria such as methicillin-resistant *S. aureus* (MRSA) or vancomycin-resistant *Enterococcus* (VRE) are much more serious and do not respond as well to antibacterial treatment. These are still quite rare but the seriousness means they can be a major problem, and the percentage of episodes caused by these is increasing.[97,99–101]

Although Gram negative infections are less common[97], they often have more serious consequences for the patient. The most common Gram negative infecting organism is *E. coli*, closely followed by *Pseudomonas*, *Klebsiella* and *Enterobacter*. Patients with these diagnoses often face poorer outcomes, in terms of catheter removal, hospitalisation and length of hospital stay, due to the more severe infection and longer treatments required (often three weeks rather than two weeks, as with more minor infections)[102–104].

1.2.3.3.2.2 Polymicrobial infections

Infections caused by multiple bacteria are not very common (only accounting for 10% of peritonitis episodes in one Australian study), with the majority caused by only two organisms. This low rate could be due to a local variations in reporting of culture results, not merely a low incidence of polymicrobial infections. The most common combinations were *S. epidermidis* and other CNS species (4.7% of cases), *Enterococcus* and *Klebsiella* (3.9%), and *E. coli* and *Klebsiella* (3.6%), with combinations of Gram positive and Gram negative more common than those with multiple organisms with the same Gram status. Polymicrobial episodes are generally more serious than those caused by a single organism, in terms of hospitalisation, catheter removal, permanent transfer to HD and death. The organisms present are important in the prognosis, with polymicrobial infections caused by only Gram positive organisms being the most mild, and those involving fungal infection the most serious.[105–107]

Polymicrobial episodes generally required hospitalisation (83% of cases), with rates of catheter removal, permanent transfer to HD and death (43%, 38% and 4% respectively) higher than in episodes caused by a single organism, although rates of relapse are lower (probably due to the high rates of technique failure so the possibility of relapse is removed). The length of hospital stay is also longer in polymicrobial cases than in single organism infections. Episodes with a bacterial and fungal component had the most serious consequences, with higher rates of hospitalisation, technique failure and longer stays in hospital than any other group. The clinical parameters are comparable to those with fungal infections alone, suggesting the virulence of the fungus is the primary causative factor in a poor outcome. Presence of an anaerobic or *Pseudomonas* species is also a predictor of poor outcome.[105–107]

1.2.3.3.3 Fungal infections

Fungal infection is an uncommon cause of peritonitis (accounts for between 1% and 15% of episodes in different studies), but it is associated with significant mortality and morbidity. Mortality from fungal peritonitis ranges from 5% to 53%, and technique failure resulting from fungal infections can be as high as 40%. Most fungal peritonitis episodes are caused by *Candida* species, accounting for 89.3% of infections in one study, with only a small number reportedly caused by filamentous fungi such as *Aspergillus* species. Clinical features of fungal peritonitis are no different from those of bacterial peritonitis described above. The likelihood of contracting fungal peritonitis is increased after antibiotic treatment. Treatment of fungal infections involves use of anti-fungal agents, such as fluconazole (for *Candida* species), for 4-6 weeks. It is recommended for the catheter to be removed immediately after fungal infectious agents have been identified, especially if there is no clinical improvement after 4-7 days.[98,108]

1.2.3.3.4 Culture negative peritonitis

Culture negative peritonitis episodes are when the standard microbiological culture methods cannot identify a causative organism. It is recommended by the ISPD that the rate of these infections do not exceed 20%. An infection can be identified as culture negative due to a number of technical or clinical reasons including recent antibiotic exposure preceding the obtaining of a sample for culture, suboptimal sample collection or culture methods, or infection with an unusual organism such as a fungus. They should be treated as all peritonitis infections are, using front line antibiotics against primarily Gram positive organisms, as it is supposed that most are caused by these bacteria, e.g. by touch contamination. If the patient is improving, then treatment should be continued, but if there is no improvement after 5 days then it is recommended to consider catheter removal. If the patient does not respond to therapy, then it might be necessary to attempt more specialised culture techniques to identify the causative organism, alongside catheter removal. There is an association between previous antibiotic treatment and culture negative peritonitis episodes. Culture negative peritonitis episodes are more likely to be resolved with antibiotic treatment, less likely to involve hospitalisation, catheter removal, permanent transfer to HD or death than culture positive peritonitis.[88,109–111]

1.2.3.3.5   Risk factors

There are many factors that increase the likelihood of having a peritonitis episode, some of which are connected to an overall increase in the probability of having an infection in general (for example, diabetes mellitus and other co-morbidities) so must be managed as appropriate. Other risk factors are specifically related to peritonitis, for example, a positive nasal carrier of *S. aureus* is more likely to have an episode caused by *S. aureus*. There are also several demographic factors that appear to correlate with an increased risk of peritonitis, such as age, gender and ethnicity. Some risk factors may also be associated with organism-specific peritonitis episodes, for example, peaks in the incidence of CNS and Gram-negative organisms in warmer seasons and *Corynebacterium* species in winter demonstrate seasonal variations in organism-specific peritonitis rates. One of the major risk factors for peritonitis episodes caused by fungal organisms is prior exposure to antibiotic therapy.[78,92,112]

1.2.3.3.6   Coagulase negative Staphylococcus

CNS is found on human skin as part of the normal microbiota. CNS was initially differentiated from *S. aureus* (a coagulase positive pathogen) as non-pathogenic, whereas it is now known that CNS causes a burden on the health system. This opportunistic pathogen is one of the major causes of nosocomial infections due to contamination from the skin, often of implanted foreign bodies such as catheters, or hip replacements. *S. epidermidis* and *S. epidermidis*-like species are the most common group of CNS bacteria and are the most common CNS group to cause infections, with significant genomic diversity compared to other CNS species. There are 18 species of CNS bacteria isolated from humans, but only five have been implicated in nosocomial infections. These bacteria colonise the body surface, with most prevalence in mucosal surfaces. Inserted or implanted foreign bodies are frequently colonised by CNS, specifically the *S. epidermidis* group, and this can be the cause of many device-related infections. These can be both local (for example, exit site infections) and systemic (i.e. bloodstream infections), although they tend to be relatively minor infections (i.e. subacute and low inflammatory component). In fact, *S. epidermidis* is now recognised as the most frequent cause of nosocomial sepsis. Clinical CNS isolates are now often resistant to the bactericidal β-lactams (such as methicillin), with growing resistance to other antibiotics due to increased exposure leading to a positive selection pressure. Treatment of these often now requires a glycopeptide, such as vancomycin, although use of this can be avoided using penicillins or cephalosporins (first

or second generation). In serious infections from implanted/inserted medical devices, if the infection is not controlled by systemic antibiotic therapy, the only effective therapy is often removal of the device, i.e. catheter removal and transition to HD. This is the most common infection in PD patients due to the biofilm often formed around or in the catheter, therefore the growing resistance is a problem in this vulnerable population.[113–115]

1.2.3.3.7   E. coli

E. coli is one of the most abundant facultative anaerobes of the human intestinal microflora, with its preferred niche in the mucosal layer of the mammalian colon, where it makes up 0.1-5% of the total gut microbial population. This Gram negative rod-shaped bacillus can have flagella or pili on its surface to aid movement and attachment to cells. *E. coli* are thought to colonise the intestinal tract of new-borns through exposure to the mother's faecal matter through birth, or through handling thereafter. This is essential to help establish the full microbiome of the infant gut. Disruptions in the normal microbiome, for example through antibiotic use, can increase the *E. coli* population and cause infection. Infections can also be caused by *E. coli* exiting the gut, either through faecal matter or disruption to the gut membrane. Potential sources of infection in peritonitis include touch contamination after poor hand hygiene (contamination from environmental sources or faecal matter), or through bowel perforation allowing the microbiome to enter the peritoneum. Leakage from the intestinal tract increases the chance of polymicrobial infections due to multiple bacteria entering the peritoneum, with *E. coli* a likely pathogenic component. There are a number of pathogenic strains of *E. coli*, as well as the ones that exist harmlessly in the intestinal tract. This highly diverse and broadly distributed species has remarkable genetic plasticity, which has been exploited for research purposes since it was first discovered, meaning they have the ability to mutate rapidly to avoid treatments, especially by generating resistance to β-lactams. Current treatments recommended include a range of aminoglycosides, ceftazidime, cefepime or carbapenem, with different combinations of each effective to treat Gram negative infections as an empirical first-line therapy. Once antibiotic-sensitivity of the infecting organism has been identified then more specific treatments can be initiated. *E. coli* infections tend to be more serious than CNS infections, with rates of hospitalisation, catheter removal, technique failure and mortality higher.[104–106,110,116,117]

1.2.3.3.8 Recurrent relapsing and repeat peritonitis

When peritonitis episodes occur close together, they can be classified as either recurrent, relapsing or repeat, and these all have worse outcomes than uncomplicated peritonitis. Recurrent infections are infections with a different organism within four weeks of completion of therapy for a prior episode, and these have a worse prognosis than relapsing peritonitis. Relapsing infections are also within four weeks of completing therapy for a previous infection but caused by the same organism (or after a sterile episode). Repeat peritonitis is an episode caused by the same organism that occurs more than 4 weeks after completion of therapy for a prior episode. Relapsing peritonitis has an associated lower rate of cure, more ultrafiltration problems, and higher rate of technique failure. Relapsing peritonitis is more common than recurrent peritonitis and is more likely to be caused by Gram positive organisms. Fungal infections are more common in recurrent infections, possibly due to antibiotic exposure increasing the risk of fungal pathogenic growth. If infections are caused by different organisms (i.e. recurrent peritonitis) then they are thought to be separate clinical entities, therefore need to be treated appropriately. Patients with recurrent, relapsing or repeat peritonitis are likely to require catheter removal and subsequent transition to HD.[110,118,119]

1.2.3.3.9 Treatment

If a patient has peritonitis for an extended period of time, they are more likely to have a poor outcome, including catheter removal. This risk is increased again with specific infections, such as *E. coli*, or when peritonitis episodes are recurrent, relapsing or repeat. It is important to prevent catheter removal, as only a low proportion of those who undergo this surgical procedure is able to return to PD therapy (with reported rates ranging from 3-13%).[78,120]

Rapid treatment of a peritonitis episode through empiric antimicrobial therapy is recommended by the ISPD as a first line. This should cover both Gram-positive and Gram-negative organisms and be used until the results of microbiological cultures can advise on a more specific treatment regime. This empiric treatment should be selected on a centre-specific basis considering the local history of organism sensitivities and frequencies in causing peritonitis episodes. To target Gram positive organisms, the use of a first-generation cephalosporin or vancomycin is suggested, whilst Gram-negative bacteria can be targeted using an aminoglycoside or ceftazidime.[78,88,121] The administration of

antimicrobial agents into the peritoneum (IP, intraperitoneal) is more effective at managing and treating peritonitis episodes than intravenous (IV) dosing.[88] Once the infecting organism has been identified microbiologically, and sensitivity to a range of antibiotics tested, then more specific treatments can be initiated. For example, many CNS species are methicillin resistant, but this will not be known until they are cultured in the presence of methicillin.[113]

Ideally, a more rapid microbiological diagnosis needs to be developed to improve peritonitis management and outcomes by facilitating timely introduction of appropriate therapy.[78,122]

1.3 Immune response

The immune system is the collection of different factors that together protect the body against foreign agents, known as antigens, as well as abnormal self, including injured or inflamed cells and tumours. These protective factors include both cellular components and molecular mediators. The immune system is classified into two complementary types of response: the adaptive and innate immune responses.[123,124] This project focuses on how the innate immune system responds to acute peritonitis episodes in PD patients.

1.3.1 Adaptive immunity

The adaptive immune response is the specific arm of host defence against pathogens, allergens and abnormal self. It consists of specialised cells, T and B cells, which can recognise potential threats to the host and instruct other cells to aid removal and destruction of potentially harmful cells or invading pathogens. The adaptive immune system is also called the acquired immune system due to the immunological memory that it possesses. This response to infection is much slower than the innate immune system but it is specific to the antigen or injury, and the memory allows a larger response to threats that have been encountered before, and this is the basis of vaccination. The innate and adaptive immune systems are closely linked, with cross-activation a key component of the host response to remove invading microbes.[123–125]

1.3.1.1 T cells

T cells, so-called because of their maturation in the thymus, are key components of the adaptive immune response and contain a T cell receptor (TCR) on their surface. There are many different types of T cells with a range of effector, regulatory and memory functions which have been characterised by different cell-surface markers.[126]

CD4$^+$ T cells are called T helper cells, and these cells help modulate other immunologic processes through cell-to-cell contact and cytokine release, including B cell maturation, CD8$^+$ T cell activation and phagocytosis. The different T helper cell subsets have different actions. For example, Th1 cells regulate the cell-mediated response typically against intracellular bacteria and protozoa, whilst Th2 cells modulate the humoral response against extracellular pathogens.[127] CD8$^+$ T cells are also known as cytotoxic T cells, due to their ability to actively kill cells that are either infected (e.g. with a virus) or abnormal (e.g. cancerous). Once activated through their TCR, they undergo clonal expansion and release a range of cytotoxins to kill the target cell.[128]

Most TCRs consist of an alpha and beta chain combined, i.e. $\alpha\beta$ T cells, but a small proportion have a gamma and a delta chain making up their TCR, so are named $\gamma\delta$ T cells. These are more similar to innate immune cells in that they recognise specific patterns that represent abnormal antigens, such as bacterial metabolites like (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP). However, they are classed as adaptive immune cells due to the rearrangement of TCR genes that is characteristic of the generation of specific TCRs by $\alpha\beta$ T cells.[129]

A key component of the adaptive immune responses is immunological memory, which is mediated through memory T cells. These long-lived cells are generated through previous exposure to an antigen, and when re-exposed to the same antigen they rapidly expand to generate a large number of effector T cells against the specific antigen. There are many different types of memory T cells including central memory (in the lymph nodes and circulation), effector memory (in the circulation) and tissue resident memory cells (found in specific tissues).[130,131]

Key in modulating and controlling immune responses, especially autoimmune reactions, are regulatory T cells (Tregs). These help maintain immunological tolerance through

suppression of autoreactive T cells that escape negative selection in the thymus. Tregs also prevent excessive immune reactions to stimuli by shutting down T-cell mediated immunity towards the end of an immune reaction.[132]

1.3.1.2 B cells

B cells are named due to their maturation in the bone marrow. They are activated by their cell surface B cell receptors (BCRs) recognising antigens, and this activation process often involves T cells. Once activated, B cells then differentiate into either plasma cells or memory B cells. Plasma cells are known as "antibody factories" due to their high production and release of antibodies. The antibodies bind to the specific antigen and coat it, to neutralise it, stick it together to promote phagocytosis (agglutination), or activate complement. Memory B cells act similarly to memory T cells, in that they respond quickly to re-exposure with the same antigen to rapidly produce specific antibodies and generate a robust defence against the same antigens. B cells can also act as antigen-presenting cells (APCs), where they take up external antigens by endocytosis and present it to T cells through cell surface major histocompatibility complex (MHC).[133–135]

1.3.2 Innate immunity

The innate immune response is the immediate response to an insult, either pathogen-derived or as a result of an injury, with the same quality and magnitude of response regardless of previous exposure to the same insult, i.e. lacking immunologic memory. Innate immune responses developed earlier in evolution and are seen even in the simplest of animals due to their high levels of conservation. This response consists of a range of cellular components, including phagocytes and cells releasing inflammatory mediators, and molecular components, such as complement and cytokines. These all are incorporated into an inflammatory reaction that causes symptomatic pain and swelling, but also aids resolution of injury and tissue repair through recruiting and modulating further immune responses.[123,124]

1.3.2.1 Innate immune recognition of pathogens

The innate immune response is built upon specific cell types detecting molecular patterns that are specific to infecting pathogens, termed pathogen-associated molecular patterns (PAMPs), by PAMP recognition receptors (PRRs) which are broadly specific for a range of

PAMPs. PAMPs are specific to microorganisms, not present in the host, essential for their survival (so they cannot evade immune surveillance) and are conserved by a range of microorganisms across a class. Common bacterial and fungal PAMPs include components of the organism's cell wall such as lipopolysaccharide (LPS) and β-glucan. Viruses are largely detected by the host through unique modifications on viral nucleic acids.[136,137]

The best characterised class of PRRs are the Toll-like receptors (TLRs). These transmembrane proteins recognise viral nucleic acids and several bacterial components including LPS and lipoteichoic acid. There are currently 10 TLRs identified in humans, and activation of these at the cell membrane initiates intracellular signalling that ends in nuclear factor kappa B (NF-κB) mediated expression and secretion of inflammatory cytokines.[138] There are a range of other PRRs that are important for recognition of infection, including dectin-1 and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs).[139,140]

1.3.2.2 Cellular components

The cellular component of the innate immune system consists of phagocytes, cells that release inflammatory mediators, dendritic cells (DCs) and natural killer (NK) cells. Phagocytes, such as neutrophils and macrophages, engulf and absorb infecting microorganisms and other small particles including cellular debris. Dendritic cells present antigens to the adaptive immune system to modulate specific immune responses. Cells that release inflammatory mediators and modulate further immune responses include basophils, eosinophils and mast cells, however all immune cells are able to release inflammatory mediators in response to specific stimuli. NK cells can also recognise infected and malignant cells, then kill them through direct mechanisms unique to this cell type.[123,124]

1.3.2.2.1 Phagocytes

Phagocytes are literally called "cells that eat" in Greek, based on their ability to take up pathogens and other debris. There are a range of "professional phagocytes", named as such due to their effectiveness at phagocytosis, which includes neutrophils, macrophages and monocytes.[141]

1.3.2.2.1.1 Neutrophils

Neutrophils are the most effective phagocytes, with the ability to engulf many particles rapidly, as well as being the most abundant in circulation. They are often the first responders to acute infection and inflammation, where they can be the cell type present in the highest numbers.[141] For these polymorphonuclear phagocytes to effectively clear infecting microorganisms they must first migrate out of the blood to the site of infection, phagocytose the particle and then kill it. The migration out of the bloodstream is called extravasation and consists of the neutrophil tethering, rolling, adhering to and crawling along the endothelium, then transmigrating either between or through the endothelial cells. This is controlled by specific markers on the endothelial cells that are induced by inflammatory stimuli. Chemotaxis along a chemokine gradient then attracts neutrophils to the site of the inflammation or infection.[142] Neutrophils recognise microbes through a receptor-mediated process, by either PRRs or specific surface receptors for opsonised microbes (that are coated in antibodies or complement proteins). Once recognised, the microbe is phagocytosed by the neutrophil in a receptor-mediated process, into a vacuole.[143]

Neutrophils can kill infecting microorganisms through degranulation, release of reactive oxygen species (ROS) and production of neutrophil extracellular traps (NETs). In degranulation, the granules in the neutrophil fuse with the vacuole containing the phagocytosed microorganism to form a phagosome. The granule contents are released into the newly formed phagosome, and these include proteases and peptidases that lyse phagocytosed microorganisms.[143] There are two main types of granules in neutrophils; azurophilic granules and specific granules. Azurophilic granules are the first to be produced (also called primary granules) and contain different serine proteases. Specific granules are produced later and contain a wide range of antimicrobial agents including lactoferrin, calprotectin and lysozymes.[143,144] There is a third type of granule called gelatinase granules (also known as tertiary granules) which contain metalloproteases such as gelatinase. These are thought to be important in neutrophil maturation.[143] ROS are bactericidal chemical species produced through an oxidative burst which is stimulated by phagocytosis. These are also released into the vacuole and act in concert with the enzymes released from the granules to help kill internalised microbes.[145]

NETs were first discovered in 2004 as a novel method of microorganism killing.[146] Neutrophils release a mesh-like structure of DNA, histones and antimicrobial peptides from the granules in a process called NETosis. This can either cause neutrophil cell death or be through a non-lytic process by the primary responders to an infection. These NETs capture nearby pathogens in the sticky decondensed chromatin mesh and expose the pathogens to a high concentration of antimicrobial peptides, killing them and recruiting other phagocytes to engulf the microorganisms.[146,147] The releases of NETs must be closely controlled due to the potential for damage to surrounding normal tissues and association with autoimmune diseases.[148]

1.3.2.2.1.2 Monocytes and macrophages

Monocytes and macrophages have a more complex pathway of differentiation and activation than neutrophils. Monocytes are circulating immune effector cells that can migrate out of the blood during infection and are able to sense and respond to both cytokines and PAMPs through receptors (including PRRs). They can differentiate into inflammatory DCs (antigen presenting cells) or macrophages in inflammation. Macrophages are resident phagocytes in a range of different tissues involved in homeostasis in normal tissues and effective phagocytes. Macrophages have a range of PRRs on their surface and can produce inflammatory cytokines in response to infection.[149,150] Macrophages are traditionally divided into two main subsets; M1 and M2. M1 macrophages are classically activated and are conventionally seen as "pro-inflammatory" due to their ability to produce inflammatory cytokines and kill infecting pathogens by phagocytosis. M2 macrophages are alternatively activated and are traditionally seen as "anti-inflammatory" with homeostatic functions. These main subtypes can be sub-divided into other types with many different roles in innate immune responses, activating the adaptive immune response and homeostasis. Macrophage subtypes also have the ability to change between phenotypes.[151] The role of pro-inflammatory macrophages will be discussed here due to their response in an acute infection. However, tissue-resident macrophages are crucial in response to inflammatory stimuli, with unique transcription factor expression controlling phenotype and proliferation potential e.g. Gata6 in peritoneal resident macrophages.[152–155]

Macrophages are effective at taking up a range of different small molecules through pinocytosis (for liquids and solutes) and receptor-mediated endocytosis (in a clathrin-

dependent manner), whilst they use an actin-dependent pathway of phagocytosis for larger particles including invading microorganisms.[156] Macrophages can recognise microbes that have been opsonised or through PRRs detecting PAMPs.[157,158] Once recognised, the microbes are taken up by actin skeleton rearrangement to allow phagocytosis and a phagosome is formed.[157] Killing of microbes inside a phagosome is similar in macrophages as in neutrophils, with ROS and antimicrobial peptides playing key roles. Macrophages do however lack many of the serine proteases found in neutrophils, therefore other proteases are more important. A key mechanism of both preventing pathogen growth and killing microbes is through generation of an acidic environment in the phagosome.[159]

Macrophages have multiple roles at the site of infection, as they can also act as coordinators of further immune responses. This includes further innate immune responses such as recruiting neutrophils through cytokine and chemokine production. Macrophages can also present antigen on their cell surface to guide adaptive immune responses.[151,159]

1.3.2.2.2 Dendritic cells

DCs are key mediators of immune responses through their function as APCs. DCs continuously sample their environment by taking up and presenting antigen on their cell surface bound to MHC II complexes.[160] This is a key to the generation of self-tolerance, both central and peripheral,[161] as well as sensing invading pathogens and stimulating effector immune cells. There are many different subsets of DCs, including CD1c$^+$ DCs, CD141$^+$ DCs and plasmacytoid DCs (pDCs), which vary in their localisation to different tissues, their PRRs and cytokine production.[162] For example, CD1c$^+$ DCs have low levels all TLRs except TLR7 and TLR9, whilst CD141$^+$ DCs have high levels of TLR3,[162,163] whilst pDCs are best characterised as strong producers of type-I interferon (IFN I).[164] The main role for DCs, once they present antigen on their surface, is to modulate adaptive immune responses. T cells, both CD4$^+$ and CD8$^+$, are strongly activated even by small numbers of DCs. B cells are also activated both indirectly through expansion of T helper cells and directly through secretion of cytokines.[160,162] DCs are important for continual monitoring of the environment and engaging a specific rapid response to infection, inflammation or abnormal self.[160,161]

1.3.2.2.3 Other cells in the innate immune system

A range of other cells are involved in the innate immune response through a variety of mechanisms, mostly by sensing and responding to inflammatory or infectious insults, then producing inflammatory mediators and modulating further immune responses.

Eosinophils have weak phagocytic activity, and are best known for their cytotoxic effector functions in parasitic infections.[165] They have PRRs to recognise PAMPs, which then activates the release of anti-microbial peptides, including cationic proteins, and mitochondrial DNA-containing "traps" similar to NETs.[165–168] Eosinophils are a minor subset of the white blood cells, but they play an important role in modulating immune responses to infecting pathogens through release of cytokines and chemokines, as well as sensing and regulating the complement pathway, amongst others.[165,166]

Another cell type involved in releasing inflammatory mediators to modulate the innate immune response are basophils. They make up only approximately 1% of peripheral blood leukocytes and had historically been identified as important in allergic disease, due to their high affinity receptor for immunoglobulin E (IgE).[169] However, it is now known that they have a range of surface receptors for many different stimuli to help modulate the immune response. For example, they have PRRs to respond to PAMPs and can be activated by a range of different stimuli to produce cytokines, especially IL-4.[169,170] They can also actively kill infecting microbes through releasing extracellular DNA.[171] Basophils may also influence the adaptive immune response, through antigen presentation and regulating B cell and T cell functions.[169]

Mast cells are another effector cell type in the innate immune system. These cells are similar to basophils in that they also contain high affinity receptors for IgE and are therefore associated with allergic disease. However, mast cells are resident in tissues which form the interface between the body and the external environment, whilst basophils are found in the blood and migrate to the site of infection.[172] Mast cells express a wide range of different surface receptors that can detect external stimuli, including PRRs such as TLRs, receptors for complement proteins, and receptors for cytokines and chemokines.[172,173] Mast cells can have direct effects on pathogens through endocytosis or direct secretion of antimicrobial peptides.[172,174] They can also be potent modulators of other leukocytes through release of soluble mediators affecting their recruitment, proliferation, survival and activation.[172,175]

1.3.2.2.4 Natural killer cells

NK cells recognise and kill cells which are infected by intercellular pathogens (e.g. viruses) or are growing abnormally (e.g. cancer cells). They are normally present in the circulation and can migrate out into tissues when they encounter chemokines.[176] The activation of NK cells is tightly controlled by a range of activation and inhibitory signals through different receptors on their surface.[176–178] One key inhibitory signal is the presence of normal MHC I, which is used by normal cells to present peptides on their surface in the generation of self-tolerance to healthy cells. When this is not present, or is abnormal, the lack of this inhibitory signal activates NK cells, as is the case in virally infected cells or those replicating abnormally e.g. cancer cells.[177] Although this is the main inhibitory signal NK cells recognise, there are a range of other signals, both inhibitory and activating, that NK cells can respond to.[177,178] For example, natural cytotoxicity receptors (NCRs), such as NKp46, on NK cells are activated by the presence of viral haemagglutinin on the surface of cells.[177,179]

Once NK cells are activated, through activation signals, the removal of inhibitory signals, or a combination of both, they can kill cells by direct secretion of components from their lysosomes across the immunological synapse to kill the target cell in a process called degranulation. The granule contents include perforin, which forms pores in the target cell membrane to allow entry of other cytotoxic proteins including granzymes. These serine proteases then activate caspases to induce apoptosis.[178,180] NK cells can also induce apoptosis of target cells through release of Fas ligand (FasL), TNF-α and TNF-related apoptosis-inducing ligand (TRAIL). When these ligands interact with their corresponding death receptors on the target cells, apoptosis is triggered.[178,181] The activation of NK cells can also stimulate other immune cells, including T cells, through release of a range of cytokines and chemokines.[182]

1.3.2.3 Soluble component

The range of different cells involved in the innate immune system also produce many different soluble factors that influence the acute immune response to pathogens.

1.3.2.3.1 Complement

Complement consists of over 30 proteins which can exist in soluble forms and on cell surfaces, and is an important component of innate immune responses. Those on cell

surfaces can either be receptors for activated complement proteins or regulators of complement activation.[183] The complement system has a key role in the defence against infection, with other important roles in the interface between innate and adaptive immune responses, and aiding disposal of waste.[183,184] The key function of complement proteins is to modulate and regulate other immune responses. For example, complement proteins can opsonise bacteria and immunogenic molecules to aid phagocytosis and recognition by the immune system, with C3 especially important in this process.[183,185] Activation of the complement cascade leads to generation of chemoattractants such as C3a and C5a.[185–189] Complement proteins can also directly kill pathogenic cells through formation of the membrane attack complex (MAC). This complex of four complement proteins forms a pore in cell membranes to disrupt the cellular processes and cause cell lysis.[190]

The complement system has a wide range of functions in innate immune responses, therefore it is tightly regulated through the different activation cascades. There are three pathways that lead to complement activation; the classical, mannose-binding lectin, and alternative activation pathways.[183] Classical activation is through sensing of antigen-antibody complexes and C-reactive protein (CRP) bound to its ligand, which leads to activation of the C1 complex. The mannose-binding lectin pathway is activated upon sensing of microbes with terminal mannose groups, whilst the alternative pathway is activated spontaneously at a low level and leads to complement deposition on any surface, including bacteria and tumour cells.[183,191] These pathways are tightly controlled to enable accurate deployment of this effective defence system against infecting pathogens. Some complement complexes are crucial in augmenting antibody responses or enhancing immunological memory, as well as clearance of immune complexes and apoptotic cells.[191]

1.3.2.3.2 Acute phase proteins

Acute phase proteins are those that are altered in an inflammatory reaction, often at a distant site to that of the acute inflammation.[192] These include CRP, the first discovered acute phase protein. This very sensitive marker of inflammation and tissue damage is a soluble PRR that binds to infecting microbes or cell debris, where it is then recognised by complement proteins to stimulate an acute immune response.[192,193] Although CRP appears to be a strong marker for general, non-specific inflammation, there does appear to be an association between CRP levels and cardiovascular disease.[193,194] Other acute phase proteins include those in the coagulation and fibrinolytic system (e.g. fibrinogen),

antiproteases (e.g. pancreatic secretory trypsin indicator), transport proteins (e.g. ceruloplasmin), participants in the inflammatory response (e.g. granulocyte colony-stimulating factor) and others including serum amyloid A and procalcitonin.[192,195,196]

1.3.2.3.3   Cytokines

Cytokines are small molecules secreted by both immune and non-immune cells that have an effect on other cells. This broad category includes interleukins, interferons, chemokines and tumour necrosis factors, and they have key roles in modulating effector cells to defend against infectious and inflammatory insults.[123] Cytokines can be broadly divided into "pro-inflammatory" and "anti-inflammatory", but often have context-dependent functions.

For example, IL-6 is a pro-inflammatory cytokine, as shown by its role in the pathogenesis of rheumatoid arthritis and how it is effectively targeted as a treatment.[197–199] However, IL-6 can be protective against infection, as shown by deficiency causing a high susceptibility to bacterial infections.[197,198,200] IL-6 has been measured in PD effluent as a marker of PD-induced inflammation, peritoneal membrane transport characteristics and PD-related peritonitis, suggesting is has an important role at the site of inflammation and infection,[201,202] with roles in neutrophil trafficking and apoptosis, and T cell function in acute infection, thereby linking the innate and adaptive immune responses.[198,203–205]

Another key cytokine in response to inflammation is the chemokine C-C Motif Chemokine Ligand 2 (CCL2, also known as monocyte chemoattractant protein 1, MCP-1). Chemokines are specialised cytokines that induce movement of cells that are attracted to them in a process called chemotaxis. This pro-inflammatory chemokine is expressed by a range of cells upon inflammatory stimuli, including epithelial and endothelial cells, as well as monocytes and macrophages. CCL2 then induces recruitment of immune cells to the site of injury or infection, specifically monocytes, as well as memory T cells and NK cells.[206] CCL2 is induced upon epithelial cell exposure to high glucose conditions,[207] infection with *Mycobacterium bovis* and *Mycobacterium tuberculosis*,[208] and is shown to increase in inflammatory conditions such as asthma[209] and arthritis.[210]

TGF-β is an important family of inflammatory cytokines that can be both pro- and anti-inflammatory, dependent on the local microenvironment. This cytokine is expressed and released by many different cell types and can have both autocrine and paracrine effects.[211]

It has known roles in many different biological processes, including growth and development.[212] TGF-β is important in controlling inflammation, as knockout animals suffer fatal inflammation shortly after birth (of the 40% than develop normally to full term).[213] TGF-β is an important regulator of T cell proliferation and differentiation, whilst it also controls elements of B cell, NK cell, DC, macrophage, mast cell and granulocyte function.[212] TGF-β has been implicated in the pathology of different inflammatory diseases including asthma,[214] autoimmune diseases[215] and inflammatory bowel disease.[216]

The complex mixture of cytokines present at the site of infection or inflammation alters the specific immune response by changing how different effector cells respond and recruitment of further immune cells. Cytokines can be produced by any cell type, with both immune cells and non-immune cells producing and responding to different cytokine combinations through cytokine receptors on their cell surface. These small molecules are essential mediators of immune responses and modulators of how effector cells resolve the infectious or inflammatory episode.[123,124,217,218]

1.3.3 Immune fingerprint in PD-related peritonitis

As discussed above, the immune response is very specific to distinct pathogens, through a range of cellular and soluble markers. It has been proposed that this could be used diagnostically in a clinical setting of acute infection, such as PD-related peritonitis.[122]

The need for a new method of identification of the causative pathogen of an infectious episode is crucial due to the problems with current culture methods that are slow and can often give negative results, despite clinical presentation of an infection. Even if a culture result is positive for infecting organisms, it cannot discriminate between pathogens that cause disease or are asymptomatic, as well as those that arise from sample contamination.[109,122] Treatment, as discussed earlier, remains empirical and has associated problems including off-target effects and the potential for resistance to occur.[219,220]

The immune system has evolved to be specific for different pathogens that express distinct molecular patterns,[221] therefore the concept of using this "immune fingerprint" to determine the infecting pathogen has been developed. This hypothesis suggests that the specific cellular and soluble biomarkers at the site of infection can be used to determine the infecting pathogen and even the clinical outcome. This has been applied first to PD-

related peritonitis due to the ease of accessing samples at the specific site of infection.[122] It has been shown that the immune fingerprint of an acute PD-related peritonitis episode can be used to distinguish between culture negative, Gram positive and Gram negative episodes, as well as those that lead to technique failure and those caused by specific microbes including CNS, *Streptococcus* and *Enterococcus*, and non-*Streptococcus* Gram positive organisms (as seen in Figure 1.1).[122,219,222,223] The use of biomarkers in PD effluent is expanding to include those for inflammation, membrane function, as well as infection, and this exciting field is expanding to develop biomarkers that will be clinically useful in point-of-care tests to be used at the bedside to aid decision making about treatments.[224]

1.4   microRNAs

microRNAs (miRNAs) are small, highly conserved non-coding RNA molecules that are post-transcriptional regulators of gene expression.[225] They are relatively novel gene regulators, with the first miRNA being discovered in 1993, in *Caenorhabditis elegans*.[226] They are now known to be very important in a wide range of cellular processes, are predicted to regulate as much as 60% of human protein-coding genes, and are involved in the pathogenesis of many different diseases.[225,227,228] These molecular regulators are key biomarkers in a range of different diseases and are a key focus of this thesis, which investigates their role in the acute response to PD-related peritonitis episodes.

1.4.1 First miRNAs

The first miRNA discovered was Lin-4 in the nematode *Caenorhabditis elegans*. The *lin-4* gene is an important negative regulator of Lin-14 but does not code for a protein. The *lin-4* gene gives two small transcripts of approximately 22 and 61 nucleotides, which had antisense complementarity to the 3' un-translated region (UTR) of *lin-14*. *lin-4* was predicted to be a novel class of regulatory gene that encodes small RNA molecules that can target the 3' UTR of specific targets. Lin-4 and Lin-14 are important in the developmental pathway, controlling temporal regulation of larval development, so it was initially proposed that these novel small RNA regulators are important in controlling development, termed small temporal RNAs (stRNAs).[226,229] Small regulatory RNA molecules were then discovered in a range of different vertebrates and invertebrates, when they were called microRNAs due to their small size.[230–232] Nearly all of the miRNAs identified in *C. elegans* appeared to have orthologs in other species, suggesting they are highly conserved regulatory elements

No growth:

8 – IL-1β
9 – Total cell count
10 – MMP-8
11 – CD14+ cells
12 – CCL4

All Gram-positives:

2 – IL-12p40
6 – IL-17A
7 – IFN-γ
8 – IL-1β
9 – Total cell count

Technique failure:

10 – MMP-8
16 – sIL-6R
20 – Calprotectin
21 – CD4:CD8
22 – TGF-β

Gram-negatives:

1 – Vδ2+ T-cells
2 – IL-12p40
3 – Vγ9+ T-cells
4 – VEGF
5 – TNF-α

Non-streptococcal Gram-positives:

6 – IL-17A
7 – IFN-γ
9 – Total cell count
13 – IL-15
14 – CCL3

Coagulase-negative Staphylococcus:

9 – Total cell count
13 – IL-15
15 – IL-16
16 – sIL-6R
17 – MMP substrate

Streptococcus, Enterococcus:

8 – IL-1β
13 – IL-15
17 – MMP substrate
18 – TNF-β
19 – Zymography

Figure 1.1: Immune fingerprint summary
Diagram showing the pathogen-specific immune responses to distinguish between different infections and clinical outcomes in PD-related peritonitis episodes. Taken from[222].

and have broad regulatory functions in many different organisms.[231,232]

1.4.2 miRBase

From this initial discovery of a few small RNA molecules that could regulate *C. elegans* development, there are now a large number of miRNAs across many different organisms. miRBase is an online repository for published miRNA sequences and associated annotation that is regularly updated to list all known and predicted miRNAs. The first version was published in 2002, with 218 entries. The most recent version (v22) was published in 2018 with 38,589 entries across 271 organisms, including 2,654 mature miRNAs predicted to be present in the human genome.[233–236] The two main aims of miRBase were to enable novel miRNAs to be assigned names without accidental overlap of gene names, and to generate a comprehensive and searchable database of all published miRNA sequences.[235] miRBase specified the accepted nomenclature for novel miRNAs (described below) and defined the relationships between closely related miRNAs across different species and distinct genetic loci in the same species. This database not only includes the agreed name for new miRNAs and the published sequences, it also includes details about the miRNA including genetic locus, homologous sequences, hairpin structure and sequence, links to literature that mention the miRNA, deep sequencing reads and crosslinks to other databases where information about the miRNA can be gathered (e.g. EntrezGene).[233–235] miRNAs are now known to have functions in almost all normal cellular processes, from development through to apoptosis, as well as in many different pathologies, including cancers.[225,237,238]

1.4.3 Nomenclature

Once miRNAs began to be discovered at a rapid rate it became important to have a system to name them consistently. miRNAs are given numerical identifiers based on their sequence similarity to others that have already been discovered, for example in other organisms. If no such sequence similarity exists, miRNAs are named sequentially i.e. if the last miRNA to be identified was miR-100, then the next novel miRNA will be miR-101. If a novel miRNA shares the same mature 22 nucleotide sequence as another miRNA in another organism, it is given the same name but with the species identifier as a prefix (i.e. hsa-miR-21 for the human miRNA and mmu-miR-21 for the mouse ortholog). If the new miRNA has the same sequence as an already identified miRNA from a different genetic locus in the same organism it is given a numerical suffix, i.e. miR-6-1 and miR-6-2. miRNAs that only

differ by one or two bases are given suffixes to identify them as such; miR-181a and miR-181b. As mature miRNAs are excised from a hairpin double stranded RNA structure, it is possible to be either from the 5' or 3' arm. There is normally one arm that is dominantly expressed, with the less predominant form named with an asterisk i.e. miR-34 and miR-34*. miRNAs can also be identified by a suffix showing which arm of the hairpin they originate from, i.e. miR-49-5p and miR-49-3p.[233,235,239]

1.4.4 Biogenesis

When miRNAs were initially discovered in *C. elegans*, there were two forms of Lin-4, a shorter 22 nucleotide form (which we now know to be the functional mature miRNA) and a longer 61 nucleotide form.[226] This longer form is the hairpin indicative of a miRNA before it has been processed to the mature form. To get from the gene itself to the mature miRNA form is a multistep process as described below and shown in Figure 1.2.

1.4.4.1 Transcription to pri-miRNA

miRNAs can be located anywhere in the genome, in exonic, intronic and intergenic locations. miRNAs are often located close to other miRNAs to form polycistronic transcriptional units, or clusters, which are generally co-transcribed (although the individual miRNAs can be subject to additional post-transcriptional regulation). If a miRNA is in an intronic region, it can share a promoter region with the host gene, although they can have distinct promoters.[240,241]

miRNA genes are generally transcribed by RNA Polymerase II (Pol II), and this is controlled by a range of RNA Pol II-associated transcription factors and epigenetic regulators. Some miRNAs can also be transcribed by RNA Pol III, but this is unusual. The long primary transcript, typically over 1kb, contains a local stem-loop structure. A typical pri-miRNA consists of a stem loop of 33-35 bp, a terminal loop structure, and single stranded sections at both the 3' and 5' ends.[240,241]

1.4.4.2 Cleavage to pre-miRNA

Whilst still in the nucleus, the pri-miRNA is subject to cleavage to generate a pre-miRNA. This is conducted by an RNase III enzyme called Drosha, which forms the microprocessor complex with DiGeorge syndrome Critical Region 8 protein (DGCR8), an RNA binding
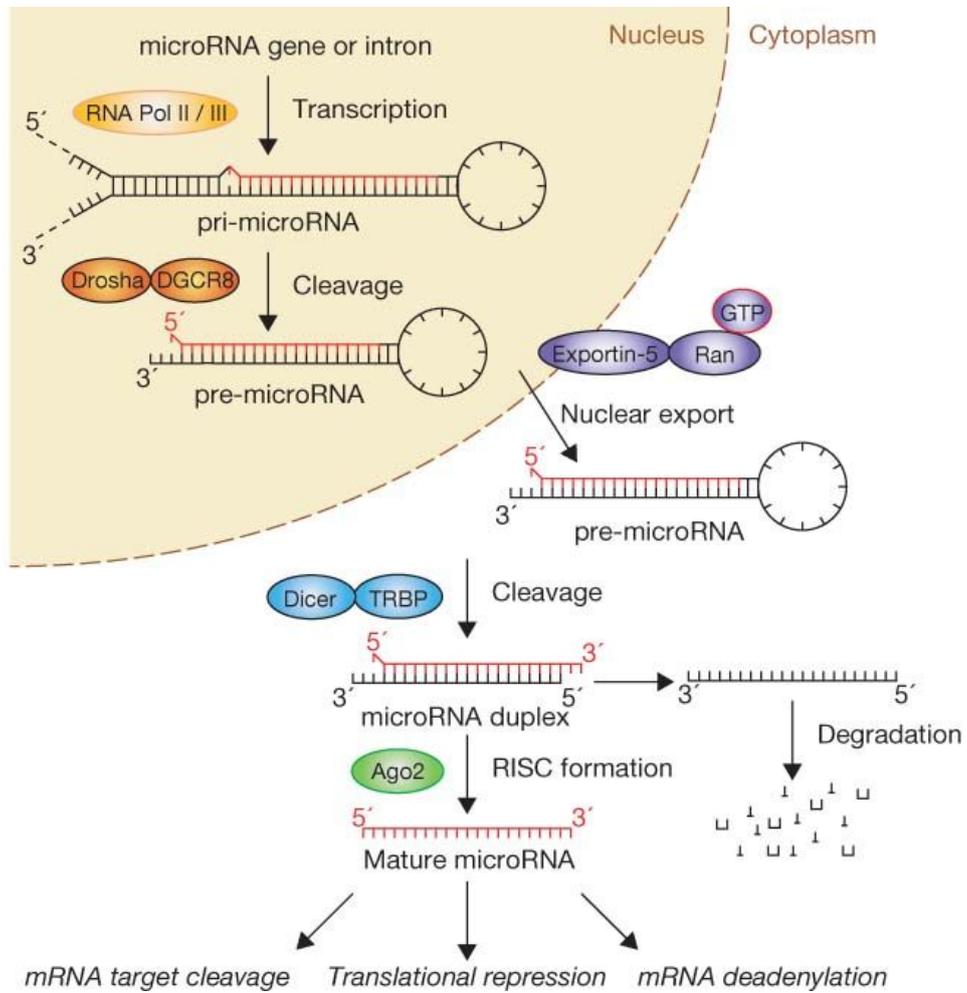
Figure 1.2: miRNA biogenesis and function
Showing the process of miRNA biogenesis from transcription to function in target
regulation, taken from[242].

protein that interacts with the C terminus of Drosha to stabilise it. The endonuclease activity of Drosha acts approximately 11 bp away from the hairpin stem-loop section of the pri-miRNA (called the SD junction) to release a hairpin structure of approximately 65 nucleotides consisting of an approximately 22 nucleotide-long stem loop and a terminal loop of approximately 48 nucleotides long. Drosha also leaves a small single stranded overhang on the 3' end of approximately 2 nucleotides. Drosha and DGCR8 can both regulate each other to control the efficiency of pri-miRNA cleavage. Cleavage can also be regulated by a range of post-translational modifications mostly altering protein stability, location and processing activity.[240,241]

1.4.4.3 Nuclear export

To complete miRNA maturation, the pre-miRNA must be exported out of the nucleus into the cytoplasm. To do this, a complex is formed between the pre-miRNA, exportin 5 (EXP5), RAN (a nuclear GTP-binding protein) and guanosine tri-phosphate (GTP). Upon translocation through the pore, GTP is hydrolysed, which initiates complex disassembly and the pre-miRNA is released into the cytosol. EXP5 also protects the pre-miRNA from degradation in the nucleus. The regulation of this export is not particularly well known, especially with regard to cofactors that may influence this pathway.[240,241]

1.4.4.4 Cleavage to miRNA duplex

Once the pre-miRNA is exported into the cytoplasm, it is subject to further cleavage by another RNase III endonuclease called Dicer. Dicer binds to and cleaves the double stranded pre-miRNA 21-25 nucleotides from the 3' overhang that is left by Drosha, to leave a small RNA duplex consisting of the miRNA guide strand bound to the miRNA passenger strand (miRNA:miRNA*). The process of Dicer cleavage is regulated by a range of cofactors, most notably by double stranded RNA binding proteins (dsRBPs) that interact with the endonuclease, for example TAR RNA-binding protein (TRBP) in human miRNA processing.[240,241]

1.4.4.5 RNA-induced silencing complex formation

miRNAs regulate genes by directing the RNA-induced silencing complex (RISC) towards specific target messenger RNA molecules (mRNAs). This is formed by two steps: miRNA duplex loading and subsequent unwinding.[240]

The miRNA duplex formed after Dicer cleavage is loaded onto an Argonaute (AGO) protein by the 5' end being anchored to the 5'-phosphate-binding pocket. The miRNA threads along the basic channel in the AGO protein until it reaches the PAZ domain (named after the PIWI, AGO and Zwille proteins in which it is conserved), which then binds the 3' end of the miRNA. The GW182 protein also binds to the AGO protein to promote its stability. Once in position, the seed sequence of the miRNA is arranged into a helix to allow for efficient scanning of target mRNAs for complementary sequences. This whole loading process requires energy input and is therefore adenosine tri-phosphate (ATP)-dependent. The most common AGO protein in humans is AGO2, although all four AGO proteins (AGO1-4) can post-transcriptionally regulate genes in the same way.[240,241]

Once the miRNA duplex is loaded onto the AGO proteins, this is called the pre-RISC. To generate a mature RISC, the passenger miRNA strand must be removed. This can be conducted by the AGO protein itself (e.g. by AGO2 in humans), facilitated by the endonuclease C3PO. If there is a mismatch in the duplex in the middle, or the AGO protein is unable to slice open the duplex (as AGO1, AGO3 and AGO4 in humans are unable to do), the duplex may unwind without cleavage. This is promoted by mismatches at positions 2-8 and 12-15. Passenger strand release is an ATP independent process.[240,241]

To select which strand of the duplex is the passenger and which is the guide strand depends on the thermodynamic stability of the different strands in the loading process. The strand with the more unstable 5' terminus is normally selected as the guide strand, followed by rapid degradation of the passenger strand. As strand selection is not a completely strict process, the strand that is not favoured (miRNA*) is still loaded and used in the RISC, just at a lower frequency. This whole process is regulated by a range of post-translational modifications on AGO that control its activity and stability.[240,241]

1.4.5 Functions

Once the mature RISC complex is formed, miRNAs regulate specific mRNA targets by binding to complementary regions in the target mRNA and regulating its translation by direct target cleavage, translational repression or mRNA deadenylation.

1.4.5.1 Target recognition

miRNAs specifically target genes due to complementarity between the miRNA (normally the seed sequence) and the target mRNA (often the 3' UTR). The seed sequence of a miRNA is a small, approximately 8 nucleotide section near the 5' end of a mature miRNA. This region is most important to determine the specificity of binding and is therefore presented when the miRNA is loaded into RISC to allow for efficient scanning of potential targets. This region is often used to predict potential mRNA targets for miRNAs through target prediction algorithms such as Diana, miRDB, miRanda and TargetScan. Some miRNAs target other sections of mRNAs including the coding sequence, not just the 3' UTR. It is possible for the rest of the miRNA to be important for target recognition, not merely the seed region, however, the majority of miRNAs target the 3' UTR of mRNAs by binding with the seed region. Binding to this 8-nucleotide region is important, but it is not necessary to have perfect Watson-Crick complementarity across all eight nucleotides, with one mismatch still allowing sufficient targeting to regulate a target. It is common for multiple miRNA target sites to be present in one 3' UTR of a gene, which increases the likelihood that the miRNA will repress the target. This complementary binding brings the RISC towards an mRNA to promote repression through multiple methods.[243,244]

1.4.5.2 Target regulation

miRNAs silence gene expression by repressing translation and accelerating mRNA degradation of targets in the cytoplasm. mRNA targets that are perfectly complementary to the miRNA in the RISC are cleaved directly by catalytically active AGO proteins. This is only possible with AGO2 in humans, therefore silencing of targets also occurs through a combination of translational repression, deadenylation, decapping and mRNA degradation, which requires the AGO protein to recruit cofactors.[244–247]

mRNA degradation is a widespread process and accounts for the majority (66-90%) of miRNA-mediated target repression at steady state. This degradation occurs via the 5'-to-3' mRNA decay pathway. Initially mRNAs are deadenylated by the poly(A) specific ribonuclease subunit complex PAN2-PAN3 and Carbon catabolite repressor 4- negative on TATA complex (CCR4-NOT) deadenylase complexes via GW182 (trinucleotide repeat containing adaptor 6A), which interacts with the deadenylase complexes and the AGO proteins. The mRNA is then decapped by the decapping protein 2 (DCP2), along with other required cofactors. The final step is degradation by the cytoplasmic nuclease 5'-to-3'

exoribonuclease 1 (XRN1). Consecutive steps in this pathway are coupled through direct interactions between subunits of the catalytic complexes, which helps prevent accumulation of intermediates.[244–248]

Repression of translation occurs at an early time point, but normally contributes a smaller proportion of effective target regulation than accelerating mRNA degradation (6-26%). However, this is an important method of regulating target gene expression, as demonstrated by repression of artificial mRNA reporters that lack a poly(A) tail and are therefore resistant to deadenylation.[245–248]

The exact molecular mechanism of translation repression has not been elucidated, but it is thought to be by inhibition of cap-dependent translation at initiation. It is possible that mRNA decay can be linked to repression of translation, due to the earlier time point of this process starting. This has not been proven as a causal link and there is some evidence disputing that repression leads to decay, but it is one possibility.[245,247,248]

The current model of translation repression involves RNA helicases, either directly or via cofactor recruitment. For example, some studies show that the RISC interacts with ribosome scanning by interfering with translation initiation factors such as eIF4A. This RNA helicase functions by unwinding secondary structures within the 5' UTR to allow the pre-initiation complexes to scan it and find the start codon to initiate translation. The molecular mechanism of preventing this is unclear, but the assembly and/or function of the pre-initiation complex is crucial for translation repression, possibly via cofactors such as DDX6.[244–246,248]

Other proposed methods of translation repression include repression of the 60S ribosomal subunit joining, blocking elongation once translation has initiated, causing ribosomes to drop-off, or direct degradation of the nascent polypeptide. There is experimental evidence for different mechanisms of translation repression but it is yet to be elucidated which one, if any, is the dominant one, with the potential for redundancy and plasticity in the mechanism of translation repression potentially an important factor in responding to different situations.[244–246,248]

1.4.5.3 Normal cellular processes

miRNAs regulate the majority of genes and are hence important in many different cellular pathways. Discussed below are some examples of their function in development, apoptosis and metabolism, as a demonstration of their ubiquitous role in regulating all aspects of normal cellular processes.

1.4.5.3.1 Development

The first miRNA discovered (lin-4) was identified as important in regulating temporal development in *C. elegans*, with other miRNAs subsequently identified that have similar functions classified as stRNAs.[226,229] Since this discovery, there has been much work looking into the role of miRNAs in development. Animals that cannot express miRNAs (i.e. Dicer knockout) do not survive or reproduce normally.[238,249–251] Their role in development and the cell cycle of embryonic stem cells has been recognised in many ways, including the identification of a group of embryonic stem cell-specific miRNAs, the miR-290-295 and miR-302 clusters, which can promote the cell cycle of embryonic stem cells and are not detectable in adult cells or organs. The role of these in maintaining pluripotency is antagonised by another miRNA, let-7, which is thought to stabilise the differentiated state.[250–253] Other individual miRNAs have an effect on embryonic stem cell differentiation.[251] For example, miR-1 and miR-133 regulate differentiation to cardiac muscle cells,[254,255] miR-134 induces pre-embryonic stem cell maturation[256] and miR-145 induces lineage-restricted differentiation.[257]

1.4.5.3.2 Apoptosis

The majority of published work on miRNAs has been conducted into cancers, therefore the role of miRNAs in relation to apoptosis is well known due to the common dysregulation of this process in tumorigenesis. Many of the important proteins in both the intrinsic and extrinsic apoptotic pathways are known to be direct and functional targets of miRNAs, and this has a functional effect in pathogenesis. For example, miR-125b can regulate the pro-apoptotic Bak protein to confer resistance of breast cancer to paclitaxel.[258,259] The anti-apoptotic protein Bcl-2 is regulated by numerous miRNAs including miR-15a, miR-16-1, miR-24-2, miR-148a and miR-204, in different cancers such as leukaemia, gastric cancer, colorectal cancer and breast cancer.[259–263] miRNAs also target important proteins in the extrinsic apoptotic pathway, including Fas ligand (FasL) which is directly targeted by miR-21 and miR-590, and Fas which is regulated by miR-146a and miR-196b. These interactions

are important in pancreatic cancer, leukaemia, osteosarcoma and autoimmune disease.[259,264–267] The role of miRNAs in apoptosis and other cell death pathways is substantial, with direct targets implicated at all stages from initiation through to effectors, meaning miRNAs may have important roles in human pathologies, considering the importance of apoptosis in many different diseases.[259]

1.4.5.3.3 Metabolism

miRNAs are not only involved in the development of tissues and cells required for energy production, utilisation and storage, but they also have roles in the regulation of insulin release, amino acid and lipid metabolism, as well as energy homeostasis.[268] For example, the islet-specific miR-375 is a negative regulator of insulin release via direct targeting of Myotrophin.[268,269] miR-29b is linked to lipid metabolism through control of branched chain amino acid catabolism by targeting branched chain α-ketoacid dehydrogenase.[268,270] Lipid energy storage and cellular lipid levels can also be altered by miRNAs, via the liver-specific miR-122[268,271,272] and regulation of cellular acetyl-CoA levels by miR-103 and miR-107.[268,272]

1.4.5.4 Diseases

miRNAs have been implicated in many different human pathologies, with the majority of published research conducted into cancers. However, they are known to have roles in many different diseases including cardiovascular disease, infectious diseases and autoimmune diseases.[250]

1.4.5.4.1 Cancers

miRNAs were first associated with cancer in 2002, when the miR-15a/16-1 cluster was identified as a tumour suppressor due to its frequent deletion in chronic lymphocytic leukaemia.[273] Since then, many miRNAs have been identified as either tumour suppressors or oncogenic (called oncomiRs) across a wide range of cancers, with one study suggesting that over half of all known miRNAs (at the time) were frequently altered in cancer.[274] The importance of miRNAs in cancer is further emphasised by the effect of a loss of Drosha or Dicer. A decrease in Dicer and Drosha mRNA was prevalent in 60% and 51% of ovarian cancer patients respectively.[275] Specific miRNAs can be involved in the pathogenesis of cancers. For example, miR-21 is a well-known oncomiR in multiple cancers including lung, oesophagus and colon cancers.[276] miR-34, on the other hand, is a major tumour suppressor in multiple cancers including colon and ovary.[277]

miRNAs can also be involved in the resistance of a tumour to therapy, e.g. chemotherapy.[278] This can be by one of five different methods; metabolism of the drug (e.g. miR-27b),[279,280] drug transport (e.g. miR-145),[281] DNA repair (e.g. miR-96),[278,282] epithelial-to-mesenchymal transition (e.g. miR-200c)[283] or regulation of cancer stem cells (e.g. miR-145).[278,284] miRNAs can also be biomarkers of cancer progression and prognosis, either in the tumour itself or in easily accessible bodily fluids, for prognosis prediction, disease classification or prediction of response to treatment, and miRNA are very good non-invasive biomarkers.[285] The first use of miRNAs as biomarkers was in chronic lymphocytic leukaemia, where a miRNA signature was defined to stratify prognosis and progression.[286] Since then, techniques have allowed more accurate screening for biomarkers, and many different miRNAs have been associated with cancer prognosis and progression. For example, microarray expression profiling has been used to identify panels of miRNAs that are significantly altered in pancreatic cancer, specifically those associated with reduced survival.[287] To classify the disease stage or tissue origin can be useful to guide treatment options, for example, subtypes of glioblastoma can be defined by miRNA signatures.[288] Predicting drug efficiency is especially useful to help clinical decisions, for example, miR-125b can be used to predict resistance of breast cancer to paclitaxel if measured before treatment.[258] As mentioned, miRNAs can be great non-invasive biomarkers for cancer, and this will be discussed later on in the context of all human diseases.

1.4.5.4.2 Cardiovascular disease

miRNAs have been identified in different pathologies related to the heart. For example, decreased miR-24, miR-29 and miR-320 (the miR-29 family) have been associated with myocardial infarction via apoptosis and fibrosis.[289–292] miRNAs have also been associated with heart failure (miR-765),[293] arrhythmias (irregular heartbeat, miR-1 and miR-133)[289,294] and hypertension (miR-155).[295,296] The role of miRNAs in cardiovascular pathologies has been well studied, showing how the molecular pathways, such as apoptosis, can have important roles in human pathologies.[289]

1.4.5.4.3 Autoimmune diseases

The role of miRNAs in immune homeostasis means they have been implicated in autoimmune disease pathogenesis.[297] For example, the establishment and maintenance of

immune tolerance is controlled by miRNAs, including miR-181 in T cells[298] and miR-185 in B cells.[297,299] One of the miRNAs that has been implicated in many different roles in the regulation of the immune system, and therefore in autoimmunity, is miR-155.[300] This miRNA is a critical modulator of immune development and responses, as it is not just a downstream member of inflammatory cascades[301] but also influences T and B cell differentiation towards an inflammatory phenotype.[302] Raised levels of miR-155 have been observed in a range of autoimmune conditions including multiple sclerosis,[303] colitis[304] and systemic lupus erythematosus.[305]

Other miRNAs that have been implicated in autoimmune pathologies include miR-21,[297] which alters the balance of T helper cells towards pro-inflammatory Th17 cells[306] and increases in APCs in response to TLR4 signalling[307] in multiple autoimmune diseases including lupus,[308] psoriasis[309] and inflammatory bowel disease.[310] miR-146a is also strongly involved in autoimmunity pathogenesis through induction of tolerance, specifically to TLR signalling,[297,311] and its broad role for controlling adaptive immune responses.[297,312,313] miR-146a has been identified as either an important biomarker or key in the pathogenesis of type 1 diabetes,[314] lupus[315] and other autoimmune disorders.[297,316]

1.4.5.4.4   Peritoneal membrane dysfunction

miRNAs have been implicated in the dysfunction of the peritoneal membrane, through fibrosis and EMT/MMT. For example, TGF-β-induced fibrosis or EMT alters a range of miRNAs including miR-15a, miR-21, miR-29b, miR-30a, miR-129 and miR-589.[317–322] Whilst high glucose/GDP-induced fibrosis in peritoneal membrane mesothelial cells alters levels of miR-15a, miR-30b, miR-34a, miR-193a and let-7e.[323–325] PD fluid itself induces alterations in miRNA levels, both in the peritoneal membrane, and released into the PD effluent, as shown by animal models and clinical studies of patients who have experienced a degree of fibrosis or EMT, for example miR-21 and miR-182.[326–329] These studies indicate miRNAs are sensitive to a range of stimuli and are altered in fibrosis and inflammation.

1.4.5.4.5   Infectious diseases

miRNAs can be involved in infectious diseases in three ways: modulating the pathogenicity of individual pathogens; affecting the efficiency of host innate and adaptive immune responses; and altering the magnitude and resolution of inflammatory responses.[250]

1.4.5.4.5.1 Viral infections

Viral infection can lead to miRNA alterations in host cells, which can be an advantage for the virus to allow replication, either by regulating antiviral mechanisms, viral latency or lytic cycles, as well as initiate defence mechanisms.[250] For example, the functions of miR-155 appear to be hijacked to promote Epstein-Barr Virus (EBV) latency.[330] A panel of miRNAs altered in EBV latency have been identified, which includes up-regulated miR-21 and miR-34a, and down-regulated miR-96 and miR-128a/b.[331] miRNAs can also be potential therapeutic targets, for example miR-122 is essential for Hepatitis C Virus (HCV) replication, therefore treatments that target miR-122 are a potential therapeutic option (e.g. locked nucleic acid-modified antagomirs).[332] Viruses themselves have been known to produce their own miRNAs (v-miRNAs) that can regulate host cell proteins to create an environment that allows viral replication, for example in EBV.[333] Due to the need for viruses to hijack host cell machinery to replicate, it is likely that the alterations in miRNAs are substantial, with only a small proportion of identified changes mentioned here.

1.4.5.4.5.2 Bacterial infections

Bacterial infections, both intracellular and extracellular pathogens, often lead to alterations in miRNAs, especially in cells involved in the immune response. For example, *M. tuberculosis* infected DCs and macrophages have a higher level of miR-99b, and this is a potential therapeutic target.[334] The miRNA alterations from *Mycobacterium* infections also cover leprosy (*M. leprae*), where infected monocytes have increased miR-21 levels, which causes a decreased IL-1β and increased IL-10 response. This is potentially used to escape the vitamin D-dependent antimicrobial pathway, and therefore advance from the self-limiting to progressive form of leprosy.[335] Gastritis patients with *Helicobacter pylori* infections have higher levels of miR-155 and miR-146a in gastric lesions.[336] Also, the levels of these miRNAs are altered in sepsis, which results from bacterial infections; miR-155 is increased and miR-146a decreased in plasma from severe sepsis patients.[250,316,337] Bacteria do not express miRNAs, as viruses can, but they do release small RNAs (sRNAs) that may function to regulate gene expression in the bacteria by complementary base-pairing, akin to eukaryotic miRNAs. These bacterial RNA molecules are larger than miRNAs (approximately 50-450 nucleotides) and have roles in numerous processes including metabolism, biofilm formation and host-cell contact. This field of research is growing rapidly, with novel mechanisms of action and physiological functions being discovered constantly.[338–340]

1.4.5.4.6 Cross-species regulation

Currently, it is thought that the miRNAs and sRNAs produced by infecting organisms are only able to act within the organism itself, but there is some evidence that small RNA molecules can be transferred to the host. For example, ingestion of rice has been shown to result in rice-derived miRNAs being present in human and animal sera, with these miRNAs able to regulate target genes functionally.[341,342] This has not yet been applied to infectious diseases, but the possibility that small regulatory RNA molecules are used by infecting bacteria and viruses to modulate host targets is very real.

1.4.5.5 Immune responses

As shown above, miRNAs are involved in many different aspects of immune responses and have been implicated in associated pathologies. Below will be a more detailed discussion of what is known about miRNAs in relation to innate and adaptive immunity, with specific focus on the inflammatory acute response. miRNAs have been identified as key regulators of haematopoiesis,[343] but this is beyond the scope of this thesis so will not be discussed here.

miRNAs can act as post transcriptional regulators of genes involved in immune responses by three principles. Firstly, miRNAs can respond quicker to stimuli than other regulators, such as proteins, due to the fact that they do not require translation to act on their targets, as they regulate targets at an earlier stage. The second principle relies on the fact that miRNAs take longer to act when their target is already in the protein form, i.e. they have to wait for new mRNA to be transcribed before they can have an effect. The third principle is that they do not cause a complete knockdown of the target, so probably 'fine-tune' the response to a stimulus. These three principles mean that miRNAs are important in all aspects of the immune response, both innate and adaptive, in the acute and chronic responses.[344]

1.4.5.5.1 Innate immune responses

miRNAs have roles in many different aspects of the innate immune system, including in the inflammatory response, in phagocytes, inflammatory cells and NK cells.

1.4.5.5.1.1 Inflammation

One of the first identifications of miRNA involvement in immune responses was that certain miRNAs can be induced by inflammatory stimuli, with several key miRNAs identified including miR-155 and miR-146a. These two miRNAs have contrasting roles in inflammatory pathways, with miR-155 seen as a "pro-inflammatory" miRNA and miR-146a as a regulator of immune responses.[344–346] miR-155 can be induced by inflammatory stimuli in monocytes, macrophages and DCs by a range of different stimuli, activating multiple pro-inflammatory pathways including via NF-κB.[301,346–350] miR-146a is also induced by different inflammatory stimuli including multiple TLRs, microbial components and pro-inflammatory cytokines, in a range of different cell types, but it controls the inflammatory response by suppressing the NF-κB pathway.[301,346,348,349,351–353] Other miRNAs are also altered after inflammatory stimuli, e.g. miR-125, with varying roles for miRNAs to both promote and dampen down inflammation dependent on the stimulus, cell type and local microenvironment.[344,354]

1.4.5.5.1.2 Neutrophils

Neutrophils are the most abundant type of immune cells in the body and respond rapidly to infectious and inflammatory stimuli. They accumulate locally as the first responders to an infection or inflammation at the site of the insult, for instance they are highly abundant in the peritoneal effluent of patients with peritoneal dialysis-related peritonitis. miRNAs have been shown to be important in many different neutrophil functions including their migration along a chemoattractant gradient, senescence, cytokine expression, responsiveness to infectious or inflammatory challenge, and differentiation.[355–357] For example, miR-451 targets p38 mitogen-activated protein kinase (MAPK) to influence the migration of neutrophils towards an inflammatory stimulus.[356,358] miR-223 is important in both the differentiation of neutrophils and in their expression of ROS.[355,356,359] miR-1959 is important in the production of antimicrobial peptides,[356,360] and other miRNAs are crucial in the expression of chemokine (C-X-C motif) ligand 8 (CXCL8) (miR-17 and miR-155)[355,361,362] and IFN-γ (including miR-142 and miR-29).[355,356,363,364] The close regulation of all aspects of neutrophil biology shows how important miRNAs are in the functions of these phagocytes.

1.4.5.5.1.3 Other innate immune cells

In addition to neutrophils, other immune cells involved in the innate immune response are similarly regulated by miRNAs. For example, miR-221 and miR-222 regulate both the resting and activated states of mast cells, with roles in the cell cycle, cytoskeleton organisation, degranulation, cytokine production and cell adherence.[365–367] The production of IFN-γ by NK cells requires both miR-155 and miR-150,[368–370] with other functions also reliant on miRNAs, including differentiation, cytotoxicity and secretion of other proinflammatory cytokines.[371]

1.4.5.5.2 Adaptive immune responses

miRNAs are involved in the normal cellular function of cells involved in the adaptive immune system through affecting their differentiation and function in a variety of ways.[372]

1.4.5.5.2.1 T cells

T cell differentiation is regulated by different miRNAs, including miR-155 which controls the lineage decisions towards different T helper cell subsets, promoting Th17 and Th1 generation,[302] while also maintaining Treg cell proliferation and homeostasis.[373] Treg cell function is also regulated by miR-146.[372,374] The role of miRNAs in different aspects of T helper cells is well defined, with their expansion being regulated by miR-182,[375] the Th2 response promoted by miR-126 in inflammation,[376] and miR-326 promotes Th17 differentiation.[377] All this shows that miRNAs are important in modulating the direction and magnitude of immune responses by different T cell subsets.[372]

1.4.5.5.2.2 B cells

The role of miR-155 in the immune system is well documented, with a proposed role for this miRNA in different aspects of B cell function,[378] including B cell activation through the germinal centre response and immunoglobulin G (IgG) class-switching.[372,379,380] miR-181b also regulates this class-switching reaction.[380,381] miRNAs have also been implicated in B cell apoptosis, including miR-125b,[379,382] and in controlling immunoregulatory B cells to prevent autoantibody formation, via miR-15a.[383,384] The role of miRNAs in B cell function is not well defined, but it is likely they are important in many other functions of these immune cells, as they are crucial in most normal cellular processes.

1.4.6 miRNAs as biomarkers

The definition of a biomarker, as decided by the Biomarkers Definitions Working Group in 2001, is a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[385,386] Through this definition, miRNAs have been identified as biomarkers through associations with a range of diseases, through increased levels in biopsies, for example in multiple solid cancers.[285] However, miRNAs offer the added bonus of potential as biomarkers in biological fluids due to their stability outside cells. This allows non-invasive tests to detect the levels of specific miRNAs as biomarkers in a range of diseases.[387–389]

1.4.6.1 Cell-free miRNAs

miRNAs have been detected in almost all biological fluids, ranging from those that require no invasive sampling procedures, including saliva and urine, to those that require more invasive procedures to acquire, including whole blood, plasma and serum.[388,389] These cell-free miRNAs have been associated with pathologies such as cancers,[390–392] diabetes,[393] infectious diseases[394] and cardiovascular diseases,[395] amongst many others.[387–389,396]

1.4.6.1.1 Release from cells

miRNAs are produced and act in the cytoplasm of cells, but they have been observed as stable biomarkers extracellularly therefore it is important to understand if this is an active process that has an intercellular communication function. It has been shown that miRNAs are stable and can withstand prolonged storage, multiple freeze-thaw cycles and incubation at room temperature. They are also resistant to RNase treatment, compared to other endogenous RNA species or "spiked-in" artificial miRNAs, which helps their function of communicating between cells without being degraded by ribonucleases that are present in normal biological fluids.[389,396–399] All of this suggests that extracellular miRNAs are protected from degradation, with the most likely protection either being associated with proteins, lipids/lipoproteins, or being encompassed in extracellular vesicles such as exosomes, microvesicles or apoptotic bodies.[389] While the nomenclature of extracellular vesicles is rapidly changing, a consistent characterisation of vesicles on size and mechanism of release from cells has been used here to attempt to discuss the current level of knowledge in this area. The formation of all three types of extracellular vesicles is discussed below and detailed in Figure 1.3.
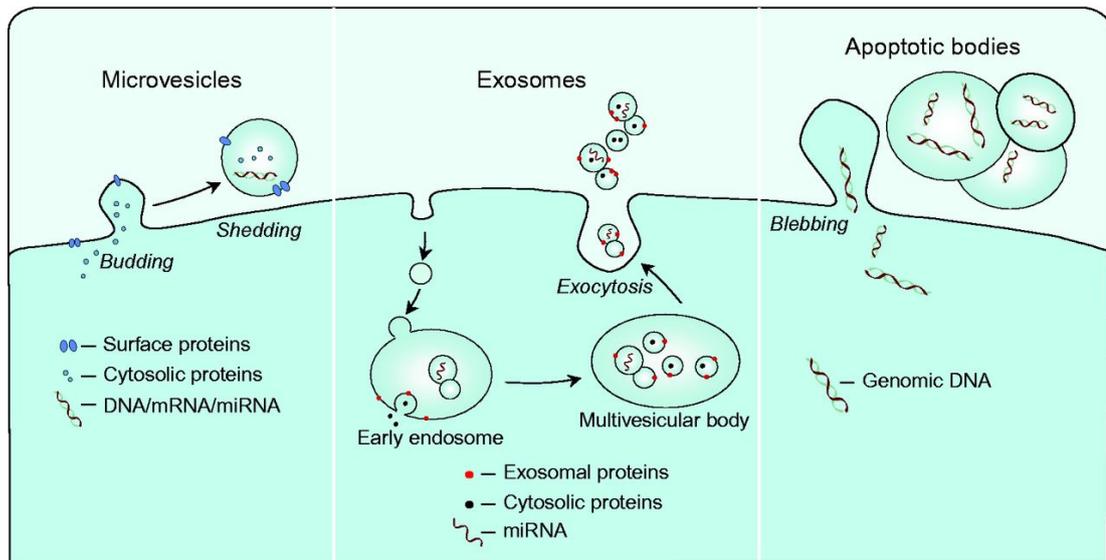
Figure 1.3: Extracellular vesicle formation
Showing how microvesicles, exosomes and apoptotic bodies are formed and released from cells, adapted from[400].

1.4.6.1.1.1 Exosomes

Exosomes are possibly the best known class of extracellular vesicles. These are small membrane vesicles (30-120 nm) released by the fusion of multivesicular bodies with the cell membrane, releasing the exosomes into the extracellular space.[389,401] They were first discovered in 1983 as small vesicles in which the transferrin receptor localised after being released from mature sheep reticulocytes.[402] Exosomes are mainly composed of lipids and proteins, with an enrichment of lipid rafts,[403] but they also contain a range of molecules, including nucleic acids such as miRNAs, that are functional and can be used to signal with and modulate recipient cells.[403–405] It is now known that the packaging of miRNAs (and other signalling components such as mRNAs, other non-coding RNA molecules (ncRNAs) and proteins) is through an active process that selects specific molecules to package into the vesicles to be released, with a number of molecular mechanisms.[406]

Exosomes are important for intercellular communication through transfer of contents, including miRNAs, between cells. Transfer of miRNAs is important due to their implication in many different cellular processes and pathologies. For example, miRNAs can be released by T cells and taken up by APCs in a functional manner during the formation of an immune synapse.[407] This type of intercellular communication is functional in a pathological state. For example, miRNA-containing exosomes released by normal bone-marrow mesenchymal stem cells inhibit the growth of multiple myeloma, whilst those from multiple myeloma patients have lower quantities of the tumour suppressor miR-15a, and therefore promote the disease progression.[408] Transfer of miRNAs between cells via exosomes has also been demonstrated in different forms of cancer and cancer cell lines,[405] as well as in heart disease,[409] lung injury or disease,[410,411] and other disorders.[387,389]

1.4.6.1.1.2 Microvesicles

Microvesicles are larger extracellular vesicles (100-1000 nm) released by the direct budding of cytoplasmic protrusions from the cell surface membrane,[401,412] meaning their lipid content and surface markers are different to exosomes, and much the same as the cellular membrane from which they originate.[389,413] When microvesicles were first identified from platelets, they were thought to be cellular debris,[414] but it is now known they have important functions in signalling between cells and altering recipient cell phenotypes.[415]

Microvesicles are similar to exosomes in that they contain a wide range of molecules for intercellular signalling, including miRNAs,[415] which are selectively packaged into the vesicles, as with exosomes.[416] The use of microvesicles to signal between cells, specifically through transfer of miRNAs has been observed in many different pathological conditions. For example, microvesicles from platelets are implicated in miRNA transfer in myocardial infarction,[417] atherosclerosis[418] and lung cancer.[419] Microvesicles containing miRNAs have also been identified in different types of cancer (e.g. glioblastoma),[416,420] and in other disease states including atherosclerosis,[421] ischemia[422] and systemic lupus erythematosus.[423] The role of miRNAs in microvesicles is not as clear as with exosomes owing to the lack of consensus on nomenclature and method of isolation of these larger extracellular vesicles, but they are thought to be important in the intercellular communication of many different normal biological processes and disease states.[389]

1.4.6.1.1.3 Apoptotic bodies

Apoptotic bodies are the third classification of extracellular vesicles. These are larger than exosomes and microvesicles (1-5 μm) and are formed through membrane blebbing during the later stages of apoptosis, therefore their lipid and surface protein makeup is representative of the apoptotic cells.[424] Apoptotic bodies also contain a range of signalling molecules including miRNAs.[389,424] The loading of miRNAs into apoptotic bodies was first identified in 2009,[425] but is not as well defined as with exosomes or microvesicles, with some enrichment of specific miRNAs over others, although this may be due to a pathological process rather than an active enrichment. What is known is that the enrichment of miRNAs in apoptotic bodies is different to that seen in other extracellular vesicles. For example, the profile of miRNAs in exosomes, microvesicles and apoptotic bodies released by keratinocytes are different across the different types of extracellular vesicles.[426]

Apoptotic bodies are less well studied than exosomes or microvesicles, and the role of miRNAs in intercellular signalling through apoptotic bodies has not been characterised as thoroughly. After the initial discover that miR-126 could be transferred from apoptotic endothelial cells to stabilise atherosclerotic plaques,[425] there have been only limited studies looking into other functional transfer of miRNAs to recipient cells. For example, one group showed miRNA transfer by apoptotic bodies in bone homeostasis.[427] Another suggested the shuttling of miR-221 and miR-222 by apoptotic bodies from macrophages

has an important role in promoting proliferation of lung epithelial cells.[428] Given that apoptosis is a key component in the pathogenesis of many diseases, it is likely that apoptotic bodies, and their packaged miRNAs, will be increasingly recognised to play a role in disease pathways.

1.4.6.1.1.4 Protein-bound miRNAs

Another way of stabilising extracellular miRNAs is through binding to proteins, either protein or lipoprotein complexes. Lipoproteins are a biochemical assembly of a single phospholipid layer with proteins embedded, which are involved in transport of molecules from the liver to peripheral tissues. These highly soluble complexes are often used to carry nucleic acids due to their ability to bind water-insoluble materials. Lipoproteins can either be high density lipoproteins (HDL) or low density lipoproteins (LDL) complexes.[389,429] They were first identified as carriers of miRNAs in 2011, by the ability of apolipoprotein A-I to carry small interfering RNA (siRNA) molecules to the liver.[430] The first confirmation of miRNA carriage by HDL came two years later, as the mechanism was suggested to be regulated by neutral sphingomyelinase, meaning it may be an active process. That study also showed a specific miRNA signature to identify familial hypercholesterolaemia.[431] Since then, HDL-bound miRNAs have been shown to contribute to an anti-inflammatory state in endothelial cells,[432] be indicative of vulnerable compared to stable coronary artery disease,[433] and be useful biomarkers for uremic patients.[434] This area of study is relatively novel, with much still to learn about the process. The majority of current work is investigating miRNA loading onto HDL, as this is the most abundant lipoprotein complex, but it is likely that LDL also carries and stabilises extracellular miRNAs.[435]

miRNAs can also be stabilised extracellularly by AGO proteins that form part of the RISC. This novel idea was only recently discovered, with the first identification by two groups in 2011.[399,436] One group suggested that only 10% of extracellular miRNA is encapsulated in vesicles, with 90% bound to protein complexes. They theorised that it might be possible for cells to release a functional RISC into the extracellular environment.[436] Cell-free miRNAs have been identified as preferentially bound to AGO1 or AGO2 in both plasma[399,437] and urine.[438] There have not been any studies investigating the functional relevance of this and how it relates to disease states, but it is likely AGO-bound proteins will turn out to be important in pathologies now that they have been identified as highly abundant in biological fluids.[437]

1.4.6.2 In biological fluids

As discussed above, miRNAs can be stabilised outside cells by multiple different complexes. The use of these extracellular miRNAs as biomarkers has been explored extensively in many different biological fluids, including tears, saliva, cerebrospinal fluid, serum, plasma, amniotic fluid, urine and bronchial lavage.[388,389] Discussed below are some examples of the use of extracellular miRNAs as biomarkers in blood (plasma, serum and whole blood) and PD effluent, as well as those of relevance in infections.

1.4.6.2.1 Circulating blood-based miRNAs

miRNAs have been identified in both serum and plasma, as well as in whole blood, in many different pathologies. miRNAs are a very good potential circulating biomarker due to their ability to be detected in samples that are routinely taken (i.e. minimally invasive), they can arise from either cell death or be actively secreted by live cells, they can be highly tissue- or disease-specific, they are highly stable extracellularly, and can easily be quantified.[439] The main body of work on circulating miRNAs has been conducted in cancer, suggesting they are relevant biomarkers for diagnosis and progression of different forms of cancer including breast, gastric, colorectal, hepatocellular, pancreatic, lung, cervical and prostate cancers.[440,441] These reviews summarise what is currently known in terms of miRNAs altered in multiple cancers, but there is also a substantial body of work looking at circulating miRNAs in other diseases. For example, circulating placenta-specific miRNAs can be used to identify pregnancy,[396] a range of inflammatory miRNAs can be indicative of type 2 diabetes,[398] and inflammatory miRNAs or miRNAs specific to cardiac tissues present in the circulation can suggest a cardiovascular disease diagnosis.[439]

1.4.6.2.2 PD Effluent

As already noted above, miRNAs are stable in most biological fluids, which includes PD effluent. This fluid is another easily accessible source of biomarkers, which can be used to assess the effectiveness of dialysis or potential reasons for a lack of efficacy such as fibrosis. As mentioned above, miRNAs have been identified as markers of fibrosis in the peritoneal membrane, but they are also useful as soluble biomarkers in PD effluent. The published work on this subject has so far only looked at whether miRNAs can be biomarkers for fibrosis of the peritoneal membrane, to prevent effective dialysis. Multiple miRNAs, including miR-21, miR-129 and miR-589, have been identified as important indicators of

peritoneal membrane transport characteristics or EMT.[317,326,327,442] Animal models have shown how merely the injection of PD fluid into the peritoneum of a mouse or rat alters the miRNA profile, due to changes in the peritoneal membrane.[328,443] Although no work has yet looked at miRNAs as biomarkers of infection in PD effluent, it is thought they will be useful biomarkers due to their role as cell-free biomarkers in other infectious diseases.

1.4.6.3 Infection

It is already known that miRNAs have an important role in the immune response, as discussed above, therefore their usefulness as circulating biomarkers in infection has been evaluated in viral, bacterial and parasitic infections. For example, a range of miRNAs have been shown to be either up- and down-regulated in patients with HIV infection.[394,444] *M. tuberculosis* infection also results in altered circulating miRNA levels, with some miRNAs able to distinguish active from latent tuberculosis.[394,445,446] miRNAs can also be effective biomarkers for diagnosis of malaria, caused by the *Plasmodium* parasites, as well as identifying more severe disease progression.[447,448] The use of miRNAs in diagnosing infectious diseases has been shown to be useful in distinguishing spontaneous bacterial peritonitis episodes in patients with peritoneal ascites,[449] showing the potential usefulness of these stable biomarkers in positively identifying an infection at the site of infection, as opposed to other inflammatory conditions. This suggests that miRNAs could be useful to distinguish an infection in PD, but this has yet to be evaluated.

1.5 Hypothesis and aims

Hypothesis

miRNAs are altered in acute PD-related peritonitis episodes, are biomarkers of infection, and play a functional role in modulating the immune responses to different pathogens.

Aims

The main aim of this study was to define the diagnostic and functional role of local miRNAs in PD effluent from patients with acute peritonitis.

To achieve this aim, the following objectives were defined:

- To characterise the miRNA alterations in well-defined acute PD-related peritonitis episodes and investigate candidate miRNA biomarkers in a patient cohort
- To characterise the cellular source of candidate miRNAs
- To identify potential mRNA targets for the candidate miRNAs
- To characterise the stabilisation of miRNAs in PD effluent from peritonitis patients

2 Materials and Methods

2.1 Ethic statement

Recruitment of PD patients and healthy volunteers for this study was approved by the South East Wales Local Ethics Committee under reference numbers 04WSE04/27 and 08/WSE04/17, respectively. It was conducted according to the principles expressed in the Declaration of Helsinki. All individuals provided written informed consent. The PD study was registered on the UK Clinical Research Network Study Portfolio under reference numbers #11838 "Patient immune responses to infection in Peritoneal Dialysis" (PERIT- PD) and #11839 "Leukocyte phenotype and function in Peritoneal Dialysis" (LEUK-PD). Fresh omentum samples from consented patients were obtained from the Wales Kidney Research Tissue Bank.

2.2 Patient info and data collections

The local study cohort comprised 131 adult PD patients admitted to the University Hospital of Wales (UHW), Cardiff, on day 1 of acute peritonitis between September 2008 and October 2018, as well as a limited number of samples from days 2-7 of infections. 20 stable individuals receiving PD for at least 3 months and with no previous infections (or at least 3 months since previous infection) served as stable, non-infected control samples. Subjects known to be positive for HIV or HCV were excluded. Clinical diagnosis of acute peritonitis was based on the presence of abdominal pain and cloudy peritoneal effluent with >100 white blood cells/mm^3. According to the microbiological analysis of the effluent by the routine Microbiology Laboratory, Public Health Wales, episodes of peritonitis were defined as culture-negative (with unclear aetiology) or as confirmed bacterial infections caused by specific subgroups of Gram-positive and Gram-negative organisms. Cases of fungal infection and mixed or unclear culture results were excluded from this analysis. All patient sub cohorts described were selected from this full cohort.

2.3 Cell isolation and culture

2.3.1 Human peritoneal mesothelial cells and peritoneal fibroblasts

Human peritoneal mesothelial cells (HPMCs) and human peritoneal fibroblasts (HPFBs) were isolated from omental tissue of non-PD, consented patients undergoing abdominal surgery. Shortly after collection from surgery, tissue was stored in a sterile plastic container with PBS and incubated at 4°C until tissue dissection. Omental tissue was subjected to 2

digestion cycles with 0.05% trypsin-EDTA solution (Invitrogen, T4174). Precisely, the tissue was washed with PBS in a Petri dish, to remove blood and other excessive fat, and cut into small pieces (depending on the tissue size). Each piece was then dissociated in a universal tube with 10 ml 0.05% trypsin-EDTA solution and positioned on a rotating wheel at 37°C for 15 min. Dissociated cells were pelleted by centrifugation at 250 g for 6 min at 20°C and supernatant was then carefully removed. The cell pellet was re-suspended and washed with 20 ml of complete M-199 medium (HPMCs) or complete Ham's F12/DMEM medium (HPFBs) by centrifuging at 250 g for 6 min at 20°C. Washed cells were re-suspended in 5 ml of fresh complete medium and transferred into T25 flask (Nunc, Thermo Scientific) (passage number 0) for further growth and expansion in culture. HPMCs and HPFBs were maintained at 37°C in a humidified incubator with 5% CO_2. Cells were harvested and transferred to T75 flask when the cultures reached confluency (≥80%) (passage 1). Prior to treatment, the growth of HPMCs was arrested by changing complete medium to M-199 medium with 0.1% foetal calf serum (FCS) (HPMCs) or Ham's F12/DMEM medium with 0.2% FCS (HPFBs) for 24 h. All the subsequent functional experiments were performed with the use of HPMCs or HPFBs expanded within 2 passages.

For HPMCs and MeT-5A cell line, medium 199 (Life Technologies) was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and 10% FCS from Invitrogen; and bovine pancreas insulin (5 μg/ml), hydrocortisone (0.4 μg/mL) and apo-transferrin (5 μg/ml) from Sigma-Aldrich.

For HPFBs, Ham's F12 nutrient mixture medium (Life Technologies) was mixed at a 1:1 ratio with DMEM medium and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 20% FCS and 100 μM NEAA from Invitrogen; and bovine pancreas insulin (5 μg/ml), hydrocortisone (0.4 μg/ml) and apo-transferrin (5 μg/ml) from Sigma-Aldrich.

2.3.2 Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood, collected from consented heathy volunteers. At the time of the collection, blood was mixed with anti-coagulant buffer made of 20 U/ml heparin and 15 mM EDTA (Fisher Scientific UK Ltd). Diluted blood was subsequently layered on top of Ficoll-Paque (Axis-Shield, 1114544) and centrifuged for 20 min at 500 g at 18°C with minimal acceleration and deceleration at

lowest level. Afterwards, the white mononuclear cell layer (buffy coat) between the plasma and red blood cells (RBCs) was collected and washed with serum-free RPMI.

2.3.3 Peripheral blood monocytes

Monocytes were isolated by magnetic activated cell sorting (MACS) from PBMCs. To block non-specific binding of monoclonal antibodies to Fc receptors, PBMCs were firstly incubated with human IgG (FcR blocking reagent, Miltenyi, 120-000-442) in MACS buffer at 4°C for 5 min, then incubated with anti-CD14 microbeads (Miltenyi, 120-000-305) at 4°C for 10 min. Monocytes were then positively selected by passing through two LS columns (Miltenyi, 130-042-201) to obtain purities of >95% CD14$^+$ monocytes (as determined by flow cytometry).

To prepare MACS buffer, 2% FCS (Invitrogen) and 5 mM EDTA were added to sterile PBS and passed through 0.22 μm filter before use.

2.3.4 Peripheral blood neutrophils

PMN neutrophils were isolated from venous blood collected from consented healthy volunteers. At the time of collection, EDTA was added as an anti-coagulant. Blood was then layered on top of Ficoll-Paque Plus (GE Healthcare, 17-1440-02) and centrifuged for 30 min at 470 g at room temperature with minimal acceleration and no brake. The PMN and RBC layer was then washed with sterile PBS. Erythrocyte contamination was reduced by RBC precipitation (by 4% Dextran in PBS) and hypotonic lysis (by 10 mM NaCl). After washing, high purity PMNs were isolated by negative magnetic separation using EasySep Human Neutrophil Enrichment Kit (StemCell Technologies, 19257) for PMNs. After washing and resuspension in culture medium, purity was measured by flow cytometry, with purities >99.5% CD15$^+$ neutrophils achieved (with <0.5% contamination of CD14$^+$ monocytes).

For isolated monocytes and neutrophils, complete RPMI 1640 medium used in cell culture was made by adding 10% FCS (Invitrogen), 50 μg/ml penicillin/streptomycin, 2 mM L-glutamine, 1% sodium pyruvate and 100 μM non-essential amino acids (NEAA) into RPMI-1640; all from Invitrogen.

2.3.5 MeT-5A cell line

MeT-5A, a human epithelial cell line, was purchased from the American Type Culture Collection (ATCC). MeT-5A cells were cultured in complete M-199 medium as described for HPMCs above.

2.3.6 Long-term cell storage

To store cells at -70°C in Cryo Tubes after cell isolation, cells were quickly re- suspended in complete medium supplemented with 40% FCS (Invitrogen) and 10% DMSO.

2.4 In vitro cell stimulation

2.4.1 HPMCs and HPFBs

HPMCs and HPFBs were cultured until confluent in a T25 flask then a T75 flask, before transfer to a 12-well plate (Nunc), with all treatments conducted in serum-reduced media (after 24 h serum-starvation) before cells reached passage 3. Cells were incubated with bacterial extracts (*E. coli* and *S. epidermidis*) at final protein concentrations of 50 µg/ml, and with PAM3 (Pam(3) CSK(4), EMC microcollections, L2000) and LPS (ultrapure from *E. coli* serotype, Enzo life sciences, O1111:B4) at a final concentration of 500 ng/ml, overnight at 37°C. Following incubation, supernatants were frozen at −80°C and cells were lysed using TRIzol (500 µl per well) then frozen at −80°C, before RNA extraction as described below.

2.4.2 Monocytes

5×10^5 primary monocytes per well were stimulated with bacterial extracts (*E. coli* and *S. epidermidis*, at final concentration of 50 µg/ml), PAM3 or LPS (at final concentrations of 500 ng/ml) in 24-well plates (Nunc) for 4 h or overnight at 37°C. Following incubation, supernatants were frozen at −80°C, and cells were lysed using TRIzol (250 µl per well) then frozen at −80°C, both stored for RNA extraction as described below.

2.4.3 Neutrophils

Primary neutrophils were plated at a concentration of 5×10^6 PMN/ml in a 12-well plate (Nunc). After resting for 20 min, they were stimulated with bacterial extracts (*E. coli* and *S. epidermidis*, at final concentration of 50 µg/ml), PAM3 or LPS (at final concentrations of 500 ng/ml) for 4 h at 37°C. Following incubation, supernatants were frozen at −80°C, and

cells were lysed using TRIzol (500 µl/well) or RLT buffer (600 µl for >10^6 cells, 350 µl for <10^6 cells) then frozen at −80°C for RNA extraction as described below.

2.4.3.1 Conditioned media (CoM) generation

After isolation and stimulation of PMNs for 4 h with 500 ng/ml LPS as above, CoM was generated by removing the cells by centrifugation at 300 *g* for 10 min at 4°C. This was stored at −80°C until use.

2.5 Cell viability assays

2.5.1 Lactate dehydrogenase (LDH) assay

LDH release was used as a measure of cell viability, proportional to total cell lysis, using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, G1782). 45 min prior to supernatant harvesting, 10 µl of Lysis solution (10×) was added for every 100 µl of target cells added as a maximum lysis control. Supernatant was removed from each well of the culture plate. In the case of non-adherent cells (i.e. PMNs and monocytes), media was centrifuged at 500 *g* for 5 min. 50 µl of supernatant was put into a fresh clear 96-well plate and 50 µl of CytoTox 96® Reagent was added to each well. The plate was incubated in the dark for 30 min at room temperature. 50 µl of Stop Solution was then added to each well, and the absorbance was measured at 490 nm within 1 h. Cytotoxicity was calculated as a percentage of the maximum lysis control.

2.5.2 AlamarBlue assay

AlamarBlue assay was used for examination of relative cell number and/or viability. This method employs a non-toxic, cell permeable, blue and non-fluorescent dye, *resazurin*, converted to the pink and fluorescent dye, *resorufin*, in response to metabolic activity of cultured cells. The fluorescent and colorimetric signal is proportional to the number of living cells in the sample. To perform the assay, cells were incubated for 1 h in new culture medium without serum at 37°C and 10% (v/v) AlamarBlue reagent (Bio-Rad, BUF012B). The medium was then transferred to a black 96 well plate (100 µl/well) and the fluorescent signal was monitored using 544 nm excitation wavelength and 590 nm emission wavelength. Medium with 10% (v/v) AlamarBlue reagent was kept in the incubator for one hour before the measurement to use as a blank.

2.6 Flow cytometry

Flow cytometry was used to assess the purity of isolated cells and expression of activation markers on their surface. Cells were firstly washed with PBS and then stained with Live/dead fixable Aqua dead cell stain kit (Life Technologies, L34957) for 12 min at RT. Subsequently, cells were washed with fluorescently activated cell sorting (FACS) buffer, centrifuged at 450 g for 3 min and incubated with IvIg (Kiovig; Baxter) at 1:1000 dilution for 10 min at 4°C to block non-specific binding of antibodies to Fc receptors . To stain for cell-surface markers, cells were incubated with a cocktail of monoclonal antibodies conjugated with different fluorochromes (Table 2.1) at 4°C for 30 min. Cells were then washed with FACS buffer and fixed using 2% formaldehyde for 10 min at 4°C. After a final wash with FACS buffer, results were acquired using BD FACSCanto II or LSR Fortessa (BD Biosciences). All analysis was performed using FlowJo (version 10.4; TreeStar Inc.) and the cells of interest were gated based on the appearance on side scatter and forward scatter area/high (SSC-A/FSC-A) for intact cells, forward scatter area/high (FSC-A/FSC-H) for single cells and live-dead/FSC-A for live cells. Monocytes were defined as $CD14^+$ live cells. PMNs were defined as $CD15^+$ live cells, with activated PMNs defined as $CD15^+$, $CD11b^{high}$ and $CD62L^-$.

To prepare FACS buffer, 2% FCS (Invitrogen) were added into sterile PBS and passed through 0.22 μm filter before use.

2.7 Bacterial culture and lysis

Clinical bacterial isolates were kindly provided by Dr Mark Toleman, Division of Infection and Immunity, Cardiff University. These isolates were chosen based on the most common Gram-positive and Gram-negative causative pathogens in PD-related peritonitis episodes encountered at UHW, Cardiff (*Staphylococcus epidermidis* and *Escherichia coli* respectively). Bacteria stored at −70°C in cryotubes with cryogenic medium (sterile PBS supplemented with sucrose, glycerol and peptone) were inoculated onto Colorex urinary tract infections (UTI) agar plate (E&O Laboratories Ltd, PP3000) and incubated aerobically for 24 h at 37°C. The following day, the growth of a single species was confirmed by the presence of uniform colonies with the same colour. Bacteria were transferred to 20 ml Luria Broth (LB, tryptone, yeast extract and NaCl) in a universal tube and incubated for 2.5 h at 37°C with shaking to facilitate bacterial division. Bacteria were harvested when the

Marker	Fluorophore	Company	Catalogue number	Clone
CD15	BV605	Biolegend	323032	W6D3
CD62L	PE-Cy5	BD	555545	DREG-56
CD11b	BV421	Biolegend	301323	ICRF44
CD14	FITC	BD	555397	M5E2
CD14	PE-Cy7	Biolegend	301813	M5E2

Table 2.1: Antibodies used for flow cytometry analysis of purity and activation of primary cells.

culture reached an optical density of 0.5-0.8 at a wavelength of 600 nm (OD_{600}). Each tube was subsequently centrifuged at 3,600 rpm for 1 h and the bacterial pellet re-suspended in 1 ml of sterile PBS before sonication. The bacterial suspensions were kept on ice during sonication, using a Soniprep 150 sonicator for 10 min with 10 sec on and 20 sec off cycles. Bacterial extracts were then transferred to Eppendorf tubes and centrifuged at 20,000 g to pellet cell debris. Collected supernatant was passed through Ultrafree-MC centrifugal filters (Durapore PVDF 0.1 µm, Millipore, UFC40VV25) and centrifuged for 10 min at 10,000 g, at 4°C, to remove larger aggregates. At this point a small aliquot was obtained to measure the protein concentration with BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, 23225), following manufacturer's protocol. Bacterial extracts were then stored at −20°C.

2.8   In vivo model

Samples for in vivo experiments were kindly provided by Dr Ann-Catherine Raby, generated as described previously.[450] Briefly, 8-12-week-old inbred C57BL/6 mice (Charles River) were injected intraperitoneally with PBS (500 µl) or *E. coli* (ATCC 25923 strain, 2 x 10^7 CFU per mouse). At indicated time points, the mice were sacrificed, and their peritoneal cavities were lavaged with 2 ml of ice-cold PBS. All experimental procedures were performed under a Home Office project license.

2.9   RNA extraction

2.9.1   Cell-free liquid samples

RNA was extracted from cell-free PD effluent, supernatant of cultured cells, peritoneal lavages from mice and all other cell-free liquid samples using the mirVana™ PARIS™ and Native protein purification kit (Life Technologies, AM1556), using the protocol to enrich for small RNAs. Samples were rendered cell-free, as in the Human Tissue Authority (HTA) guidelines. 500 µl of sample was mixed with an equal volume of the 2× denaturing solution at room temperature. 100 pM of *Caenorhabditis elegans* miR-39 (cel-miR-39; Life Technologies, MC10956) was spiked into each sample. 1 ml of Acid-Phenol Chloroform was then added, thoroughly mixed and centrifuged at maximum speed (20,000 g) to separate the mixture into aqueous and organic phases. The aqueous phase was then transferred to a separate tube and 1/3 volume of 100% ethanol was added and mixed thoroughly. For PD effluent samples only, a second separation was performed by adding nuclease-free water

to the original tube to replenish the aqueous phase removed, mixing thoroughly and separating by centrifugation at 20,000 g or 15 min. This aqueous phase was also transferred to a separate tube and 1/3 volume of 100% ethanol was added and mixed thoroughly. For all samples, this mixture was then passed through a filter cartridge, with the flow-through discarded. The filter cartridge was washed with 700 µl of miRNA Wash Solution 1 and 500 µl of Wash Solution 2/3 twice, with the flow-through discarded after each wash. The RNA was eluted into a fresh collection tube by adding 90 µl of nuclease-free water, pre-heated to 95°C, to the centre of the filter cartridge. After incubating at room temperature for 5 min, this was centrifuged and the flow-through was stored at −80°C.

2.9.2 Cultured cells using TRIzol^{TM}

RNA was extracted from cells cultured in standard tissue culture plates using TRIzol^{TM} Reagent (Invitrogen, 15596018). Supernatant of cells was removed, and RNA extracted from these liquid samples as above. Sufficient TRIzol was added to the sample to lyse all cells present, as in the manufacturer's protocol. For example, 500 µl of TRIzol was added per well of a 12-well plate and 1 ml was added per 5-10 × 10^6 cells in samples from PD effluent or cells in suspension. This lysate was then transferred to a tube and left at room temperature for 5 min to permit complete dissociation of the nucleoproteins complex. 200 µl of chloroform was added per 1 ml of TRIzol used, mixed well and incubated for 2-3 min. This was then centrifuged for 15 min at 4°C and 12,000 g, to separate the phases, and the aqueous phase was carefully transferred to a new tube. To precipitate the RNA, 500 µl of isopropanol was added to the aqueous phase per 1 ml of TRIzol reagent used for homogenisation, then left at room temperature for 10 min. This was centrifuged for 10 min at maximum speed (20,000 g), at 4°C to form a small white pellet containing the RNA. Supernatant was carefully removed, and the pellet was washed three times with 1 ml of 75% ethanol, where the pellet was re-suspended then centrifuged at maximum speed for 5 min at 4°C, with the supernatant discarded after each wash. The pellet was then air dried for 30-45 min and re-suspended in 10 µl of nuclease-free water. RNA yield was determined using a NanoDrop 2000 Spectrophotometer. 1 µl of RNA was measured for absorbance at 230 nm, 260 nm and 280 nm, with concentrations given used to determine the amount of RNA to add into RT reactions. RNA was stored at −80°C until use.

2.9.3 Cultured cells using RNeasy Mini kit

RNA was extracted from cells cultured in standard tissue culture plates using an RNeasy kit (Qiagen, 74034). Supernatant of cells was removed, and RNA extracted as above. 350 μl of RLT buffer was added for <10^6 cells (600 μl for >10^6 cells), left for 1-2 min, then pipetted up and down several times. This lysate was transferred to a tube and homogenised by passage through a 29-gauge needle, attached to a plastic syringe, 5-10 times. Homogenate was transferred to a genomic DNA (gDNA) Eliminator spin column and centrifuged, the flow-through was retained and transferred to a new tube. 1.5x volume 100% ethanol was added (i.e. 900 μl if 600 μl lysis buffer used) and mixed well. This mixture was then passed through an RNeasy Mini spin column, with the flow-through discarded. The spin column membrane was washed three times with 500 μl of Buffer RPE, discarding the flow-through each time. The spin column was dried by another centrifugation step, then placed in a fresh collection tube. 30 μl of RNase-free water was used to elute the RNA. RNA yield was determined by NanoDrop 2000 Spectrophotometer. RNA was stored at -80°C until use.

2.10 TaqMan Low density array (TLDA)

2.10.1 Reverse transcription

RNA was reverse-transcribed to complementary DNA (cDNA) using the Megaplex™ RT Kit (Life Technologies, 4366596 and 4401009). The RT master mix included: Megaplex™ RT Primers, dNTPs with dTTP, MultiScribe™ Reverse Transcriptase, RT buffer, $MgCl_2$, RNase inhibitor and nuclease-free water. 4.5 μl of the RT master mix and 3 μl of total RNA were added to individual tubes in a MicroAmp® 8-Tube Strip. The amplifications were carried out with 40 cycles of 2 min at 16°C, 1 min at 42°C and 1 sec at 50°C, followed by 5 min at 85°C and a cooling step at 4°C.

2.10.2 Pre-amplification

cDNA was amplified before the PCR reaction using the TaqMan PreAmp Kit (Life Technologies, 4391128). The master mix included: TaqMan® PreAmp Master Mix, Multiplex™ PreAmp Primers and nuclease-free water. 2.5 μl of each RT product and 22.5 μl of PreAmp master mix were added to individual tubes in a MicroAmp® 8-Tube Strip. The amplifications were carried out for 10 min at 95°C, 2 min at 55°C and 2 min at 72°C, followed by 12 cycles of 15 sec at 95°C and 4 min at 60°C, an enzyme inactivation step of

10 min at 99.9°C and a cooling step at 4°C. Product was diluted with 75 µl of nuclease-free water per tube.

2.10.3 Quantitative PCR (qPCR)

cDNA was detected using a pre-loaded 384-well plate-based microRNA array (Life Technologies, 4398965). 9 µl of diluted preAmp product was combined with TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 2× (Life Technologies, 4440040) and nuclease-free water, then loaded into the ports in the array. After centrifugation to distribute into the individual wells, this was run on a ViiA 7 real-time PCR system (Life Technologies) using the 384 well TaqMan Low Density Array default thermal-cycling conditions. The comparative $2^{-\Delta\Delta Ct}$ method was used to calculate sample-specific threshold cycle (Ct), using the RQ (relative quantification) software provided on the Thermo Fisher Cloud (https://apps.thermofisher.com/apps/dashboard/). The expression of target genes was normalized to the global expression of all targets across the plate, using standard settings in the Thermo Fisher software.

2.11 RT-qPCR of individual samples

2.11.1 Reverse transcription

RNA was reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, 4368813). The RT master mix included: RT buffer, dNTPs, MultiScribe Reverse Transcriptase (all Life Technologies) and RNase inhibitor (New England BioLabs® Inc, M0307). miRNA-specific primers (Life Technologies, see table 2.2 for details) were also added to the master mix.

High-Capacity cDNA Reverse Transcription Kit (Life Technologies) was used, containing RT master mix made of nuclease-free water, 10X RT buffer, 100 mM dNTPs, 50 U/µl MultiScribe Reverse Transcriptase, 10X RT random primers (all Life Technologies) and 40 U/µl RNase inhibitor (New England BioLabs ® Inc), Quantitative PCR was performed using TaqMan® Universal Master Mix II, no UNG, with specific TaqMan primers (all Life Technologies). The volume of RNA and RT master mix varied dependent on the sample type and extraction method, as detailed in Table 2.3.

miRNA	Catalogue number
hsa-miR-191-5p	Part number 4427975 Assay ID 002299
hsa-miR-16-5p	Part number 4427975 Assay ID 000391
sno-135	Part number 4427975 Assay ID 001230
snRNA U6	Part number 4427975 Assay ID 001973
hsa-miR-223-3p	Part number 4427975 Assay ID 002295
hsa-miR-139-5p	Part number 4427975 Assay ID 002289
hsa-miR-197-3p	Part number 4427975 Assay ID 000497
hsa-miR-25-3p	Part number 4427975 Assay ID 000403
hsa-miR-27a-3p	Part number 4427975 Assay ID 000408
hsa-miR-203a-3p	Part number 4427975 Assay ID 000507
hsa-miR-21-5p	Part number 4427975 Assay ID 000397
hsa-miR-31-5p	Part number 4427975 Assay ID 002279
hsa-miR-199a-3p	Part number 4427975 Assay ID 002304
hsa-miR-100-5p	Part number 4427975 Assay ID 000437
hsa-miR-99a-5p	Part number 4427975 Assay ID 000435
mmu-miR-31-5p	Part number 4427975 Assay ID 000185
cel-miR-39-3p	Part number 4427975 Assay ID 000200

Table 2.2: Product codes for TaqMan miRNA assays used in RT-qPCR.

Sample	Cell-free liquid	Cells (cultured or from PD effluent)	
Extraction method	mirVana^{TM} PARIS^{TM} kit for small RNAs	TRIzol^{TM} reagent	RNeasy Mini kit
Vol of RNA added to RT	9.26 µl	5 µl (plus 4.26 µl water)	5 µl (plus 4.26 µl water)
Concentration of RNA added to RT	Unknown	2 ng/µl	2 ng/µl
Vol of RT Master mix	5.74 µl	10 µl	10 µl

Table 2.3: Volumes of RNA and RT master mix to add to MicroAmp® 8-Tube Strip for reverse transcription reaction.

The amplifications were carried out with thermal cycling of 10 min at 25°C, 2 h at 37°C and 5 sec at 85°C, followed by a cooling step at 4°C. Generated cDNA was diluted with 30 µl water prior to use as a template for qPCR.

2.11.2 Quantitative PCR

miRNA qPCR was carried out by mixing 10 µl of TaqMan 2× Universal PCR Master Mix, No AmpErase UNG (Life Technologies, 4440040) with 1 µl specific PCR primers (all Life Technologies, see table 2.2 for details). 11 µl of miRNA specific master mix was added to 9 µl of diluted cDNA (for cell-free samples) or 4 µl cDNA further diluted with 5 µl water (for samples with cells). This was then plated in an Optical 96-Well Fast Plate (Life Technologies). The reaction was performed on ViiA7 Real-Time PCR System (Life Technologies) and the cycling parameters were: hold for 10 min at 95°C, 40 cycles of 15 sec at 95°C (denature step) and 1 min at 60°C (anneal/extend steps). Ct values were calculated as the cycle number when the threshold was reached. The expression of target genes was normalized to the expression of small nuclear RNA U6 (snRNA U6) endogenous control and a control sample via the comparative $2^{-\Delta\Delta Ct}$ method to give sample-specific relative quantification values, or represented as 40-Ct (40 minus the raw Ct value) as appropriate.

2.12 Extracellular vesicle (EV) characterisation

2.12.1 Differential centrifugation for isolation of EVs

Samples were cleared of live cells and cell debris with preliminary centrifugation steps at 300 g and 2000 g respectively, both for 10 min at 4°C. The supernatant from these steps was then centrifuged at 10,000 g for 1hr, at 4°C to isolate larger EVs. Ultracentrifugation of the resulting supernatant at 100,000 g for 1hr, at 4°C isolated smaller EVs. For RNA extraction and miR-223 detection, 5 ml was used for each centrifugation step and each pellet (except the initial cell pellet, which was lysed with TRIzol and extracted as above for cell-based samples) was resuspended in 500 µl of endotoxin-free PBS (Sigma Aldrich, TMS-012), with a 500 µl aliquot of each supernatant also taken for RNA extraction. Extraction was performed as above for cell-free liquid samples.

2.12.2 RNase and proteinase treatments

500 µl aliquots of samples were all treated with 100 pM of *Caenorhabditis elegans* miR-39, then treated as required with either RNase A, proteinase K or both. RNase A (Life Technologies (Ambion) AM2272 or EN0531) was used at a concentration of 0.1 mg/ml, at 37°C for 30 min. Proteinase K (P-2308, Sigma-Aldrich) was used at 50 µg/ml, at 55°C for 30min. Samples were also treated with RNase A followed by proteinase K, and the reverse, as well as temperature controls. This protocol was optimised and described in detail previously.[438]

2.12.3 Size-exclusion chromatography

Pre-packed size exclusion columns (Cell GS, Exo-Spin™, EXO4-20) were washed with 6 mM EDTA in PBS, then 2 ml of samples were added, and 15 × 1 ml aliquots were collected. RNA was extracted from 500 µl of each aliquot as above (for cell-free liquid samples). The remainder was used for the plate-based immuno-assays to detect relevant tetraspanin markers and CD15 (PMN surface marker).

2.12.4 Plate-based immuno-assay

500 µl of column fractions were diluted 1:1 with 6mM EDTA in PBS, then bound to protein-binding ELISA plates in duplicate (100 µl per well, Greiner Bio-One, 756071). After overnight coupling and then blocking (with 1% (w/v) BSA in PBS for 2 h at room temperature), the bound material were labelled with primary antibodies against proteins including CD9 (R&D systems, JOK0713081), CD81 (Bio-Rad, MCA1847EL) and CD15 (BD Biosciences, 557895) or HSA (human serum albumin) (250 ng/ml) (R&D systems, MAB1455) for 2 h at room temperature on a plate shaker. After three washes, goat anti-mouse-biotinylated antibody (Perkin Elmer, NEF823001EA), diluted 1:2500, was added for 1.5 h. After three washes, Europium-conjugated streptavidin (Perkin Elmer, 1244-360) was added for 45 min. After a final six washes, a signal was obtained using time-resolved fluorometry (TRF), measured using a Wallac Victor-II multi-label plate reader (Perkin Elmer).

2.13 miR-223 reporter assay

The luciferase-based reporter assay for miR-223 uptake was generated as shown in the schematic diagram in Figure 2.1 and detailed below.
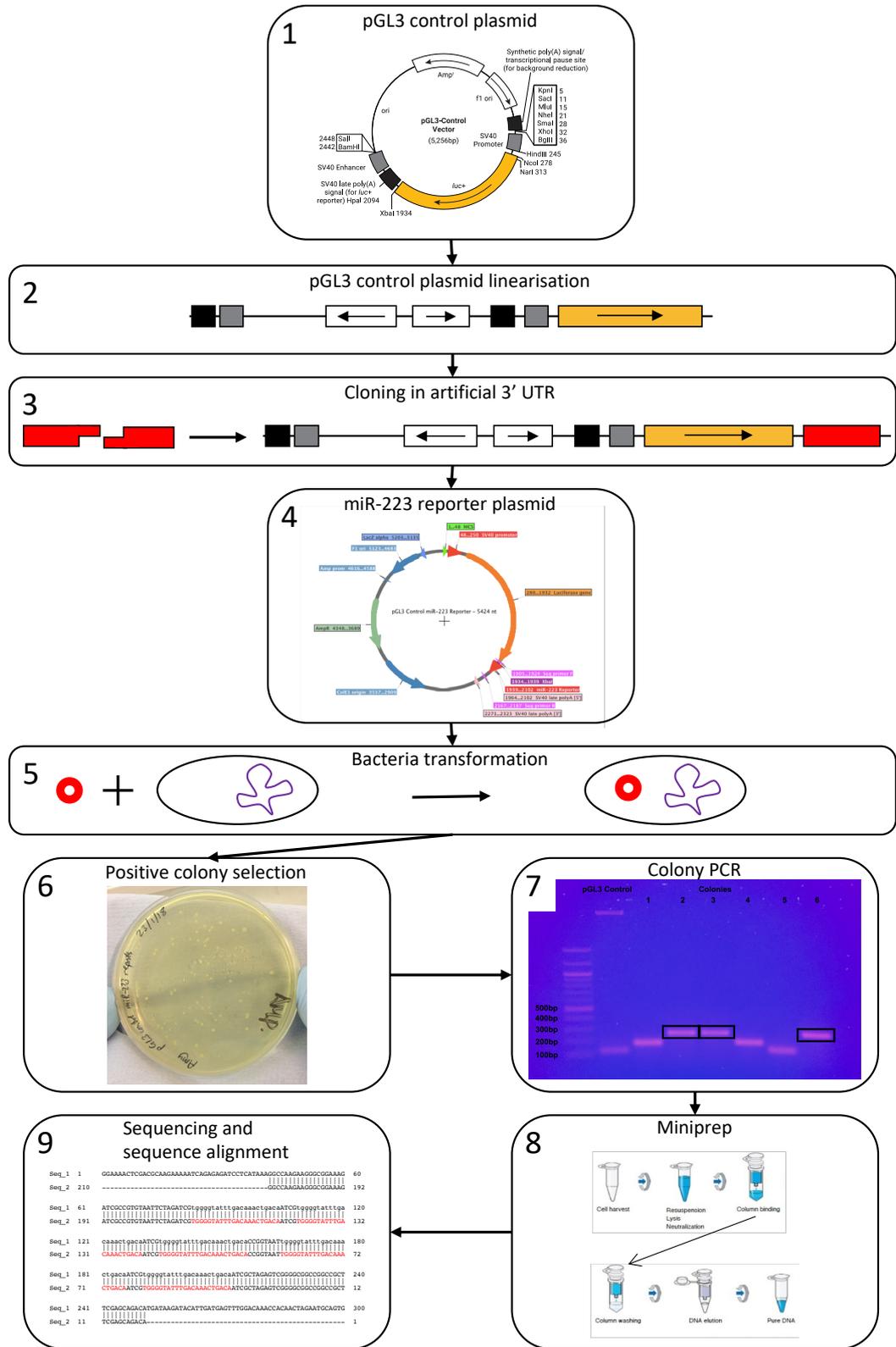
Figure 2.1: Schematic diagram of miR-223 reporter assay generation.

2.13.1 Plasmid generation

An artificial 3'UTR (3' untranslated region) reporter was designed containing multiple perfect binding sites for miR-223-3p. The binding sites were separated by a spacer of 4bp. The 3'UTR reporter was cloned into the pGL3-Control vector using the In-Fusion HD cloning kit (Clontech, 638909) according to the manufacturer's instructions. The 3'UTR reporter was positioned immediately after the firefly luciferase coding sequence.

First, the vector was linearised downstream of the firefly luciferase coding sequence using *XbaI*, a restriction endonuclease that recognises T^CTAGA sites and cuts leaving a 5' overhang. The restriction endonuclease digestion was performed at 37°C for 2 h in a block heater. The linerised vector was separated on a 1% agarose gel. A band corresponding to approx. 5.2kb was excised from the gel. The DNA was extracted and purified using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, 740609) according to the manufacturer's instructions. The eluted DNA was quantified by NanoDrop 2000 spectrophotometer.

Second, the 3'UTR reporter was contracted by annealing two pairs of overlapping oligonucleotides into two double stranded DNA fragments, which contained a 15bp overlapping sequence with the linearised plasmid. The overlapping oligonucleotides were annealed by heating to 90°C for 3 min then at 37°C for 15 min, before cooling on ice. The cloning reaction was performed by combining the annealed oligonucleotides (3ng) and linearised vector (100 ng) in a molar ratio of 3:1, along with 5X In-Fusion HD Enzyme Premix containing T4 DNA ligase (Clontech, 638909), adjusted to a final volume of 10 µl using molecular grade water. The cloning reaction was incubated at 50°C for 15 min, before cooling on ice.

2.13.2 Bacterial transformation and selection of positive colonies by colony PCR

The product of the cloning reaction was used for transformation of Stellar competent *E. coli* (Takara, 636763) by heat shock. Briefly, 1 µl of product was added to 50 µl Stellar *E. coli* cells and incubated on ice for 30 min. This mixture was then heated to 42°C for 45 sec (heat shock), followed immediately by recovery on ice. 450 µl of SOC medium was then

added, followed by incubation for 90 min at 37°C on a shaking platform. The bacterial culture was then plated out and grown on a 2x YT agar supplemented with 100 µg/ml ampicillin overnight at 37°C, to select positive colonies. The next day, 6 colonies were selected for colony PCR. Primers were designed to surround the insert:

Colony PCR forward primer: CAAGAAGGGCGGAAAGATCG

Colony PCR reverse primer: ACCACAACTAGAATGCAGTGA

Colony PCR was conducted using OneTaq Quick load 2x mastermix with standard buffer, with the reaction size scaled down to 15 µl. PCR reactions were performed with an initial denaturation step of 94°C for 30 sec, followed by 25 cycles of 94°C for 30 sec, 46°C for 30 sec and 68°C for 40 sec, with a final extension step of 68°C for 5 min then 4°C continuously. The final PCR product was analysed on a 1% agarose gel to confirm successful cloning of insert into vector. Successful cloning was distinguishable from the vector without an insert by different sized bands – 280bp for successful cloning versus 115bp for unsuccessful cloning. 3 of the 6 colonies selected showed correct sized bands.

2.13.3 Purification of plasmid DNA and sequencing

The positive colonies were grown up in 5 ml 2x YT broth supplemented with 100 µg/ml ampicillin overnight at 37°C on a shaking platform. The next day, 1.6 ml was mixed with 400 µl glycerol to make a long-term bacterial stock, which was stored at -80°C. The remainder of the culture was used to purify plasmid DNA using the QIAprep Spin Miniprep Kit (Qiagen, NA0160), following the manufacturer's instructions. The eluted plasmid DNA was quantified by NanoDrop 2000 spectrophotometer. The fidelity of the pGL3-miR-223 3'UTR reporter insert was confirmed by Sanger sequencing (Eurofins MWG) using the colony PCR reverse primer.

Stock quantities of plasmid DNA were prepared for pGL3-miR-223 3'UTR reporter, pGL3-Control and pRL-SV40. pRL-SV40 expresses Renilla luciferase under the control of the strong, constitutive SV40 promoter. 100 µl of the bacteria stocks was added to 50 ml of 2x YT broth, supplemented with 100 µg/ml ampicillin, at 37°C overnight, on a shaking platform. The next day, bacteria were pelleted, and plasmid DNA extracted by Midiprep (Qiagen, NA0200), according to the manufacturer's instructions. The eluted plasmid DNA was quantified by NanoDrop 2000 spectrophotometer. A working stock was kept at -20°C, whilst the main stock was stored at -80°C.

2.13.4 Transfections and treatments

MeT-5A pleural mesothelial cells was grown to approximately 70% confluency in a 48-well plate, before transfection with the pGL3-miR-223 3'UTR reporter. This was co-transfected with a the pRL-SV40 plasmid in a ratio of 1:9 (pRL-SV40[renilla]:pGL3[firefly]), to act as a control for transfection efficiency. Lipofectamine LTX with PLUS reagent (Life Technologies, 15338-100) was used according to the manufacturer's protocol. Briefly, 40 μl of Opti-MEM™ (Gibco, 31985-070) was combined with 0.2 μl of PLUS reagent, 0.5 μl of LTX, 20 ng of pRL-SV40 and 180 ng of pGL3-miR-223 3'UTR reporter or pGL3-Control plasmid. The mixture was incubated at room temperature for 30 min then added to cells in serum-free media.

After 24 h, the cells were treated with either conditioned media (CoM) from PMNs (as generated above), samples from differential centrifugation of PD effluent (as described above) or transfected with *mirVana miRNA mimics* as a control. Transfection of the small miRNA mimics was performed with lipofectamine RNAi max (Life Technologies, 13778-075) according to the manufacturer's protocol. Briefly, 25 μl of Opti-MEM™ was combined with 0.75 μl of RNAi Max and 0.25 μl of the miRNA mimic (equivalent to final concentration of 10 ng, either miR-223-3p or a miR-Control mimic). This was added to each well alongside fresh serum-free media. After 48 h, the cells were lysed with passive lysis buffer from the luciferase kit used below (Promega, E1910) for assay of luciferase expression.

2.13.5 Luciferase assay

The activity of the firefly (*Photinus pyralis*) luciferase was used as an output for the reporter assay generated in this study. The co-transfection with a control plasmid containing the coding sequence for Renilla (*Renilla reniformis*) luciferase acts as a transfection efficiency control. The different origin, structure and substrate requirements allow discrimination between the bioluminescent reactions, using a Dual-Luciferase Reporter Assay System (Promega, E1910), in a single sample.

Briefly, the transfected and treated cells were washed with PBS, incubated with 65 μl of Passive Lysis Buffer at 37°C for 15 min. 20 μl of cell lysate was transferred into a white 96-well plate. 50 μl of Luciferase Assay Reagent II (LAR II, substrate for firefly luciferase) was

added prior to measurement of firefly luminescence on FLUOstar OPTIMA plate reader. Subsequently, 50 µl of Stop & Glo Reagent (quenches previous reaction and substrate for *Renilla* luciferase) was added, before measurement of Renilla luminescence (representative of transfection efficiency). The results were expressed as a relative ratio of Firefly:*Renilla* luminescence.

2.14 Statistical analysis

Statistical analyses were performed using Excel 2013 or GraphPad Prism 6.0. All variables were tested for normal distribution using D'Agostino & Pearson omnibus normality test. Comparison between two non-paired normal distributed groups (parametric) was performed by one-sample t test, whereas paired t test was used for matched data. For the data that did not pass the normality test (non-parametric), Wilcoxon test was performed for paired data and Mann-Whitney test was used for unpaired data.

Comparison among three or more groups was performed with ordinary one way-ANOVA for unpaired parametric data, with repeated measure one-way ANOVA for paired parametric data. While the comparison between matched non-parametric groups was performed by Friedman test, Kruskal-Wallis test was used for the comparisons between unpaired non- parametric groups. Descriptive statistics were described as mean ± standard error of the mean (SEM) unless otherwise stated.

All statistical tests were two-tailed; differences were considered statistically significant as indicated in the figures and tables: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2.15 In silico target prediction

miRNA target prediction programs are routinely used to find miRNAs that may regulate genes of interest and/or to identify possible targets of specific miRNAs. Diana, miRanda - mirSVR, miRDB and TargetScan are between the algorithms most commonly used for predictions and were chosen to identify possible new targets of the miRNAs of interest. An actualized summary Table 2.4 is displayed below to compare these tools. The threshold used for each one of these algorithms and comparison was Diana = 0.9, miRanda – mirSVR = - 0.95, miRDB = 80 and TargetScan = - 0.24. The use of these tools as well as their quality and reliability will be further discussed in Chapter 5.

Criteria	Diana	miRanda	miRDB	TargetScan
Seed match	+	+	+	+
Conservation	+	+	+	+
Free energy	+	+	+	
Site accessibility	+	+	+	
Target site abundance	+			
Machine learning	+	+	+	
References	451,452	453,454	455,456	457,458

Table 2.4: Features of different target prediction algorithms

Showing the main criteria used by the 4 target prediction algorithms utilised here to predict mRNA targets for specific miRs, adapted from[459].

3 Identification of altered microRNAs in PD-related peritonitis

3.1 Introduction

PD, as a treatment for end stage renal disease, is an effective renal replacement therapy due to the ultrafiltration and osmosis of waste products and water across the semi-permeable peritoneal membrane from the blood into the dialysis solution.[2] However, the presence of a permanent catheter, used for the exchange of dialysis solutions, greatly increases the risk of peritoneal infections, primarily by bacteria. The accompanying inflammatory reaction by the body can be detrimental for the patient, and ultimately cause technique failure if it is not treated effectively and rapidly. Current methods of identifying the causative pathogen, through culturing the organism, take a couple of days, meaning the patient is exposed to an empirical first-line treatment of broad-spectrum antibiotics, which come with possible drug-related side effects and the potential for anti-microbial resistance to develop.[78] Identification of the causative organism more rapidly is essential to be able to treat peritonitis episodes more accurately and rapidly, to enable the patient to remain on PD for a longer time, as the most convenient and cost-effective renal replacement therapy for many patients.[122,222]

It has been previously shown that the specific immune response evoked at the site of infection ('immune fingerprint') can be indicative of the causative pathogen, through soluble and cellular markers present in the PD effluent of patients, on day 1 of an acute peritonitis episode.[122] It is suggested that local miRNAs may play a role in this specific immune response as they are involved in many major cellular processes, including the response to infection and inflammation.[460,461] miRNAs in the PD effluent are stable and important in inflammation-related fibrosis in the peritoneum.[327] These small, endogenous, non-coding, single-stranded RNA molecules are post-transcriptional gene regulators that are estimated to control 30-60% of human genes. Their stability, location and possible functions in pathways involved in response to infection and inflammation suggests they may contribute to the immune fingerprint model already established.[222]

To investigate the potential of local microRNAs as pathogen-specific biomarkers, a screening of 377 miRNAs was conducted in PD effluent from patients presenting at UHW, Cardiff on day 1 of peritonitis (with a well-defined single-organism bacterial infection), comparing the most common Gram positive and Gram negative infections (coagulase

negative *Staphylococcus* and *E. coli*, respectively) with uninfected patient samples. 11 candidate microRNAs were identified as altered in one or both types of infection, compared to uninfected samples. This shortlist was refined to four candidates through a small validation cohort of patient samples, and these four were then investigated in 109 infected PD effluent samples, compared to 20 stable patient samples, collected from September 2008 to December 2017. These data generated show the potential of miRNAs to act as pathogen-specific biomarkers in patient PD effluent samples with levels of four miRNAs altered in infection.

3.1.1 Aims:

The aims of this chapter were to:

1. Identify candidate miRNAs altered in infection through screening of PD effluent samples with well-defined infections
2. Validate these findings in a small cohort of patients with well-defined infections and refine the candidate biomarker list
3. Validate changes in candidate miRNAs in full cohort of 129 PD effluent samples from patients infected by a broad range of bacterial organisms

3.2 Results

3.2.1 Optimisation

Cell-free samples present specific difficulties on normalisation of miRNA data, with no standard protocol or reference gene widely used.[462] Instead of normalisation to a reference gene, it may be possible to control for the amount of total RNA added into the RT-qPCR reaction at the start. However, this was not possible due to two specific modifications of the RNA extraction procedure for cell-free samples. Firstly, the extraction procedure is optimised to extract small RNA molecules, and as consequence many larger RNA molecules including mRNA are lost during the procedure, meaning the total RNA extracted is not the total RNA originally present in the sample. Also, the use of a "spike-in" control of a *C. elegans* miRNA not present in human samples (cel-miR-39) to assess the efficiency of the RNA extraction procedure skews the perceived RNA content if measured by standard methods. With cell-free samples and a column-based extraction method, there is the possibility for variation in the efficiency of extraction, therefore this is an important method of standardisation.

Here, I tested different candidate referencing methods to determine the best way of analysing miRNA data from cell-free PD effluent, including using different microRNAs, other small RNAs or the cel-miR-39 added in the extraction as internal reference, as well as presenting the raw, un-normalised data. In preliminary experiments with 5 infected and 5 uninfected PD effluent samples (Figure 3.1 A-D), 4 different potential reference genes were assessed; miR-191, miR-16, sno-135 and snRNA U6. miR-191 and miR-16 have been previously used for normalisation of miRNA data in other cell-free biological fluids such as urine, whilst sno-135 (small nucleolar RNA 135) and snRNA U6 (small nuclear RNA U6) have also been used in-house as reference genes for miRNA work. It was shown that snRNA U6 was the most consistent between the groups and was therefore used as reference to normalise the target miRNA data. Although snRNA U6 is different between stable and infected samples when a larger selection of patient samples was used (15 stable patients compared to 12 with Gram +ve and 12 with Gram -ve infections, Figure 3.1E), there is no difference between the two groups of infections tested here, suggesting this method of normalisation will be useful when comparing levels of miRNAs between different infections. Figure 3.1F shows there is no difference between cel-miR-39 levels across all three groups, suggesting the extraction is consistently performed, and that this also a possible reference gene.

To avoid problems with normalisation, data can be presented as raw data, or 40-Ct. This is the standard way of presenting raw RT-qPCR data where the Ct value has an inverse relationship with the amount present (i.e. high Ct means not very much is in the sample, and vice versa). The raw data is therefore presented as 40 (total number of cycles conducted in the qPCR reaction) minus the Ct value. However, as mentioned above, it was impossible to accurately detect the amount of RNA starting in these samples (after extraction), so any differences seen with this method could be due to different amounts of total RNA, or different efficiencies of RNA extraction. Nevertheless, any difference due to the efficiency of RNA extraction would also appear in the amounts of cel-miR-39 present, as the same amount of this was added at the start of the RNA extraction, and no difference in cel-miR-39 levels was detected (Figure 3.1F). Due to the problems with possible different amounts of total RNA present, a normalisation procedure was generally used, where possible. As described above, snRNA U6 was deemed the best of multiple options tried as a reference gene.
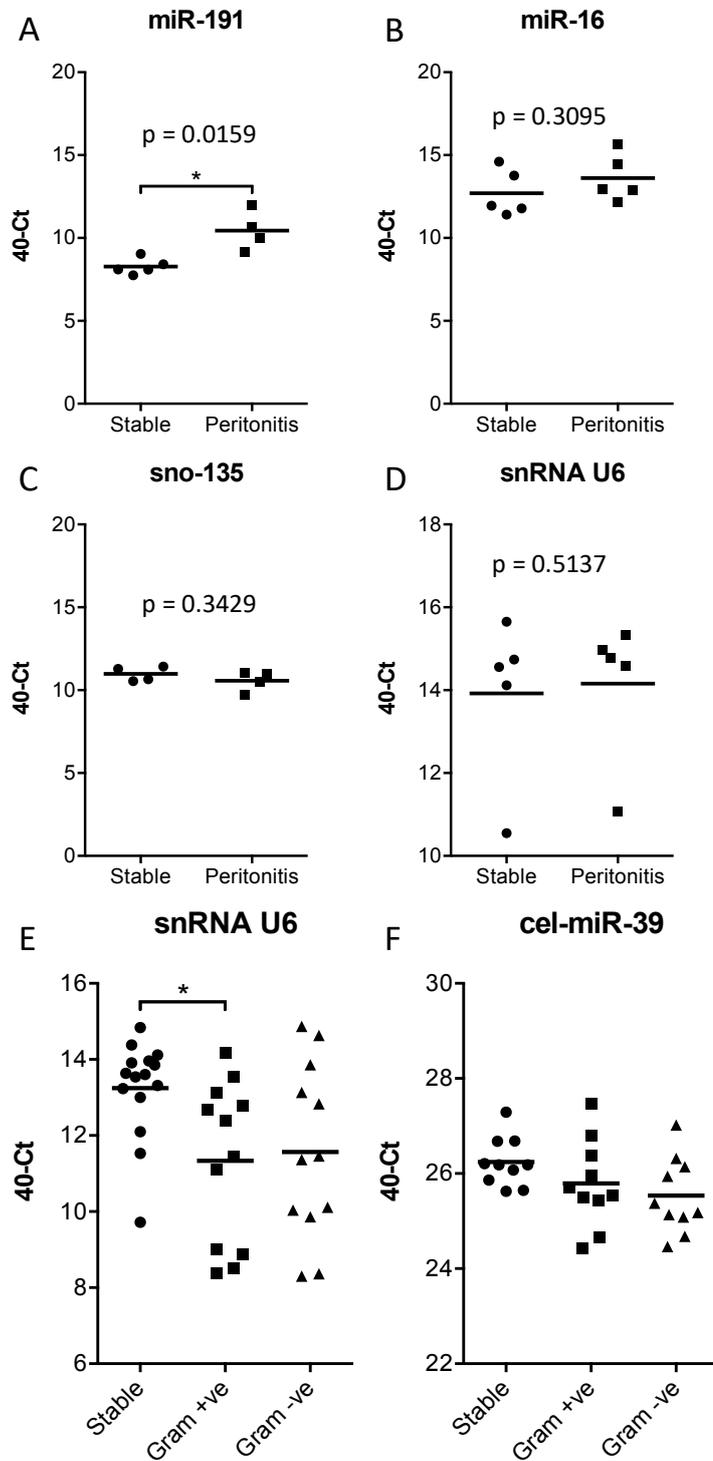
Figure 3.1: Screening of candidate reference genes in PD effluent samples
5 stable and 5 peritonitis PD effluent samples were tested for miR-191 (A), miR-16 (B), sno-135 (C) and snRNA U6 (D). E shows snRNA U6 levels and F shows levels of cel-miR-39 ("spike-in", control for the extraction procedure) in a larger patient cohort consisting of 15 stable PD effluent samples, 12 from patients with Gram +ve (Coagulase negative *Staphylococcus*, CNS) infections and 12 with Gram -ve (*E. coli*) infections. Data were analysed by Mann-Whitney test (A-D) or Kruskal-Wallis test (E-F) and significant differences are indicated: *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001.

3.2.2 Identification of infection-associated candidate microRNAs through unbiased screening

To study the potential role of miRNAs as pathogen-specific biomarkers in PD-related peritonitis episodes, I aimed to identify candidate miRNAs which may have important roles in this disease pathway. TLDA cards were used to look at the expression patterns of 377 miRNAs (see Table in appendix 9.2) in 30 PD effluent samples. Prior to testing these 30 samples, optimisation of the experimental conditions was required, by determining whether an extra amplification step, after the reverse transcription, was necessary. This is often the case in samples where the RNA content is low, and due to the inability to accurately quantify the levels of RNA extracted from PD effluent samples, this was thought to be necessary. To test this, 10 stable PD effluent samples were pooled and run on separate TLDA cards both with and without the pre-amplification step. When no pre-amplification took place, only 12 miRNAs amplified above the limit of detection, whereas 122 amplified when the pre-amplification step was included (Figure 3.2). This indicated that the extra processing was necessary to be able to detect as many miRNAs as possible, and therefore was included in future experiments.

For the subsequent TLDA screening, 10 PD effluent samples each from 3 groups of individuals receiving PD were pooled together: uninfected patients (stable), those with a CNS infection (Gram +ve) and those with an *E. coli* infection (Gram –ve). Samples were pooled to attempt to average out the inter-sample differences, as well as to be mindful of limited resources and sample volumes at this preliminary, discovery stage. CNS is the most common Gram +ve organism in PD patients, whilst *E. coli* is the most common Gram -ve organism. These two organisms cause the majority of peritonitis episodes at this site (UHW, Cardiff), and at this stage of the project they were considered sufficiently representative of other Gram +ve and Gram -ve bacteria. All infected samples were taken from the first cloudy bag on day 1 of presentation at hospital, before antibiotic treatment commenced. Uninfected patients had not experienced an episode of peritonitis for at least 3 months prior to inclusion in this study.

As shown in Figure 3.3, changes in the abundance of 11 miRNAs were detected, as defined by greater than two-fold changes in relative expression compared to stable samples,
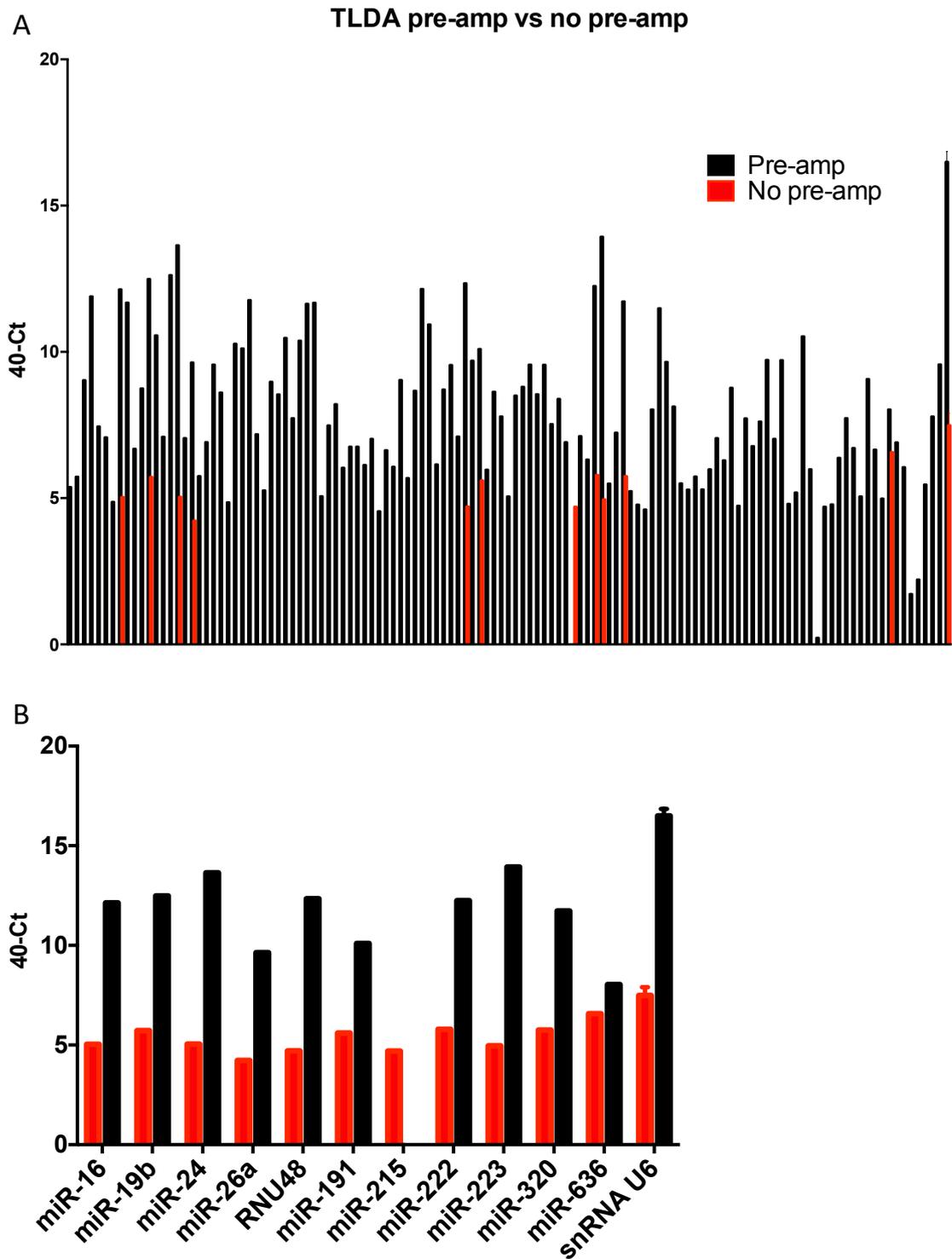
Figure 3.2: Optimisation of TLDA methodology
10 pooled stable PD effluent samples were run on the TLDA system, both with (black) and without (red) a pre-amplification step, to assess whether this extra step was necessary. (A) shows all miRNAs that amplified across both plates, with 122 miRNAs amplifying when the pre-amplification step was used, as opposed to 12 miRNAs when it was not used. (B) shows the 12 miRNAs that amplified without pre-amplification and the differences observed when pre-amplification was employed.
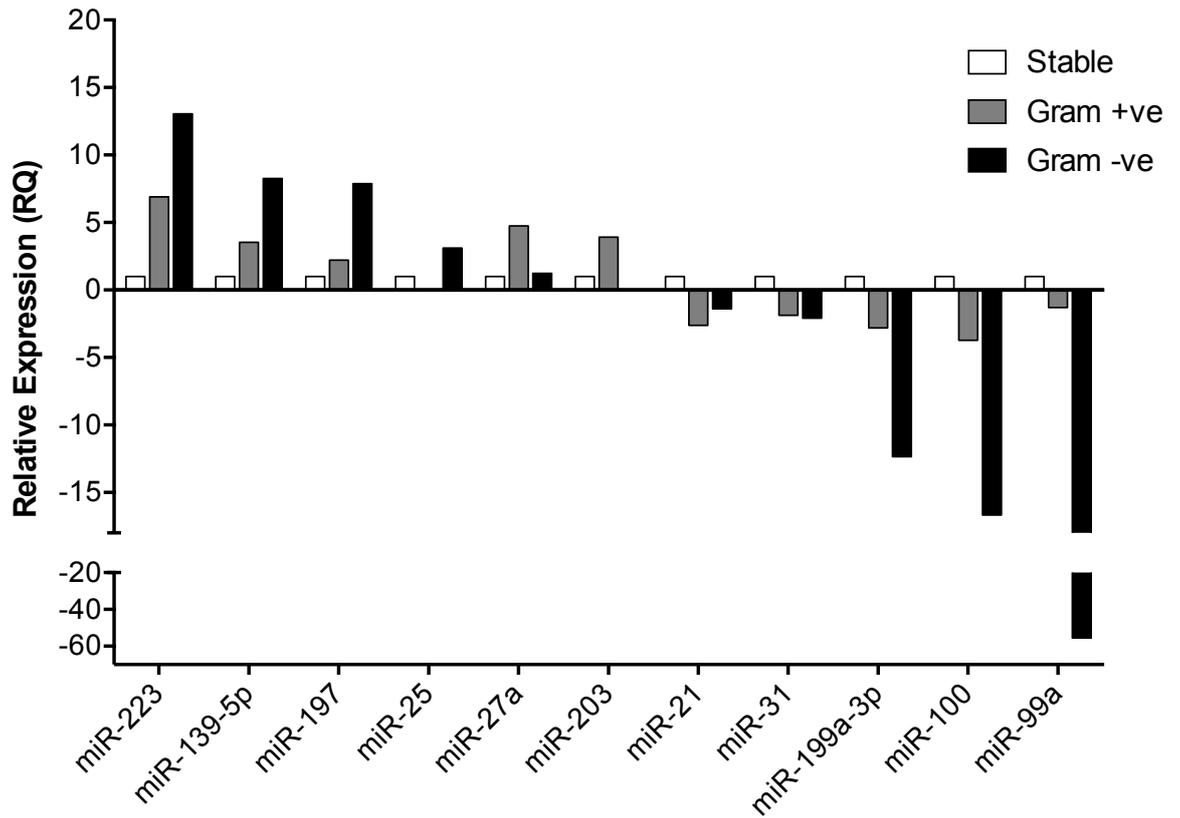
Figure 3.3: Screening of microRNA candidates
10 pooled samples from stable, Gram +ve (Coagulase negative *Staphylococcus*, CNS) infected and Gram −ve (*E. coli*) infected patient PD effluent samples screened for 377 human microRNAs using a TLDA (TaqMan low density array) card. 11 candidate microRNAs identified with altered levels in different infections and compared to uninfected PD effluent. Data shown as mean values from global normalisation.

normalised to global expression of all targets across the whole plate. As is standard for TLDA cards with patient samples, global normalisation was used for these data, not normalisation to snRNA U6 which will be used for individual patient PD effluent samples in future experiments, due to the variability both within a plate and across multiple plates. Levels of three miRNAs (miR-223, miR-139 and miR-197) were increased in both types of infections, and with higher levels in Gram -ve than Gram +ve infected PD effluent. Two miRNAs were detected in two of the sample groups only; miR-25 was only detected in stable and Gram -ve infected samples, whilst miR-203 was only detected in stable and Gram +ve infected samples. Increased miR-27a was detected in Gram +ve infected samples whilst there were comparable levels in the Gram -ve infected and stable samples. Detected levels of two miRNAs (miR-21 and miR-31) decreased in both types of infections compared to uninfected samples. Three miRNAs (miR-199a, miR-100 and miR-99a) also showed decreased levels in both types of infection, with lower levels in Gram -ve infected samples, compared to Gram +ve infected samples. This confirmed that miRNA levels are altered in acute PD-related peritonitis episodes, compared to uninfected PD effluent, and that they may be altered in different types of infection. Taken together, this screening resulted in the identification of 11 candidate miRNAs to investigate further for their potential to serve as diagnostic biomarkers.

3.2.3 Validation of candidate microRNA changes in individual patient PD effluent samples

To validate the changes in the 11 candidate microRNAs seen in the pooled samples, they were measured individually in a set of 48 well-characterised samples, consisting of 10 stable samples, 20 with well-defined CNS infections (Gram +ve) and 18 with confirmed coliform infections (Gram -ve, 15 *E. coli*, two *Enterobacter* and one *Klebsiella* infections). 30 of these samples were those already used for the TLDA cards, whilst a further 18 were added to increase the power of this validation. The aim of these experiments was to validate the changes observed with pooled samples, on the high-throughput screening TLDA card, in individual samples with separate RT-qPCR reactions for each miRNA.

As shown in Figure 3.4Ai, miR-223 levels were increased in both types of infection, compared to stable samples, although there was no difference in the levels between the two types of infection (Figure 3.4Aii). In contrast, miR-139 and miR-197 did not show the
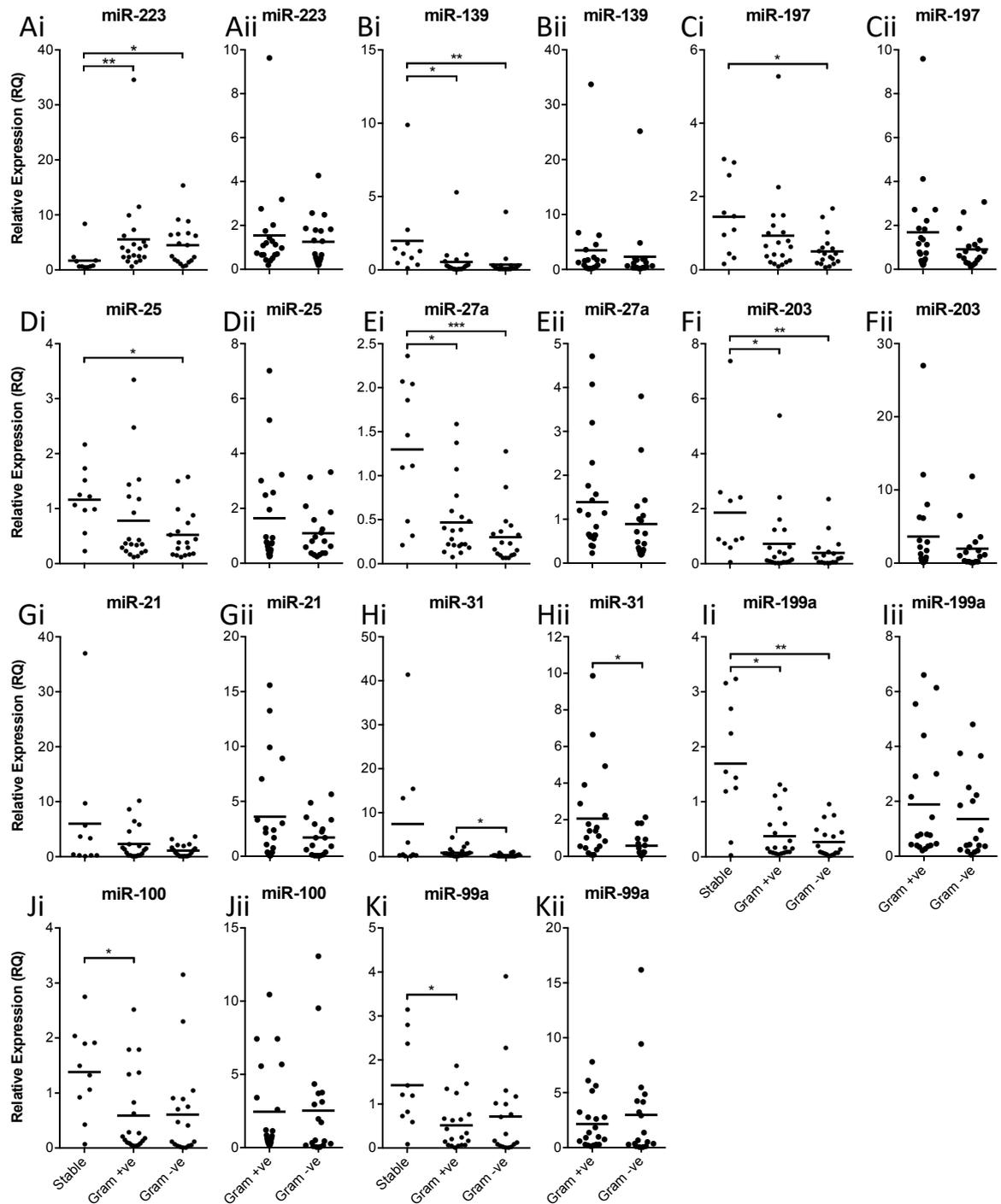
Figure 3.4: Using a small patient cohort to identify the 4 most promising miRNA candidates

11 miRNAs identified as promising candidates from TLDA screening were tested in a validation cohort of 10 stable, 20 Gram +ve (CNS) and 18 Gram –ve (15 with *E. coli*, 2 with *Enterobacter* and 1 with *Klebsiella* infections) infected patient PD effluent samples. Each miRNA was normalised to snRNA U6 and either stable samples (i) or Gram +ve (ii) infected samples. 11 candidates were miR-223 (A), miR-139 (B), miR-197 (C), miR-25 (D), miR-27a (E), miR-203 (F), miR-21 (G), miR-31 (H), miR-199a (I), miR-100 (J) and miR-99a (K). Data were analysed by Kruskal-Wallis test and significant differences are indicated: *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001.

same increase as observed **in** the TLDA screening (Figure 3.4B-C). These two miRNAs were therefore excluded from further analysis. miR-25 and miR-203 were also excluded from further analysis as they had been undetectable in one of the three groups in the screening dataset but were present in all individual samples tested here (Figure 3.4D and 3.4F). For miR-199a, miR-100 and miR-99a, the decrease in infection seen in the TLDA screening could be reproduced using individual samples (Figure 3.4I-K). However, the levels of these three miRNAs was low, near the detection limit, with not all samples amplifying (data not shown), therefore these three were excluded from further analysis. The pattern of miR-27a seen in the screening dataset could not be replicated here (Figure 3.4E). Previously it was increased in Gram +ve infections compared to both other sample types, however the data from the individual samples suggested that miR-27a was decreased in both types of infection. This miRNA was, however, still included due to its high overall levels, and the strong decrease in infected PD effluent samples, compared to uninfected stable samples. miR-21 and miR-31 were present at high levels in all samples, as well as a trend to decreased levels in infection for both (Figure 3.4G-H). Despite these changes not being statistically significant, these two miRNAs were taken forward for subsequent analysis, given their previously shown strong association with peritoneal inflammation and fibrosis.[327,463] Notably, miR-31 was the only miRNA to show a difference between the two types of infections (Figure 3.4H), with lower levels in Gram -ve infected PD effluent compared to Gram +ve infections. This important observation suggests that miRNAs may be useful as pathogen-specific biomarkers to help distinguish different causative organisms and inform treatment decisions more accurately. The final shortlist of four candidate miRNAs (miR-223, miR-27a, miR-21 and miR-31) was examined in more detail in the remainder of this thesis.

3.2.4 Validation of candidate microRNAs in a real-world patient cohort

To confirm whether these four candidate biomarker miRNAs could be used in patients to predict early peritonitis and even distinguish between different types of infections, the miRNA analysis was extended to the full cohort of patients presenting at UHW, Cardiff on day 1 of infection, between September 2008 and December 2017. The purpose of this was to investigate a large number of samples, which included a range of different Gram +ve and Gram -ve infections as seen in clinical practice. To reach this aim, more samples were needed to increase the power of this study and to see if the differences observed in the relatively small dataset with well-defined CNS and *E. coli* infections were still valid in a large cohort representative of the variability across the PD community in the UK and beyond.

This cohort consisted of 109 infected PD patients presenting with acute peritonitis caused by a range of organisms: Gram +ve organisms (n=77), including CNS (n=40), *S. aureus* (n=9) and coryneform species (n=2); confirmed Gram -ve organisms (n=32) included *E. coli* (n=18), other coliform species (n=4), *Pseudomonas spp.* (n=3), *Acinetobacter spp.* (n=3), and others (see Table in appendix 9.1 for full list of samples and types of infections). These 109 samples were compared to 20 stable, uninfected PD effluent samples from PD patients who had not had a peritonitis episode for at least 3 months prior to inclusion in this study.

As shown in Figure 3.5A, miR-223 was greatly increased in both Gram +ve and Gram -ve infections, compared to stable uninfected PD effluent samples. miR-27a showed a significant decrease in levels in both types of infection, compared to stable samples (Figure 3.5C). These data confirmed the earlier findings, demonstrating that the observed changes in miRNA levels in well-defined infections caused by two representative organisms translates to the full cohort of patients infected by a wide spectrum of organisms. Both these miRNAs showed no difference in detected levels between Gram +ve and Gram -ve infections (Figure 3.5B and 3.5D). miR-21 showed a similar pattern as before, with lower levels in both types of infected samples compared to those without an infection (Figure 3.5E). Earlier data with a smaller number of patient samples suggested that miR-31 levels were lower in Gram -ve infected patient samples (Figure 3.4H), compared to Gram +ve. This was not seen here (Figure 3.5H), instead there was a difference in the levels of miR-21 between the two different types of infection. There were significantly lower levels of miR-21 in samples from patients with a range of Gram -ve infections, compared to those with infections caused by Gram +ve organisms (Figure 3.5F). miR-31 was decreased in both types of infections, compared to stable samples (Figure 3.5G), confirming earlier results, despite there being no difference in levels between the different types of infections (Figure 3.5H). When the samples from patients infected with CNS and *E. coli* were removed from the analysis, the same directions of altered levels were seen, but the limited numbers of samples meant the magnitude of differences was greatly reduced (data not shown), indicating it is not merely an effect of the main causative organisms skewing the data. These findings mirror the trends from the screening data, with small alterations in magnitude of changes, and therefore confirmed the diagnostic potential of these four microRNAs in patients presenting with acute PD-related peritonitis.
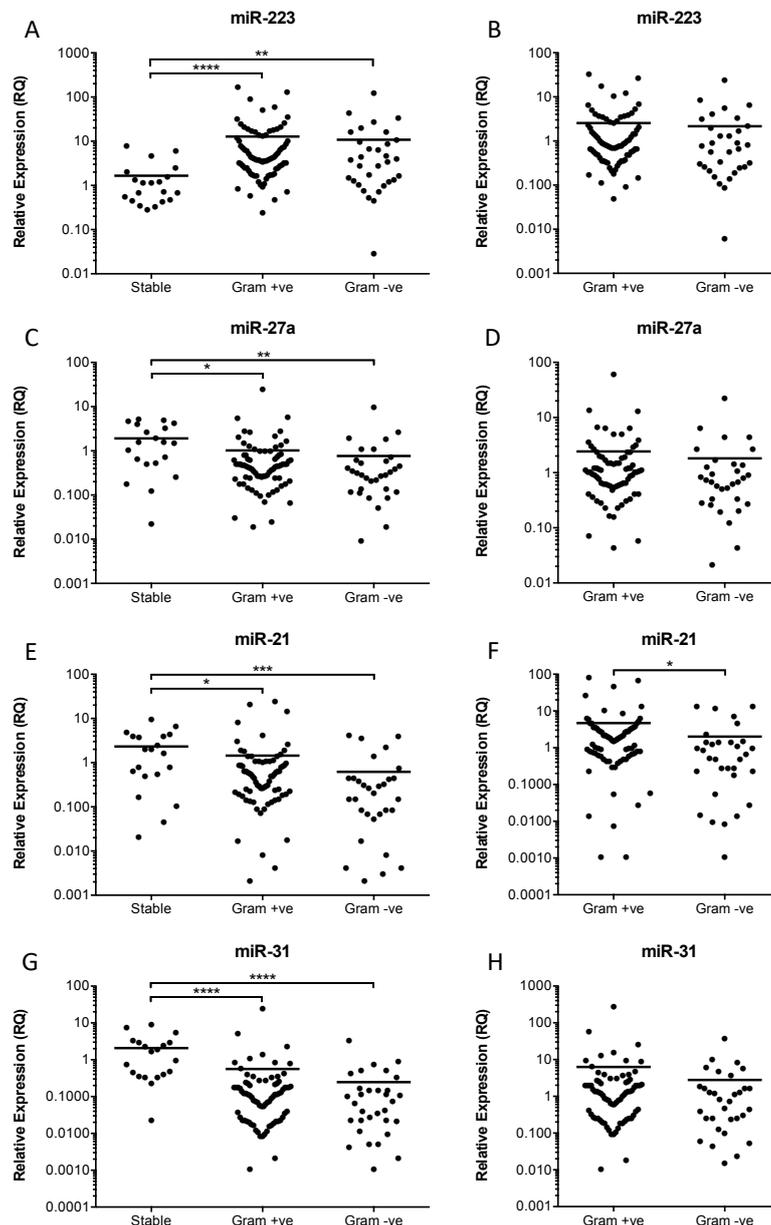
Figure 3.5: 4 candidate microRNAs are altered in infections, in cohort of patient PD effluent samples

Four candidate miRNA biomarkers were measured in a real-world patient cohort consisting of 20 stable uninfected PD effluent samples, 77 with Gram +ve infections and 32 with Gram -ve infections. miR-223 (A-B), miR-27a (C-D), miR-21 (E-F) and miR-31 (G-H) were normalised to snRNA U6 and either stable samples (A, C, E and G) or Gram +ve infected samples (B, D, F and H). Data were analysed by Kruskal-Wallis test and significant differences are indicated: *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001.

3.2.5 miRNAs in resolution of infection

As the levels of the four candidate miRNA biomarkers are altered in infection, it was interesting to investigate whether they could be indicative of the resolution of an acute peritonitis episode. Using longitudinal PD effluent samples from three patients over the first week of infection, the four candidate miRNAs were measured, normalised to snRNA U6, and compared to the levels in a stable sample from the same patient before presenting with peritonitis. Each patient, when they enrol on the clinical study which these samples are from, has a stable PD effluent sample taken as a baseline to measure against. This was used for the three patients shown in Figure 3.6. miR-223 (Figure 3.6A) showed a strong decrease across the first week of infection towards the levels seen in the respective stable PD effluent samples, in all three patients. This confirmed what we expected to see, as we predicted miR-223 would be a representative a marker of the acute response to infection and therefore would decrease with the resolution of the acute immune reaction. It is thought that the reduction in miR-223 is representative of the resolution of infection, with decreased inflammatory mediators and immune cells present. The other three miRNAs showed more variability across the three patients. miR-27a (Figure 3.6B) levels showed an increase towards the level in the stable PD effluent levels in one patient, a decrease in another patient and no change in the third patient. miR-21 (Figure 3.6C) levels showed similar variability as miR-27a, whilst miR-31 (Figure 3.6D) showed an increase in two patients over the week towards the level in the stable PD effluent sample, whilst one patient had the opposite pattern of expression. This inconsistency between samples was not unexpected due to the variability seen with patient samples, and the number of factors that contribute to the difference between individuals. The small number of patients followed in these experiments meant they could only be used as a preliminary investigation into the role of miRNAs in the resolution of infection. Nevertheless, miR-223 looked promising as it robustly decreased across all three patients investigated during the course of a week from the start of an acute peritonitis episode towards resolution.

3.2.6 Correlations to cellular data

miRNAs are both produced and act inside cells, although they can be found outside cells, either associated with protein or extracellular vesicles, where they are involved in intercellular signalling. It was therefore thought that the peritoneal levels of the candidate miRNA biomarkers may be indicative of the levels of different cell types present in PD
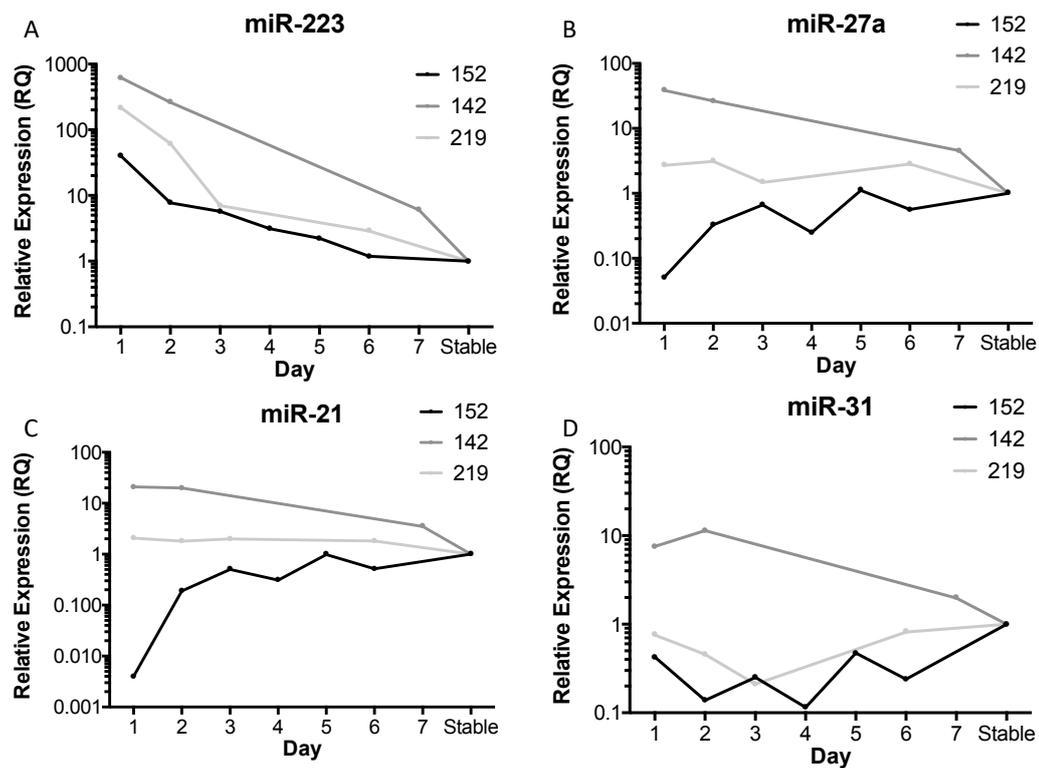
Figure 3.6: miRNAs in resolution of infection
Showing how the four miRNA candidate biomarkers change during the resolution of three acute peritonitis infection episodes from three PD patients. miR-223 (A), miR-27a (B), miR-21 (C) and miR-31 (D) were measured in PD effluent samples from patients across the first week of a peritonitis episode, as well as a stable PD effluent sample from before they had an infection. All data are normalised to snRNA U6 and the corresponding stable sample from each patient. Individual patient samples are plotted separately.

effluent. This could help to identify the potential cellular source of the cell-free miRNAs detected. Both the raw and normalised levels of the four miRNAs were compared to the total cell count, the number of live macrophages (CD14+) and the number of live neutrophils (CD15+) in the PD effluent. Total cell count was selected to eliminate the possibility that these miRNAs were merely surrogate markers of the total number of cells present. Neutrophils are the most abundant cell type present in infected PD effluent, and it is conceivable that they may be a major source of the cell-free miRNAs. As macrophages are another very abundant cell type, they were also investigated for correlations with the miRNA data. Data were compared for 82 patient samples, where all cellular and miRNA data needed for such analyses were available. The cellular data was kindly generated and shared by Dr Ann Kift-Morgan. Linear regressions were plotted for all four miRNAs (40-Ct and RQ data) and snRNA U6 (used for normalisation, 40-Ct only), with graphical representation shown in Figure 3.7, and the statistical analysis shown in Table 3.1.

For all correlations, the r^2 values (representative of how closely the data fit the linear regression line) were very low. This was due to the significant variation associated with patient samples and indicated that any correlations were not particularly strong. However, there were multiple statistically significant correlations ($p < 0.05$), notably between miR-223 (40-Ct) and all cellular levels (total, macrophages and neutrophils) (Figure 3.7Gi-iii). This suggests that miR-223 may be important and relate to the cells that infiltrate the peritoneum during infection. miR-27a (40-Ct) also showed the same pattern of correlating with the cellular levels (Figure 3.7Ei-iii), with similar conclusions. Notably, raw snRNA U6 levels (40-Ct) also correlated with the levels of total cells and macrophages (Figure 3.7Ii-iii). As snRNA U6 is present inside cells in the spliceosome and is not normally released, this is a logical correlation to observe. As snRNA U6 levels were used for normalisation, this would explain why there were no further correlations between the cellular data and the normalised miRNA data. It is important to note that all correlations, despite being statistically significant, were not very strong with low r^2 values. These r^2 values are the main indicator of the possible importance of a correlation as it shows the amount of variance that the linear regression (correlation) accounts for, in the dataset. This is best shown by the graphical representations, where any statistically significant correlations were almost indistinguishable from those that are not significant, suggesting they might be an artefact of the statistical tests with a variable and relatively small cohort of patient samples, together with considerable patient to patient variation.

Ai miR-21 (40-Ct) vs total cells

Aii miR-21 (40-Ct) vs macrophages

Aiii miR-21 (40-Ct) vs neutrophils

Bi miR-21 (RQ) vs total cells

p = 0.0296*
r² = 0.05148

Bii miR-21 (RQ) vs macrophages

Biii miR-21 (RQ) vs neutrophils

Ci miR-31 (40-Ct) vs total cells

Cii miR-31 (40-Ct) vs macrophages

p = 0.0327*
r² = 0.05576

Ciii miR-31 (40-Ct) vs neutrophils

Di miR-31 (RQ) vs total cells

Dii miR-31 (RQ) vs macrophages

Diii miR-31 (RQ) vs neutrophils

Ei miR-27a (40-Ct) vs total cells

p = 0.0042**
r² = 0.08768

Eii miR-27a (40-Ct) vs macrophages

p = 0.0049***
r² = 0.09485

Eiii miR-27a (40-Ct) vs neutrophils

p = 0.0287*
r² = 0.05844

Fi miR-27a (RQ) vs total cells

Fii miR-27a (RQ) vs macrophages

Fiii miR-27a (RQ) vs neutrophils

96

Figure 3.7: Correlations of miRNA data with cell data

Showing raw data (40-Ct) of miR-21 (A), miR-31 (C), miR-27a (E), miR-223 (G) and snRNA U6 (I), alongside miRNA data normalised to snRNA U6 levels for miR-21 (B), miR-31 (D), miR-27a (F) and miR-223 (G), correlated to the total cell count (i), number of live CD14+ macrophages (ii), and the number of live CD15+ neutrophils (iii). Data shown on $\log_{10}$ scale for cell counts and normalised miRNA data, with data for 82 PD effluent samples available, from patients with acute peritonitis episodes. Colours represent types of infections, red for Gram +ve infections, blue for Gram -ve infections and green for no growth in culture results. Linear regression was plotted, with statistics (p and r^2 values) shown on panels where statistical significance was reached (p<0.05) and in Table 3.1.

	Total cells		Macrophages		Neutrophils	
	p value	r^2 value	p value	r^2 value	p value	r^2 value
miR-21 40-Ct	0.5111 ns	0.004812	0.4090 ns	0.008537	0.6642 ns	0.002368
miR-21 RQ	0.0296 *	0.05148	0.1147 ns	0.03081	0.0539 ns	0.04565
miR-31 40-Ct	0.0773 ns	0.03428	0.0327 *	0.05576	0.1649 ns	0.02397
miR-31 RQ	0.3673 ns	0.009039	0.6421 ns	0.002713	0.4067 ns	0.008622
miR-27a 40-Ct	0.0042 ***	0.08768	0.0049 ***	0.09485	0.0287 *	0.05844
miR-27a RQ	0.0500 ns	0.04201	0.1379 ns	0.02731	0.2018 ns	0.02028
miR-223 40-Ct	0.0007 ****	0.1198	0.0388 *	0.05230	0.0120 *	0.07623
miR-223 RQ	0.4807 ns	0.005540	0.5949 ns	0.003551	0.4694 ns	0.006563
snRNA U6 40-Ct	0.0042 ***	0.08732	0.0078 **	0.08526	0.0724 ns	0.03979

Table 3.1: Statistics from linear regressions of correlations between miRNA levels and cellular markers
Showing the p values and r^2 values from the linear regressions conducted between raw miRNA levels (40-Ct) or normalised relative quantification (RQ) to snRNA U6, of 4 miRNAs (miR-21, miR-31, miR-27a and miR-223), and total cells, number of macrophages or number of neutrophils. Data from PD effluent from 82 patients with acute peritonitis. Significant correlations are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$; ****, $p<0.001$.

The original immune fingerprint model (Figure 1.1) suggests that the total number of cells is important to distinguish Gram +ve infections from all other types of infections. However, what is clear to see from Figure 3.7, is that this factor alone cannot absolutely separate these types of infected samples (represented by red symbols) from all others. This indicates the power of the immune fingerprint is due to multiple interacting factors, not merely one important difference.

3.2.7 *In vivo* models of peritonitis

In human patients, the exact time point of the beginning of the infection is unknown when a patient presents at hospital, the microbiological culture results may be erroneous, different dwell times and different dialysate solutions may affect peritoneal concentrations of immune cells and soluble mediators, and patients may have underlying conditions that might affect miRNA expression in the peritoneal cavity. To circumvent these limitations, we took advantage of a well-defined mouse model that allowed us to study the time course of miRNA expression after the onset of infection, in the absence of other confounding factors. We therefore measured the levels of the four miRNAs of interest in a model of peritoneal infection established by Dr Anne-Catherine Raby and colleagues.[464] Briefly, C57BL/6 mice aged 8-12 weeks had intraperitoneal (i.p.) injection of either PBS or live *E. coli*, and the mice were sacrificed at the indicated time points. All four miRNAs were detected in peritoneal lavages of these mice, both in the infected group and in mock-treated controls. Increased abundance of miR-223 was detected in infected mice (Figure 3.8A), as is seen in the human PD effluent samples from infected patients (Figure 3.5A). miR-27a levels were comparable in infected mice and controls (Figure 3.8B), in contrast to the decrease seen in PD effluent samples from infected patients (Figure 3.5C). miR-21 levels increased in infected mice (Figure 3.8C), which differed from the patient PD effluent samples, where levels decreased with infection (Figure 3.5E). Finally, there was a modest decrease in the levels of miR-31 in infected mice (Figure 3.8D), similarly to infected patient samples (Figure 3.5G). While the mouse model data did not exactly mirror the human pathology for all miRNAs studied, these findings suggested that miR-223 and miR-31 may have similar roles in the response to infection in humans and in mice. However, these findings also highlighted the limitations of using mouse models to understand human pathology as they indicated there are important differences in the regulation of miRNA expression between humans and mice, at least in the specific infection model used in the
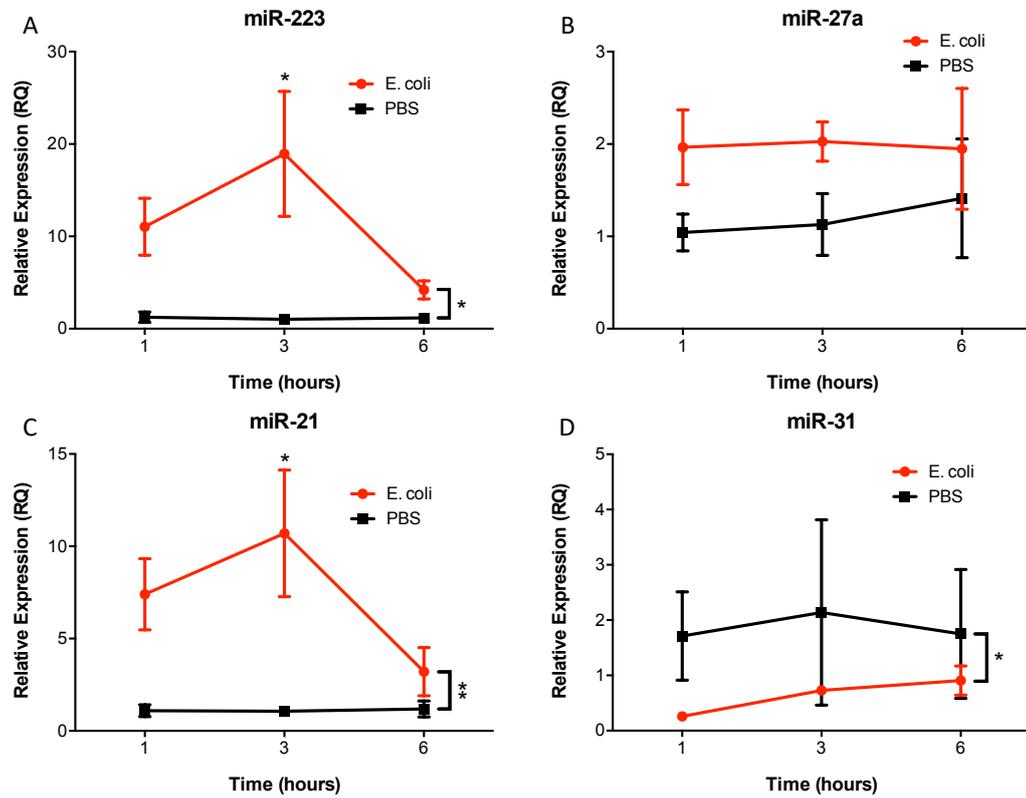
Figure 3.8: Candidate miRNAs are altered in peritoneal *E. coli* infections in mice
6 mice with i.p. injection of live *E. coli* bacteria (red) and 3 mice with i.p. injection of PBS (black) were culled at the specified time-points and the peritoneal cavity (site of infection) was washed with PBS, the 4 miRNAs were then detected in these peritoneal lavages. miR-223 (A), miR-27a (B), miR-21 (C) and miR-31 (D) were normalised to snRNA U6 and the PBS control. Data were analysed by two-way ANOVA and significant differences are indicated: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$. Data shown as mean values $\pm$SEM.

present study. Ultimately, these data reiterate the importance of using human samples and models to assess changes that will be relevant to patients, and transferable to the clinic.

3.3   Discussion

In this chapter, I show that miRNAs can be detected in PD effluent from both un-infected and infected patients, and that levels are altered in PD-related peritonitis episodes. miRNAs show potential as pathogen-specific biomarkers in PD-related peritonitis, with the aim of eventually helping to speed up diagnosis of the causative organism in each episode. This will reduce the exposure of the patient to broad-spectrum antibiotics and hopefully allow more patients to recover from peritonitis episodes and resume PD as their RRT.

3.3.1   Reference miR choice

An important consideration when looking at miRNA data is the normalisation strategy. There is no consensus on a standard normalisation procedure, as there is with the quantification of messenger-RNA (mRNA) levels where 'house-keeping' genes such as GAPDH (glyceraldehyde 3-phosphate dehydrogenase) or β-actin are often used.[462] Detection of cell-free miRNAs poses even more of a problem in finding a suitable reference gene, due to the variety of RNA species released by cells, with varying levels depending on the type of stimulus. For this study, it was important to find a suitable reference gene that did not change between different infections, as the primary aim was to identify miRNAs that were altered in response to different microbes. Also, it is highly likely that the radical changes seen in PD effluent during infection, compared to uninfected PD effluent, would mean it might not be possible to find a small RNA molecule that is unchanged between infected and stable PD effluent. From a small initial screening cohort, it was predicted that snRNA U6 would be the most appropriate reference gene to normalise the miRNA data to, as it did not appear to change much in infection. However, when a larger cohort was used, this was not the case and the levels were significantly different between stable and infected PD effluent. When different infections were compared, there was no difference between the levels in PD effluent from patients with Gram +ve or Gram -ve infecting organisms. Due to the problems mentioned above for finding a suitable reference gene for cell-free miRNAs, it is not uncommon to use a "spike-in" control to normalise the data to. This method controls only for the consistency of the RNA extraction, RT and qPCR procedures, rather than the amount of RNA in the sample to start with. When observed in these

samples, the levels of cel-miR-39, which was spiked in during the RNA extraction procedure, were consistently high and not altered between samples. These data show the technical reproducibility and reliability of the protocols from RNA extraction through to RT and qPCR. However, this is not the best way to normalise data as it does not take into account the total RNA level in the different samples to start with, therefore snRNA U6 was used for the majority of samples analysed.

3.3.2 miRNAs in PD effluent

As shown previously, miRNAs are stable in PD effluent and have the potential to be useful biomarkers.[327] Much work has been done on their role in PD-related peritoneal membrane fibrosis and in relation to the peritoneal membrane transport characteristics[70,442] and a number of miRNAs have been associated with changes including miR-15a, miR-17, miR-21, miR-30, miR-192, miR-221, miR-327, miR-377, and miR-589.[326,329,465] A number of miRNAs have also been associated with EMT/MMT. Some miRNAs are promoters or markers of increased peritoneal membrane fibrosis or EMT/MMT, including miR-9, miR-21, miR-23, miR-34a, miR-122, miR-182, miR-199, miR-200c, miR-214, miR-292, miR-296, miR-488 and miR-589.[319,322,323,327,466–470] Other miRNAs are downregulated with fibrosis, including miR-29b, miR-30a, miR-31, miR-93, miR-100, miR-129, miR-152, miR-192, miR-194, miR-200a, miR-200b and miR-497.[317,318,321,443,468] The majority of these previous studies have looked at cellular miRNAs, with limited study on cell-free miRNAs. Those that have investigated miRNAs in the circulation show serum miRNAs can be used as a marker of peritoneal fibrosis in a mouse model and in PD patients,[329] emphasising the importance of extracellular miRNAs. Whilst cell-free miRNAs in PD effluent have been used as markers of peritoneal fibrosis,[327] there are no known published studies relating to cell-free miRNAs as markers of infection in PD effluent, although one group has looked at miRNAs in ascites as markers of spontaneous bacterial peritonitis.[449] This current work shows the potential of cell-free miRNAs as biomarkers of infection, with increased levels of miR-223 and decreased miR-21, miR-31 and miR-27a in cell-free PD effluent on day 1 of an acute PD-related peritonitis episode, compared to levels in stable non-infected patients.

3.3.3 Candidate biomarkers

Identified here are four candidate biomarkers that can be used to distinguish infected PD effluent from uninfected PD effluent. These changes were first identified through screening

a wide range of miRNAs and replication in individual patient samples. The validation in a large patient cohort has shown the importance of these four miRNAs as potential biomarkers in acute PD-related peritonitis, at the site of infection.

3.3.3.1 miR-223

miR-223 increases with infection, regardless of the infecting organism. This miRNA, located on the X chromosome, has been studied widely in a range of different diseases, with particular emphasis on its role in cancer.[471] miR-223 is exclusively expressed in cells of a myeloid lineage, and consequently shows a strong association with leukaemia and lymphoma.[471–473] However, the potential for miRNAs to be transferred between immune and cancer cells, alongside the role of this miRNA in inflammation, indicated that miR-223 has a role in solid tumours.[471,473] Most interesting for this study is the role of miR-223 in inflammatory disorders, with a wide range of studies implicating miR-223 as a biomarker for diagnosis or progression, a role in the pathogenesis of inflammatory diseases, and in the response of the host.[473,474] For example, its role in sepsis has been widely studied, with the majority of studies suggesting miR-223 is a good prognostic biomarker and can be used to distinguish sepsis from Systemic Inflammatory Response Syndrome (SIRS) and healthy controls.[474–476] There is, however, some contradictory evidence suggesting this is not always the case.[477] Several groups have also looked at miR-223 in the context of rheumatoid arthritis (RA). They suggest that miR-223 may have a role in the pathogenesis via an IL-6 feedback loop, with an important role as a systemic biomarker, with the potential to distinguish osteoarthritis (OA) from RA accurately.[478–481] The limited number of studies investigating miR-223 in infection are mostly investigating animal models. miR-223 is increased in HIV, HCV, *Staphylococcus* and *Streptococcus* infections, with an important role in the regulation of inflammation.[482–486] miR-223 knockout (KO) animals show spontaneous inflammation and an aggravated inflammatory response to LPS or lung injury, suggesting miR-223 has a role in controlling inflammation.[359,359,410,473,487] Another study showed that miR-223 is able to distinguish between spontaneous bacterial peritonitis and peritoneal carcinomatosis, suggesting the inflammatory reaction to infection is different to that induced by cancer.[449] This, along with the role for miR-223 in macrophage polarisation and neutrophil maturation,[359,473,488–491] suggests miR-223 may be an important regulatory of the acute inflammatory response to infection, as is seen here with the increase in response to bacterial peritonitis. This may be a non-specific upregulation due

to acute inflammation rather than specific bacterial infection, which is why there is no difference between Gram +ve and Gram -ve infected patients.

3.3.3.2 miR-27a

This thesis shows a decrease in miR-27a in infected PD effluent, compared to stable PD effluent, regardless of infection type. miR-27a is part of the intergenic miR-23a/24-2/27a cluster on chromosome 19p13.13, which is highly conserved between vertebrates.[492] miR-27a has been implicated in a wide range of pathologies, from cancer to CVD. It is thought to be an oncomiR (i.e. oncogenic miRNA) in many cancers including breast cancer, hepatocellular carcinoma (HCC) and prostate.[493–496] However, there is contradicting evidence that miR-27a may act as a tumour suppressor in some cancers, including leukaemia, colorectal cancer and glioma.[497–499] One much-studied aspect of miR-27a is the different polymorphisms and how they relate to disease risks. One specific single nucleotide polymorphism (SNP) that has been associated with increased risk of breast cancer and heart disease is rs895819, where a G is substituted for an A and the level of total miR-27a is altered.[493,500] miR-27a is also altered in inflammatory pathologies, autoimmune conditions and infectious diseases. For example, miR-27a is increased in CD4$^+$ T cells from multiple sclerosis (MS) patients during an acute relapse,[501] whilst it has a protective role against inflammation- or sepsis-induced organ injury.[502–504] miR-27a can inhibit some viral infection models,[505,506] but it is increased in parasitic infections such as cerebral malaria.[507] This well-established role in regulating inflammation and innate immunity is supported by the present finding that miR-27a is altered in acute peritonitis. However, not enough is known about its role in response to bacterial infections to infer whether it is functional in modulating the immune response at the site of infection.

3.3.3.3 miR-21

miR-21 was identified here as a miRNA that was decreased in infected compared to uninfected PD effluent and is altered between different infections. This miRNA is one of the most abundant miRNAs present in most mammalian cells, located on chromosome 17. It has been widely studied in cancer and is one of the most consistently identified oncomiRs.[508–513] miR-21 has been identified as a diagnostic and prognostic biomarker in a range of cancers, including breast, colon, lung and pancreas.[514] A substantial amount of research has also shown the important of miR-21 in CVD, where it is implicated in the pathogenesis of a range of heart conditions and can be used as a diagnostic and prognostic

biomarker.[421,515–519] Due to the inflammatory nature of many cancers and CVD, and the wide range of cancers which show upregulated miR-21, it is thought to play a part in the inflammatory component of these disorders.[516,520–523] There has also been much work looking into miR-21 and other inflammatory conditions, including psoriasis, colitis and MS where it is suggested to play a role in the pathogenesis.[522,524,525] Due to the fact that it is so highly expressed and was one of the first miRNAs to be characterised, there are also a wide range of pathways that miR-21 has been shown to influence. For example, programmed cell death protein 4 (PDCD4) is probably the best characterised target of miR-21 and is important in a range of conditions, including different types of cancers and fibrosis, due to its crucial role in the control of apoptosis, which is an essential part of the pathogenesis of many disorders.[508,509,512,513,515,522,523,525–530] The regulation of miR-21 expression has been well studied, with both transcriptional and post-transcriptional regulatory mechanisms well described, showing miR-21 is often involved in feedback loops which include its own target mRNAs.[526,528] miR-21 is also increased in immune cells upon their activation,[512,522,525,529,531] which could be another reason why it is increased in so many different conditions. With regards to infection, miR-21 influences the immune response to viral infections,[508,522] and it alters the environment for intracellular pathogens such as *Listeria monocytogenes*, where it reduces the intracellular niche to control the infection in macrophages.[527] A knockout mouse for miR-21 (miR21$^{-/-}$) has been developed and studied in a range of conditions. miR-21 deficient animals are viable, fertile, born in the expected Mendelian ratios, and have no major phenotypic differences to wild-type (WT) mice of the same background. However, some of the predicted miR-21 targets are upregulated in miR-21$^{-/-}$ mice, and the pathogenesis of multiple cancers and CVD is altered.[532–534] Despite the majority of the literature suggesting miR-21 increases in inflammatory conditions, the data presented in this thesis show a decrease in miR-21 during acute peritonitis. This may primarily be due to the dilution factor, where miR-223 dominates the total miRNA content of a cloudy bag. This means that other miRNAs appear to decrease, when they are in fact just decreasing in relative terms, when compared to snRNA U6, which the miRNAs of interest are normalised to and which increased with increased numbers of cells present.

3.3.3.4 miR-31

In this thesis, miR-31 levels were reduced in response to infection in PD effluent. This miRNA, located on chromosome 9, has been identified as an epithelial-specific miRNA[535] and has been studied most widely in cancer. However, the role of this miRNA is more

varied, with it being an oncomiR in some cancers, whilst a tumour suppressor in others. The variation is not only between different cancers, but also between different stages of the same cancer.[536] For example, miR-31 is one of the most deregulated oncomiRs in colorectal cancer, and it is increased in many different studies across all clinical stages, including pre-malignant polyps.[536–538] However, it is decreased in breast cancer and acts as a tumour suppressor, by suppressing genes involved in cell motility and invasion.[536,537,539] This is not universal though, with some evidence that miR-31 acts as an oncomiR in breast cancer.[540] miR-31 has also been studied in heart disease, with varying roles in different pathologies and response to different types of CVD.[541–547] This miRNA has not been as widely studied as miR-21 or miR-223, but it has been implicated in inflammatory pathways, especially via NF-κB,[548] as well as a range of inflammatory conditions including psoriasis,[549,550] inflammatory bowel disease (IBD) or colitis,[551–553] sepsis[554] and allergic disease.[555] miR-31 is responsible for host responses to infection, specifically viral infections such as hepatitis B virus (HBV),[556] influenza[557] and respiratory syncytial virus (RSV)[558]. One of the proposed roles for miR-31 is in recruitment of neutrophils in allergic airway disease (AAD),[548] whilst it is thought to alter T cell responses by either skewing the Th1/Th2 balance,[554] or regulating the Treg development.[537,555] It is also important in development, even from very early stages,[537] which may be the reason why there are no reported miR-31 full KO animal models, only animals with a miR-31 knockdown or conditional knockout, where the effects of a reduction in miR-31 vary between different tissues from inflammatory (e.g. in colon epithelium) to reduced viability (e.g. in lung cancer cells).[535–537] The variety of roles of miR-31, and the pleiotropic functions across and within different conditions show that this miRNA can have many different functions, depending on the specific stimuli present and local microenvironment. One study shows that miR-31 in cell-free PD effluent is altered in peritoneal membrane fibrosis caused by extended PD.[70] All of the reported evidence suggests that the decrease in miR-31 in acute PD-related peritonitis is to be expected as it appears to be very sensitive to a range of stimuli, so we would expect alterations. Further work would be needed to identify why it decreases in PD-related peritonitis.

3.3.4 miRNAs in the resolution of infection

Due to the altered levels of the four miRNAs on day 1 of infection, it was proposed that these could be markers of the severity of inflammation or acute immune response, therefore would gradually go back to baseline levels during the resolution of infection. The

variability seen with a limited number of patient samples means it is difficult to interpret these results further than preliminary investigations. However, miR-223 appears to reduce back to baseline levels robustly across all three patients, in parallel with the resolution of infection. The implication of miR-223 in inflammatory responses and acute inflammation[410,449,473,474,489] is supported with the present observation that miR-223 reduces with the resolution of the acute phase, as the number of innate immune cells and the inflammatory components reduce. The more contradictory roles of miR-21,[508,510,522,528] miR-31[537,548,550] and miR-27a[494,496,502,505] suggest there is no clear consensus on the role of these miRNAs in acute inflammation and bacterial infections, which is corroborated by the results shown here, which are not as clear in terms of resolution of infection and altered levels of the miRNAs in response to it. More work needs to be done to analyse the roles of these miRNAs in the resolution phase of PD-related peritonitis.

3.3.5 Correlations to cell data

Due to the pronounced influx of cells into the peritoneal cavity during an infection, it was proposed that miR-21, miR-27a, miR-31 and miR-223 may just be indicators of either the total number of cells present, or of a specific abundant cell type. Due to the variation of patient samples and the cell-free nature of the miRNAs, there were very few significant correlations between the data sets. The levels of miR-223 and miR-27a appeared to correlate with the total levels of immune cells present. This was not unexpected, especially for miR-223 as this miRNA is restricted to expression of cells from a myeloid lineage.[473,474] The levels of peritoneal immune cells before infection are low, therefore when further cells are recruited into the peritoneal cavity in response to the acute infection, it is conceivable that the levels of miR-223 also increase.[449] The role of miR-27a in response to acute infection is less well defined, and the expression patterns across different cell types are not as clear-cut as miR-223. However, there are reports that suggest miR-27a has a role in the acute inflammatory/innate immune response, with altered levels in CD4$^+$ T cells in MS during acute relapses.[501] Also, miR-27a seems to be protective to essential organs during inflammation-induced injury,[502–504] and control viral infections by preventing replication.[505,506] These studies suggest that the role for miR-27a may be important during the initial phase of infection or inflammation, but the cellular source and role is less clear. The lack of correlations with miR-21 and miR-31 could be due to a number of reasons. Primarily, these miRNAs are altered in a range of inflammatory disorders and changed in both directions (i.e. up- and down-regulated), therefore there is less consensus on the role

of miR-21 and miR-31 in the acute response.[508,548] Also, the levels of miR-21 are high in almost all cell types, therefore any changes related to specific cell types may be difficult to distinguish, due to the mixture of different cell types and stimuli present during an acute PD-related peritonitis infection. miR-31 is expressed at higher levels in epithelial cells than other cell types.[535] As the correlations attempted here were with total cells and immune cells which have infiltrated the peritoneal cavity, the lack of correlations with miR-31 was expected, as these cell types are not predicted to express and release high levels of miR-31. The primary reason why there are few correlations between the levels of the four candidate miRNA biomarkers is very likely the fact that infected PD effluent is a highly variable ill-defined mixture of cells of different types, as well as a cocktail of soluble factors that could all influence the levels of miRNAs measured in the cell-free effluent.

3.3.6 *In vivo* models

Many studies on miRNAs and translational research use animal models, often mouse or other small rodents, to further assess the function of novel discoveries in a whole-body system. The pharmacokinetics of novel treatments or the unintentional off-target effects cannot be identified through *in vitro* models alone.[559] Although there is a growing tendency to reduce research on animals (using the principle of the 3 R's – replacement, reduction and refinement), there is still a considerable need for this kind of research.[560,561] *In vivo* experiments can use KO animals to investigate the role of a specific gene, where the target gene is removed either from the animal completely before birth through genetic manipulation and breeding strategies, or specific knockouts, either site-, cell- or stimulation-specific deletion of a gene of interest,[562,563] as has been seen with the specific candidate miRNA biomarkers studied here.[410,487,533,535] As approximately 90% of the human genome and 60% of the miRNA loci are shared with the mouse, in addition to the substantial physiological similarities, mice are often used as experimental models for miRNA research.[233,564] However, this also means there are approximately 40% of human miRNAs that are not shared with mice, and the data shown here emphasises these inter-species differences. Although there were some similarities between the data seen in patients and those in the mouse model (i.e. increased miR-223 and decreased miR-31), there were also some differences. miR-21 was decreased in infected patients, but increased in the mouse peritonitis model, whilst miR-27a decreased in patient PD effluent but slightly increased in mice *in vivo*. These differences could be due to the lack of evolutionary conservation in these miRNAs and the pathways they control, or which control their

expression. They could also be due to the fact that the mouse model employed here is not completely representative of the patient population. The patients recruited in the present study have comorbidities through their underlying ESRD diagnosis, that are not represented in the mouse model, and have a basal inflammatory state induced by the dialysate, which is then followed by an acute inflammatory reaction to a bacterial infection. The mouse model utilised perfectly healthy laboratory mice infected with a controlled bacterial dose. There have been a range of PD models[565] and peritonitis models[566] which could be explored in the future as appropriate alternative animal models which may be more relevant to PD-related peritonitis in patients. The main reason behind the differences seen here is unknown, however these do suggest these four miRNAs might be best studied in human models for translational research aiming towards better patient outcomes. It may be important to investigate these differences between mouse and human miRNAs further in the future, and to understand better whether it may be useful to study some miRNAs (e.g. those that show similar changes in infection in both mouse and human) in mouse models, either the one demonstrated here or alternative ones.

3.4   Conclusion

In summary, this Chapter shows optimisation of the methodology for detection of miRNAs in cell-free PD effluent, screening to identify miRNAs of interest and confirmation of four miRNAs (miR-223, miR-27a, miR-21 and miR-31) that are altered in acute PD-related peritonitis. The levels of miR-223 returned to basal levels over the resolution of infection. Finally, a mouse model of peritoneal infection was also assessed for its use to study miRNA levels during acute inflammation, but turned out to be of only relatively little relevance for our understanding of human disease mechanisms.

4 Cell-specific expression patterns of candidate microRNAs

4.1 Introduction

When a patient has a PD-related peritonitis episode, they experience pain, inflammation and an acute immune response. This includes a range of soluble factors, like cytokines and chemokines, as well as a cellular response. These infiltrating cells are the reason for the cloudy appearance of the effluent bag and are part of the clinical diagnosis of peritonitis, based on a dialysate cell count of >100 white blood cells/mm^3, of which >50% granulocytes, alongside abdominal pain and positive culture results.[567] To investigate the miRNAs of interest identified in the preceding Chapter further, *in vitro* models were set up where four cell types present in the inflamed peritoneal cavity and important in the immune response to infection were investigated. Neutrophils, monocytes, HPMCs and HPFBs were isolated and cultured in the presence of inflammatory stimuli, then miRNAs of interest were investigated.

The main cell type that is present in a cloudy bag are neutrophils. These polymorphonuclear (PMN) granulocytes are key components of the innate anti-microbial immune response and are the most abundant leukocytes in the blood (50-70% of circulating leukocytes). These short-lived cells are recruited to the site of infection and their primary function is to kill infecting microbes by phagocytosis, degranulation and neutrophil extracellular traps (NETs). Phagocytosis is the major mechanism used by neutrophils (and other "professional phagocytes") to remove pathogens and cell debris, where the particle to be destroyed is internalised into a phagosome, which then fuses with granules that contain anti-microbial agents including anti-microbial peptides such as α-defensins, lysozyme and lactoferrin, as well as reactive oxygen species (ROS). Neutrophils use degranulation to create an environment that is inhospitable to invading pathogens by release of granule components into the environment around the inflammatory site including anti-microbial peptides and ROS. These granular components also play a role in signalling to and recruitment of subsequent inflammatory cells. NETs are formed when neutrophils undergo an active cell death pathway and release fibrous structures that trap microbes and kill them, which is thought to be through exposure to high concentrations of anti-microbial agents and facilitating subsequent phagocytosis. NETs contain histones and a range of granular and cytoplasmic proteins encased in decondensed chromatin.[142,568,569]

Another key inflammatory cell that is recruited to the site of infection are macrophages. These "professional phagocytes" efficiently engulf and clear infecting microbes by phagocytosis. Macrophages are present in many tissues as tissue-specific macrophages, as well as in secondary lymphoid structures, as a reservoir for rapid response to infection, and in immune privileged sites. Once monocytes are recruited from the peripheral blood into the peritoneum upon infection, they then differentiate into either inflammatory or anti-inflammatory macrophage subsets, dependent on the local microenvironment they encounter. Macrophages are one of the first cell type to encounter infecting pathogens and therefore not only have a role in the clearance of bacteria, but also in recruiting other inflammatory cells to aid speedy resolution of the infection. Macrophages are able to recognise microbes by their surface pattern recognition receptors (PRRs), that recognise molecular patterns not present on healthy normal self cells, including pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS). They can also recognise opsonised microbes or cell debris, as they have receptors for antibodies and complement. Once engulfed into a phagosome inside the macrophage, this fuses with a lysosome to form a phagolysosome. The microbe is then degraded by a combination of ROS, reactive nitrogen species (RNS) and microbicidal proteins (e.g. matrix metalloproteases, MMPs), which require an acidic pH to act. As well as this phagocytic activity, macrophages recruit other inflammatory cells, such as neutrophils, both directly through release of neutrophil attractants and indirectly by releasing cytokines that stimulate epithelial cells to produce chemokines. The different stimuli that macrophages encounter can determine which subtype of macrophages is preferentially differentiated, as they can be both pro- and anti-inflammatory. During the acute response to infection they are normally pro-inflammatory to attempt to resolve the infection rapidly, as is seen in acute PD-related peritonitis episodes.[156,570–575]

The normal, healthy peritoneum consists of a layer of mesothelial cells over a structural basement membrane and interstitial layer. These HPMCs have surface microvilli and are joined by intercellular junctions. The peritoneum covers >100 m^2 in adults and is lubricated by typically 50-100 ml of peritoneal fluid containing water, electrolytes, solutes, proteins and cells. During PD, this membrane is used for ultrafiltration of waste products and excess water from the blood, which passes through blood vessels within the peritoneal membrane.[567,576] Mesothelium was historically thought to have two main roles – a protective barrier and a frictionless interface for movement of organs and tissues.

However, it is now known that mesothelial cells can also have a range of different functions including transport of fluid and cells, inflammation initiation and resolution, and tissue repair.[577] The role of HPMCs in PD-related peritonitis is therefore more complex than just acting as the barrier for ultrafiltration to occur, it is likely they have a role in recruitment of leukocytes to the site of infection, as well as secretion of pro- and anti-inflammatory cytokines to alter the inflammatory pathway.[577–581]

In a healthy peritoneum, fibroblasts are part of the submesothelial interstitial layer. However, during fibrosis (induced by PD and increased by peritonitis) there is a thickening of this layer caused by an increased deposition of extracellular matrix (ECM) components by fibroblasts that have been transformed into activated myofibroblasts. Peritoneal membrane fibrosis is also characterised by EMT/MMT, during which mesothelial cells acquire a fibroblast-like phenotype, where they become motile, and invade the underlying stromal layer.[582,583] All this means that, during PD-induced fibrosis, there is an increase in the number of HPFBs. As fibrosis is increased further during peritonitis due to the inflammation induced, there are even more HPFBs during acute infections. Fibroblasts are also effector cells in the inflammatory reaction to infection, as they can release chemokines to attract leukocytes, such as neutrophils and macrophages, as well as some cytokines, to help regulate acute inflammatory reactions.[582,584–587]

The range of cells present in acute PD-related peritonitis and the huge influx of cells in a cloudy bag suggest the miRNAs identified previously as candidate biomarkers may be important in specific cell types. It has been shown that all four cell types investigated release miRNAs in response to a variety of stimuli,[317,410,588–590] therefore it was proposed that the miRNAs detected in cell-free PD effluent may have a specific cellular source, where infection or inflammatory stimuli influence this expression.

4.1.1 Aims:

The aims of this chapter were to:

1. Culture cells present in a cloudy bag with high purity and activate them *in vitro*
2. Identify miRNAs expressed by and released by neutrophils, monocytes, HPMCs and HPFBs
3. Assess whether different inflammatory stimuli alter miRNA expression levels

4.2 Results

4.2.1 Optimisation of high purity cell culture

HPMCs and HPFBs were isolated from omentum samples using protocols optimised in our lab previously.[578] Positive identification of pure cultures was assessed by light microscopy, with HPMCs having a typical "cobblestone" appearance and close proximity to neighbouring cells, with regular sized cells indicating a healthy culture (representative image shown in Figure 4.1A). HPFBs were identified by observation of elongated cells that often overlapped by light microscopy (representative image shown in Figure 4.1B).

CD14$^+$ monocytes were isolated from peripheral blood of healthy volunteers. The purity was $\geq$ 95%, as assessed by flow cytometry, with the gating strategy and representative flow plots shown in Figure 4.1C. Neutrophil isolation from peripheral blood of healthy volunteers was more challenging due to the sensitive nature of neutrophils and how easily they are activated. To isolate resting neutrophils required optimisation using a protocol designed by our collaborators in Verona, Profs Flavia Bazzoni and Marco Cassatella.[591] Purities of $\geq$ 99% were achieved, with contamination by CD14+ monocytes <0.5% (Figure 4.1D). The amount of RNA produced by monocytes is 10 times higher than neutrophils, therefore it was important to minimise the contamination with these cells, as any results could be skewed by a small amount of monocyte RNA present. As a result of these optimisation steps, all four cell types of interest could be isolated at high purity and viability and cultured *in vitro*.

4.2.2 Assessment of *in vitro* activation

The activation of HPMCs and HPFBs was assessed by ELISA for the chemokine C-C motif chemokine ligand 2 (CCL2; alternative name: monocyte chemoattractant protein 1, MCP-1), which is released upon activation of these cells. As shown in Figures 4.2A and 4.2B, stimulation of these cells with *E. coli* extract showed a large increase in CCL2 released into the medium. Although not significant for the other conditions, due to the small sample size (n=5 for HPMCs and n=3 for HPFBs) and the preliminary nature of these experiments, it was taken that stimulation with a range of different inflammatory stimuli did activate both HPMCs and HPFBs, as detected by CCL2 ELISA.
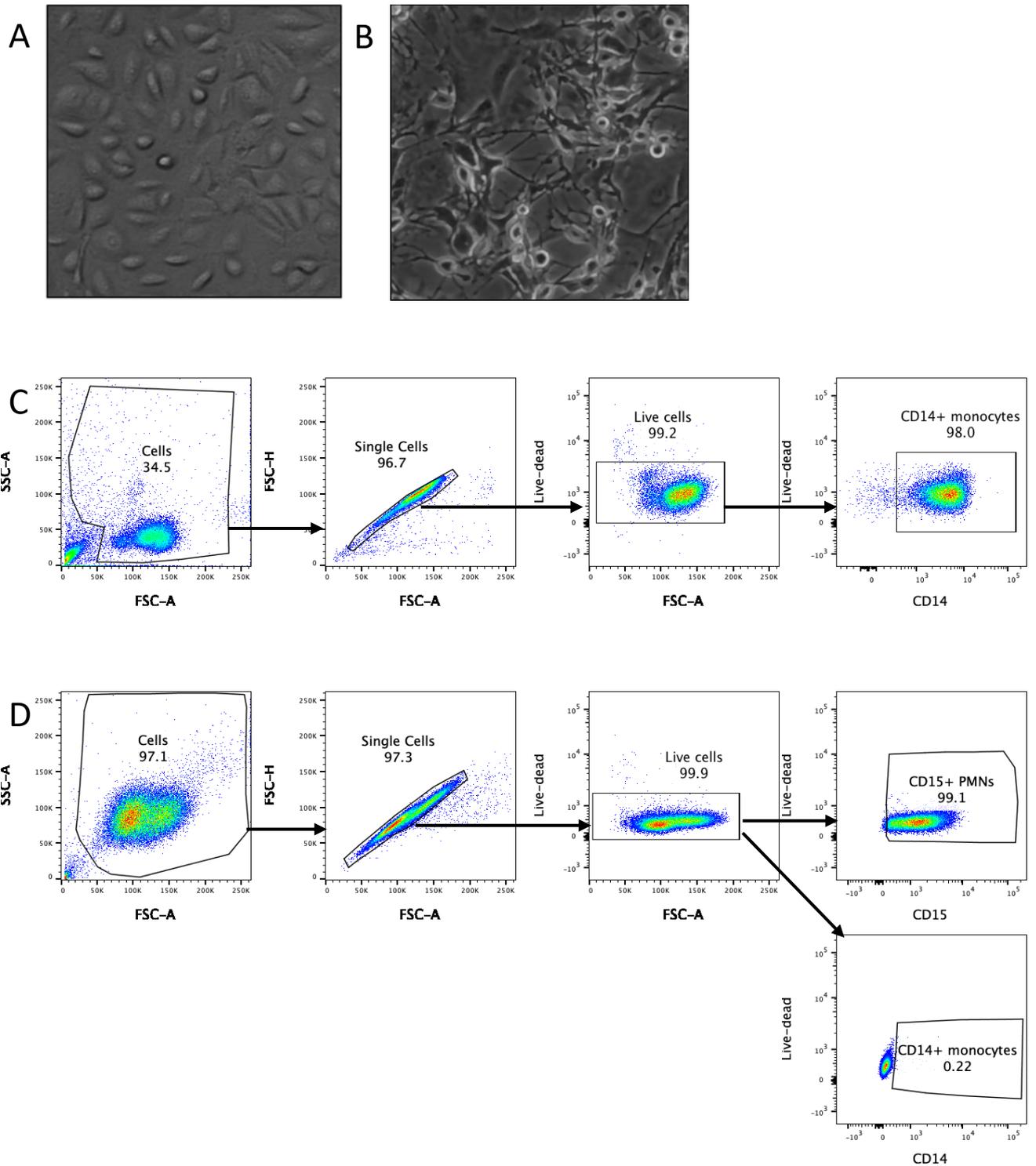
Figure 4.1: Purity of cells used in *in vitro* assays
HPMCs (A) and HPFBs (B) were isolated from omentum samples, with purity assessed by light microscopy. Monocytes and neutrophils were isolated from peripheral blood of healthy individuals and assessed for purity by flow cytometry, with gating strategy shown in C (monocytes) and D (neutrophils). Purity of CD14$^+$ monocytes $\geq$ 90%. Purity of CD15$^+$ neutrophils $\geq$ 95%, with contaminating CD14$^+$ monocytes <0.5%. All data are representative of replicates (n=5 for HPMCs, n=3 for HPFBs, n=5 for monocytes and n=4 for neutrophils).
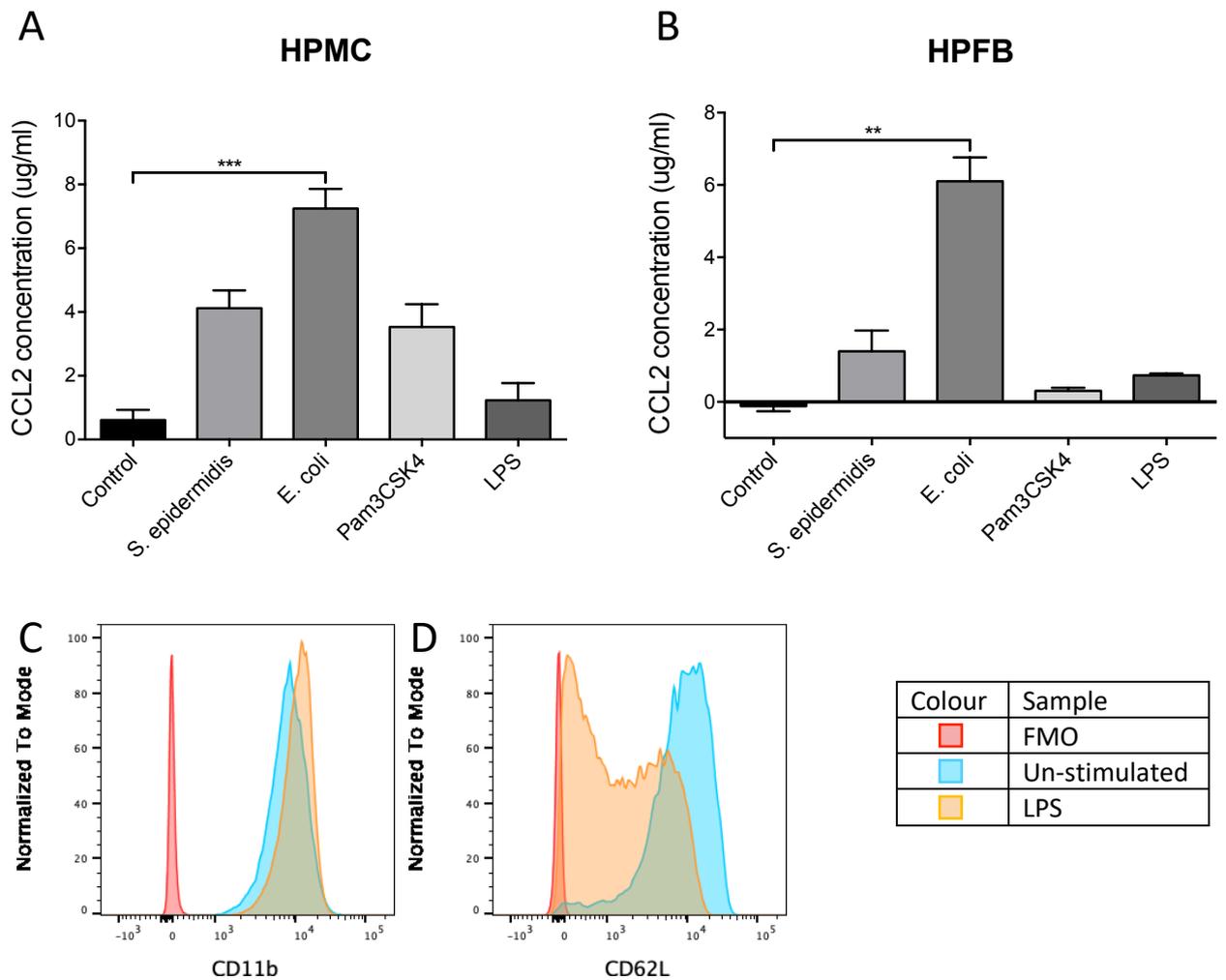
Figure 4.2: Activation of cells in *in vitro* assays
Activation of HPMCs (A) and HPFBs (B) was assessed by ELISA for CCL2 released into the media. Cells were stimulated with bacterial extracts (*S. epidermidis* or *E. coli*, protein concentration of 50 μg/ml), Pam3CSK4 or LPS (500 ng/ml) overnight. Data presented as mean ± SEM, with n=5 for HPMCs and n=3 for HPFBs, analysed by Friedman test with multiple comparisons, significant differences are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$; ****, $p<0.001$. Neutrophil activation was assessed by flow cytometry detection of CD11b (C) and CD62L (D) between un-stimulated cells (blue trace) and those stimulated with LPS (500ng/ml, orange trace) for 4hr, with representative plots shown.

Activation of neutrophils was assessed by increased CD11b and decreased CD62L expression on the cell surface. As observed in Figures 4.2C and 4.2D, the level of CD11b increased, whilst the level of CD62L decreased upon LPS stimulation. This confirmed that the neutrophils isolated were resting, and that they could readily be activated *in vitro* by LPS stimulation.

4.2.3 Levels of miRNAs produced and released by cells

All four cell types were cultured in the presence of a range of inflammatory stimuli (*S. epidermidis* and *E. coli* bacterial extract, Pam3CSK4 and LPS) to assess whether they expressed any of the miRNAs of interest, and whether that expression changed upon stimulation. Data were presented as 40-Ct due to the variable nature of primary cells. To confirm this was a valid representation of the data, control small RNA molecules were also detected. snRNA U6 was used as a control for the intracellular detection of miRNAs, to confirm consistent extraction and technical reproducibility across different cell types. This showed good reproducibility across all cell types and all culture conditions (Figure 4.3I). For extracellular miRNA detection, cel-miR-39 was used to confirm consistent extraction and technical reproducibility, which was essential due to the inability to detect the amount of RNA in the samples. The amount of variability in the levels of cel-miR-39 was minimal (Figure 4.3J), confirming the 40-Ct values can be used to observe any possible changes of miRNA levels released by cells into the medium.

The levels of miR-223 both expressed by and released into the media supernatant by neutrophils and monocytes was high, regardless of the culture conditions or inflammatory stimuli used (Figure 4.3A-B). Expression of miR-223 by HPMCs and HPFBs was below the limit of detection for RT-qPCR (Ct $\geq$ 35). Levels of miR-27a and miR-21 both expressed and released were consistent across all four cell types, regardless of the inflammatory stimuli (Figure 4.3C-F). miR-31 had the opposite expression pattern to miR-223. This miRNA was expressed and released at higher levels by HPMCs and HPFBs, with the levels expressed and released by neutrophils and monocytes near or below the limit of detection (Figure 4.3G-H). The different inflammatory stimuli did not affect the levels of any of the miRNAs either within the cells or released into the cellular supernatant, above the natural variation seen with primary cells and natural donor-to-donor variation. These data show that the four miRNAs identified have cell-specific patterns of expression and release, with no significant alterations due to a range of inflammatory stimuli.
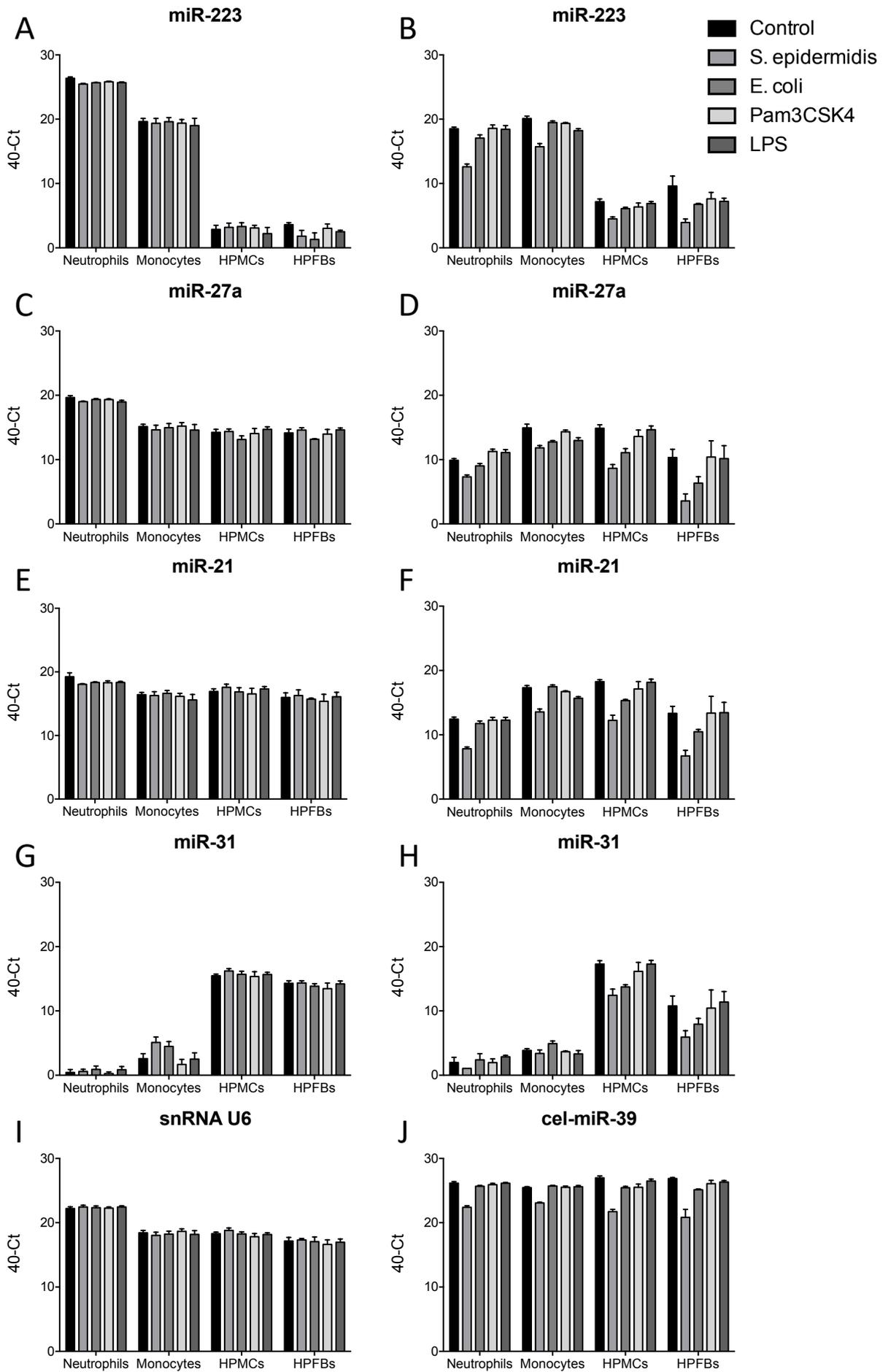
Figure 4.3: Cell-specific expression and release of miRNAs of interest

Showing the levels of four miRNAs of interest – miR-223 (A and B), miR-27a (C and D), miR-21 (E and F) and miR-31 (G and H) – alongside snRNA U6 (I) and cel-miR-39 (J) as reference genes. Data representative of levels detected in cellular extracts (A, C, E, G and I) or released into culture medium (B, D, F, H and J) after culture for 4hr with bacterial extracts (*S. epidermidis* or *E. coli*), or other inflammatory stimuli (Pam3CSK4 or LPS). The miRNAs show a cell-specific pattern of expression. Data shown as mean ± SEM with n=4 for neutrophils, n=5 for monocytes, n=5 for HPMCs and n=3 for HPFBs.

4.3 Discussion

4.3.1 Cell purity and activation

Culturing the four cell types to be investigated in this Chapter under optimum conditions was crucial for the establishment of relevant *in vitro* models, in order to assess the expression of miRNAs and the effect of microbial stimuli. One consideration when setting up the *in vitro* assays was to isolate resting cells at high purity and viability, to be able to activate them in a controlled manner.

For HPMCs and HPFBs, this was relatively straightforward as these resident cells are not particularly sensitive to activation through isolation procedures. A lack of CCL2 released by HPMCs and HPFBs, until they encounter inflammatory stimuli suggests that they are not activated by the isolation procedure or the culture conditions. CCL2 is so-called due to the C-C motif that defines similarly structured chemokines, and it recruits immune cells to the site of infection. It is expressed in most tissues and is responsible for migration of monocytes, as well as other immune cells including memory T cells and natural killer cells. CCL2 also has a proposed role in tissue fibrosis, e.g. in response to prolonged HPMC exposure to high glucose in PD dialysate, mediated by Toll-like receptor 4 (TLR4) activation.[207,579,592] Pleural mesothelial cells infected with *M. bovis* and *M. tuberculosis* also produced CCL2 to trigger immune responses and attract monocytes to help resolve the infection.[208] CCL2/MCP-1 is also released by fibroblasts in response to spider venom (in skin fibroblasts),[593] thrombin in lung fibrosis[594] and in the transformation of normal fibroblasts to cancer-associated fibroblasts (CAFs, in lung cancer).[595] These all suggest that activation of HPMCs and HPFBs can be monitored *in vitro* by assessment of CCL2 release as positive control. This chemokine may play a role in the immune response to infection and shows the importance of resident peritoneal membrane cells in the acute phase of infection.

Neutrophils are extremely sensitive to a range of stimuli and become activated very easily, which makes them effective at responding to acute inflammatory or infectious insults, but difficult to work with. To avoid activating them prematurely, the protocol designed by our collaborators at the University of Verona[591] was optimised for our lab. Using this gentle approach of negative selection, my data confirmed that such neutrophils were not activated after isolation and could be activated in a controlled manner by LPS stimulation, based on monitoring upregulation of CD11b expression and shedding of CD62L. CD11b is

also known as integrin subunit alpha M (α_M), a leukocyte-specific cell-surface integrin that is important in many processes including adhesion, phagocytosis and leukocyte extravasation, which is upregulated on the neutrophil surface upon activation.[596–598] CD62L is also known as L-selectin and is another adhesion molecule, which is constitutively expressed in leukocytes and functions in the binding and rolling of leukocytes on endothelial cells to facilitate their migration out of the blood to a site of inflammation. After activation, CD62L is shed from the cell surface and becomes a functionally active soluble form of the protein in the blood, resulting in a loss from the cell surface of neutrophils upon activation.[599,600] Both the upregulation of CD11b and the loss of CD62L can be seen upon activation of neutrophils *in vitro* with inflammatory stimuli.

This all shows that the positive and negative controls have been successfully established to enable investigation into the expression and release of the candidate miRNAs in both resting and functionally activated cells.

4.3.2 Cell-specific expression and release of miRs

This Chapter provides evidence that miR-223 is expressed and released only by neutrophils and monocytes, but not by HPMCs or HPFBs corroborating earlier reports that this miRNA is only expressed by cells from the myeloid lineage.[472,473] This high expression by infiltrating immune cells probably explains the large increase in detected levels in cell-free PD effluent in infected compared to uninfected patients (Figure 3.5A). miR-223 has also been seen to increase in a range of inflammatory and infectious diseases, suggesting it has a role either as a biomarker of the immune response or a functional role in the response.[473,474] For example, miR-223 is increased in sepsis[474,475] and rheumatoid arthritis (RA),[478,479] as well as viral infections[482,484,485] and animal models of bacterial infections.[483,486] The important discovery that miR-223 is increased in spontaneous bacterial peritonitis in ascites confirms what is seen here, in that there is a large influx of neutrophils and accompanying increase in miR-223 in the peritoneum upon infection.[449] This suggests that miR-223 may be important in the acute immune response to infection. It is important to investigate now whether this miRNA is functional, or merely a surrogate marker of the immune cell infiltration to the site of infection.

As shown in this Chapter, miR-31 is expressed only by resident peritoneal membrane cells, HPMCs and HPFBs, but not neutrophils and monocytes. This is in in line with reports that

miR-31 is an epithelial-specific miRNA,[535] although there is some evidence of miR-31 expression in other cells types such as Tregs.[537] The pleiotropic role of miR-31 in immune responses suggests that it is very sensitive to a range of different stimuli, including in a variety of inflammatory disorders[548,549,553–555] and infectious diseases.[557,558] There is a proposed role for miR-31 in the recruitment of neutrophils in allergic airway disease, and in the alteration of important inflammatory pathways including NF-κB.[548] miR-31 was only produced by the resident HPMCs and HPFBs, and it appears to be downregulated in cell-free PD effluent (Figure 3.5G). It is known that HPMCs and HPFBs produce many immunomodulatory factors,[578,579,581] and miR-31 is important in fibrosis in PD.[327] Together these studies suggest that miR-31 may be crucial in controlling or modulating the immune response in acute peritonitis.

miR-27a and miR-21 were expressed by all four cell types at similar levels. miR-21 is one of the most highly expressed miRNAs across most cell types under different conditions.[508,510,522,523,528] The expression pattern seen in this Chapter agrees with the expression patterns of this miRNA across many experimental and clinical scenarios. miR-27a is less well studied, but there is no current evidence to suggest it is restricted to specific cell types, with expression altered in a range of disorders including multiple sclerosis, sepsis and viral infections.[501–506] This also explains why the expression levels and amounts released in this Chapter were consistent across all cell types.

4.3.3 Effect of inflammatory stimuli on miRNA expression

This Chapter shows that a range of different inflammatory stimuli do not alter either the expression or release of four miRNAs of interest. Any alterations in the amount of the four miRNAs expressed was minimal. There was more variation in the amount of the miRNAs released, due partly to the technical challenges in working with cell-free fluids for miRNA work. The extraction method used did not allow quantification of the RNA content, therefore consistent amounts of RNA could not be used for RT-qPCR reactions. The use of cel-miR-39 as a control for the extraction, RT and qPCR showed that any small variation in the levels of the miRNAs released into the media was not due to the different culture conditions. The lack of effect of inflammatory stimuli on levels of miRNA expression and release was unexpected due to the sensitive nature of miRNAs and their ability to respond to a wide range of stimuli. In this respect, the findings in this Chapter do not support previous reports showing alterations of miRNAs levels in response to inflammatory stimuli.

For instance, after treating macrophages with LPS, expression profiling showed alterations in 41 miRNAs, which included decreased levels of miR-31 and miR-27a, two miRNA studied in this thesis.[601] It may be the case that any differences were masked due to the issues with donor-to-donor variation, as well as technical variations observed across the board.

4.3.4 Proposed model of miRNA release in PD-related peritonitis

These data demonstrate that the four miRNAs of interest are expressed and released in a cell-specific pattern, and that inflammatory stimuli do not affect this expression significantly. The potential cellular sources of the miRNAs identified here may help explain why the miRNAs are altered in cell-free PD effluent in infection, as depicted by the proposed model in Figure 4.4, where each cell type expresses distinct patterns of miRNA expression. For example, HPMCs and HPFBs express miR-21, miR-31 and miR-27a, whilst neutrophils and monocytes express miR-21, miR-27a and miR-223.

Taken together, these data suggest that the increase in miR-223 in infection observed in the previous Chapter can be partly explained by the increased number of neutrophils and macrophages, which infiltrate the peritoneum, as these cell types express and release high levels of miR-223. On the other hand, the observed decrease in miR-31 levels during acute peritonitis could be due to the fact that the infiltrating immune cells do not express detectable levels of this miRNA. As consequence, miR-31 levels may appear to go down in infection as there are large numbers of infiltrating cells that release other miRNAs (including miR-223), meaning miR-31 is diluted out. miR-21 and miR-27a are expressed by all cell types, but the levels of these are decreased in infection. This could also be due to a dilution by miR-223 and other leukocyte-specific miRNAs not investigated in this study.

Overall, these data suggest a potential cell-specific expression pattern for the four miRNAs of interest, as shown in Figure 4.4. According to this model, miR-223 is only expressed and released by the immune cells which infiltrate the peritoneum upon infection, whilst miR-31 is only expressed and released by the resident cells of the peritoneal membrane. miR-21 and miR-27a are expressed and released by all four cell types at relatively consistent levels. This model partly explains the alterations in miRNA level seen in patient PD effluent, with increased miR-223 due to an increase in the infiltrating immune cells which express and release it. The other three miRNAs are decreased due to the fact they are diluted out, compared to miR-223. Further work is required to investigate whether these miRNAs are
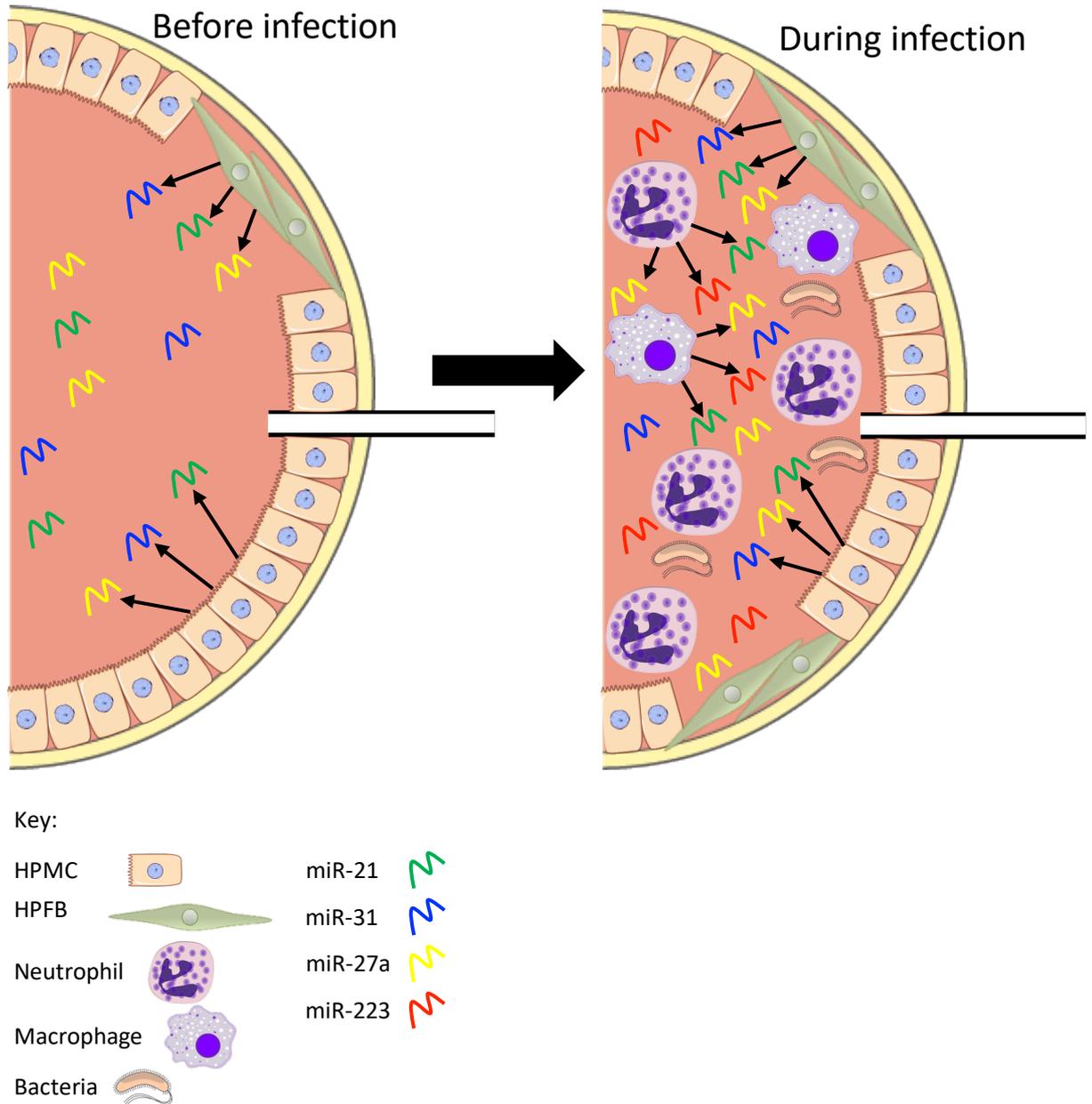
Figure 4.4: Diagram proposing model of miRNA expression in PD effluent
Showing a proposed model of the cell-specific expression pattern of miR-21, miR-31, miR-27a and miR-223 in PD effluent both before and after a bacterial peritonitis infection. miR-21 and miR-27a are expressed by all cell types, miR-31 is only expressed by resident HPMCs and HPFBs, whilst miR-223 is only expressed by infiltrating neutrophils and macrophages.

functional in the acute response to PD-related peritonitis infections, or if they are merely biomarkers of infection.

5   In silico target prediction

5.1   Introduction

One of the most important factors in the elucidation of the biological relevance of miRNAs (miRs) is the identification of the biological pathways they influence, primarily by the mRNAs they regulate. This can be done in multiple ways. Often an initial step is to predict potential mRNA targets through appropriate algorithms.[459] There are multiple algorithms that have been developed to identify mRNA targets that take into account different features thought to be important in the binding of miRs to their target mRNAs. Due to the relatively high rates of false positives resulting from each algorithm, it is common practice to use multiple algorithms and then attempt to validate targets which have been identified by complementary methods.[602,603] In this study, four different algorithms were used to identify a list of potential targets for the four candidate miRs previously identified; miR-223, miR-21, miR-31 and miR-27a. The algorithms used were all freely available online, and results could be ranked by the strength of the prediction.

The four methods used were Diana,[451,452] miRanda,[453,454] miRDB[455,456] and TargetScan. The differences and similarities of the four algorithms will be discussed in detail later on. Briefly, they all look at the seed sequence, identifying the sequences that match with the miR seed sequence (or wider miR sequence) in the 3' UTR (or whole coding sequence) of the mRNA. This is required for the miR to bind to the mRNA they regulate, and is therefore an important criterion that all algorithms use to identify predicted targets.[459,602,604,605] The next main criterion for identifying predicted targets is the conservation between species of the target site, and the surrounding region. This is important due to the fact that miR target sites are often more highly conserved than other regions of the gene. However, due to the variations in pathways that miRs regulate across different species, even closely related ones, this may miss some interactions. Regulatory elements, such as regions of the 3' UTR targeted by miRs, are more likely to be evolutionarily conserved, therefore this is an important consideration when attempting to predict functional regulation of targets.[459,602,604–606] The final component used to identify predicted miR targets is the energetic evaluation of the predicted interaction, i.e. the free energy of the miR binding to the mRNA and the accessibility of the target site, in terms of secondary structure. These parameters are important to evaluate if a potential interaction is likely to be energetically favourable, therefore more likely to be "real".[459,602,604,605] These different criteria have been

used by the different algorithms to rate potential targets of the miRs of interest, and the lists generated can be compared to find targets that are predicted by multiple algorithms, as these are most likely to be functionally relevant.

This bioinformatics-based approach of target prediction is well established in the literature; however, it does not show any functional relationships between a miR and its potential mRNA target. To identify functional changes regulated by miRs requires experimental validation.[603] One way to do this is by transfecting cells of interest with the candidate miRs, and then conducting 'omics approaches (RNAseq or proteomics analysis) to find targets whose expression levels are changed when the miR is present. This will identify both direct and indirect targets, but may pull out whole pathways which are altered, therefore those that are functionally important.[607,608] It is thought that the effect on individual mRNAs by a specific miR may be only marginal, and that phenotypic changes are due to modest targeting of multiple mRNAs in the same pathway to produce a large effect,[609] and 'omics-based technologies can help assess this.

Once predicted targets have been identified using bioinformatics or 'omics approaches, it is important to validate these interactions to identify which are "real" interactions and have functional consequences. To do this, the region of the gene that is predicted to be targeted by the miR is normally cloned into a reporter assay system, often luciferase-based, to identify whether the readout changes when the miR is present.[607,608,610] To confirm this interaction is specific, the region of interest is often subject to point mutations to confirm the interaction is lost when the specific binding cannot take place between the miR and the target region of the mRNA. This is the best direct method of confirming an interaction between a miR and a mRNA is functional.[610]

Another possible way is to transfect cells with the miR of interest and see if there is a change in the protein level of the predicted target. This is a simple way of validating the pathways that are thought to be important and are regulated by the miR, however it does not confirm if the interaction is a direct one or merely indirect.[610,611] This must normally be accompanied by a reporter assay system as described above, to identify the specific gene that is regulated by the miR.[610]

In this Chapter, a list of predicted targets for miR-223, miR-21, miR-31 and miR-27a was generated, then cross-referenced to identify candidates that are predicted to be targeted by multiple miRs. A select number of predicted targets were evaluated in the context of what is already known about these proteins in infection, inflammation and immune responses, as well as which interactions with miRs is already known.

5.1.1 Aims

The aims of this chapter were to:

1. Identify predicted targets for miR-223, miR-21, miR-31 and miR-27a using four complementary prediction algorithms

2. Identify targets predicted to be targeted by multiple miRs

3. Discuss a select number of predicted targets for their potential relevance in biologically relevant situations

5.2 Results

5.2.1 Target prediction for individual miRs

To predict potential mRNA targets for the four miRs identified previously, four different algorithms were used: Diana,[451,452] miRDB,[455,456] miRanda[453,454] and TargetScan.[457,458] Each uses different criteria to predict potential targets, therefore the most likely to be real mRNA targets of miR-223, miR-21, miR-31 and miR-27a were thought to be those predicted by multiple algorithms. Stringent criteria (defined in the Materials and Methods) were used to generate lists of potential targets from each algorithm, and Venn diagrams were used to show these predicted targets.

For miR-223 there were 160 predicted using Diana, 385 using miRanda, 64 using miRDB and 146 using TargetScan. There were 49 predicted targets which were identified from at least 3 of the 4 algorithms, with 16 targets predicted by all 4 algorithms (Figure 5.1, Table 5.1).

miR-21 had 108 predicted targets using Diana, 421 using miRanda, 67 using miRDB and 126 using TargetScan. Of these, 53 targets were predicted by 3 or more algorithms, and 19 targets predicted by all 4 algorithms (Figure 5.2, Table 5.2).
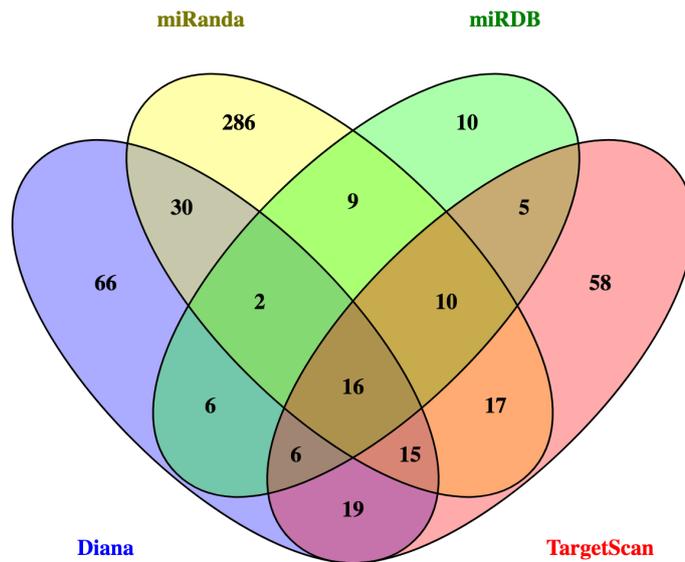
Figure 5.1: Predicted targets of miR-223
Venn diagram showing the predicted targets of miR-223 generated from four different prediction algorithms (Diana, miRanda, miRDB and TargetScan).

Diana, miRanda and miRDB	Diana, miRDB and TargetScan	Diana, miRanda and TargetScan	miRanda, miRDB and TargetScan	All 4 algorithms
DUSP10	GALNT18	GTSF1	HSP90B1	FBXW7
RRAS2	LACC1	LELP1	SEPT6	FBXO8
	SMARCD1	WDR62	INPP5B	SP3
	RAB8B	DNAJB13	CRIM1	RHOB
	SIAH1	FAM46A	INPP4A	ARMCX1
	TMEM178B	SORBS1	SLC39A1	NUP210
		C18ORF54	NLRP3	PRDM1
		CBLB	ANKRD17	PTBP2
		ATP2B1	ATP1B1	SLC4A4
		CYTIP	F3	IL6ST
		ATP10D		ATP7A
		NXF1		CDK17
		USP16		ACSL3
		SYAP1		LMO2
		RASA1		ECT2
				KPNA3

Table 5.1: Predicted targets of miR-223
Table showing the names of miR-223 targets as predicted by three or more of the four target prediction algorithms (Diana, miRanda, miRDB and TargetScan). Colour of cells shows corresponding section of the above Venn diagram they represent.
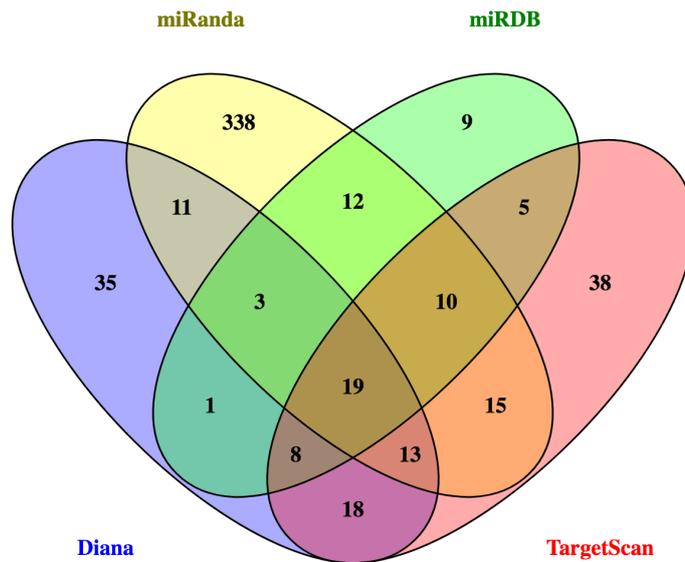
Figure 5.2: Predicted targets of miR-21
Venn diagram showing the predicted targets of miR-21 generated from four different prediction algorithms (Diana, miRanda, miRDB and TargetScan).

Diana, miRanda and miRDB	Diana, miRDB and TargetScan	Diana, miRanda and TargetScan	miRanda, miRDB and TargetScan	All 4 algorithms
KLF3	YOD1	RECK	NTF3	ZNF367
VCL	FGF18	CCL1	PITX2	SKI
YAP1	SPRY2	PDCD4	TIAM1	PELI1
	JAG1	SOX5	C10orf12	STAG2
	HIPK3	IL12A	SATB1	GPR64
	PLAG1	GLIS2	MAP3K1	PBRM1
	ARHGAP24	SMAD7	PAN3	PLEKHA1
	NFIA	MATN2	PCBP1	SCML2
		FASLG	GLCCI1	ZSWIM6
		SLC16A10	BEST3	TGFBI
		KLF5		KRIT1
		LRRC57		ARMCX1
		RASGRP1		CASKIN1
				PPP1R3A
				CPEB3
				SPRY1
				PCSK6
				ELF2
				RBPJ

Table 5.2: Predicted targets of miR-21
Table showing the names of miR-21 targets as predicted by three or more of the four target prediction algorithms (Diana, miRanda, miRDB and TargetScan). Colour of cells shows corresponding section of the above Venn diagram they represent.

miR-31 was predicted to target 116 mRNAs by Diana, 340 by miRanda, 113 by miRDB and 209 by TargetScan. 55 of these targets were predicted by at least 3 algorithms, with 14 targets predicted by all 4 algorithms (Figure 5.3, Table 5.3).

For miR-27a, there were 726 predicted targets using Diana, 282 using miRDB, 706 using miRanda and 498 using TargetScan. 213 of these targets were predicted by at least 3 algorithms, with 75 predicted by all 4 algorithms (Figure 5.4, Table 5.4).

5.2.2 Predicted targets for multiple miRs

The above analysis resulted in a large number of potential targets for each miR to investigate further. As three of the four miRs investigated here were decreased in the acute response to PD-related peritonitis (miR-21, miR-31 and miR-27a), it was interesting to investigate whether there were any common targets predicted to be regulated by multiple miRs, suggesting possible redundancy in the acute immune response. Although miR-223 is increased in infection, whilst the other three miRs are decreased, it is interesting to see if any of the same mRNAs are regulated by this miR, to attempt to understand the molecular signalling during the acute response to infection. This was done using Venn diagrams comparing the lists of targets predicted by 3 or more of the 4 algorithms. To this end, there were very few targets predicted to be regulated by multiple miRs (Figure 5.5B):

miR-21 and miR-31 = ZSWIM6

miR-223 and miR-21 = ARMCX1*

miR-31 and miR-27a = VAV3, CCNC and PAX9

miR-223 and miR-31 = RASA1

miR-21 and miR-27a = SPRY2 and ALC16A10

miR-223 and miR-27a = FBXW7*

* = identified when lists of targets predicted by all four algorithms were also compared (Figure 5.5A)

This lack of crossover between predicted targets fits with the different direction of changes in miR levels in infected PD effluent (i.e. miR-223 increases, whilst miR-21, miR-31 and miR-27a decrease), therefore they are likely to be important in different components of the acute response to peritonitis infection. However, it is interesting that the miRs that are all decreased in infection do not appear to target the same mRNAs. This is likely due to the
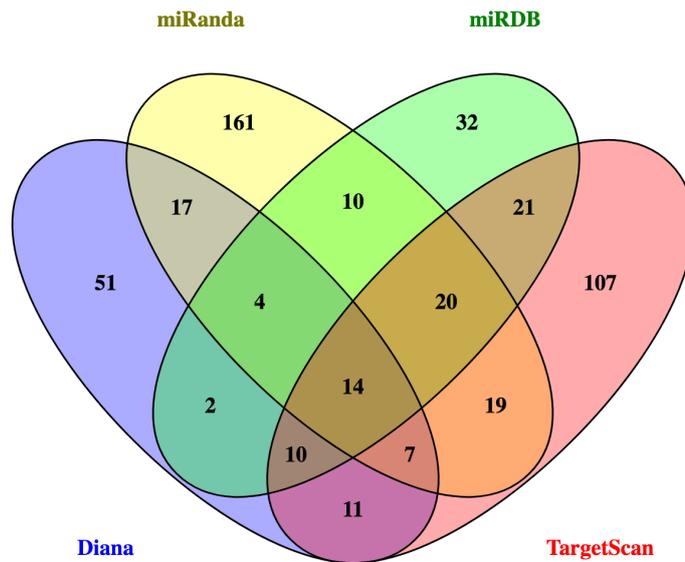
Figure 5.3: Predicted targets of miR-31

Venn diagram showing the predicted targets of miR-31 generated from four different prediction algorithms (Diana, miRanda, miRDB and TargetScan).

Diana, miRanda and miRDB	Diana, miRDB and TargetScan	Diana, miRanda and TargetScan	miRanda, miRDB and TargetScan	All 4 algorithms
FAM134B	IL34	SATB2	KHDRBS3	RSBN1
ZNF512	NR5A2	STARD13	HIAT1	RHOBTB1
HSD17B6	PDZD2	RGAG1	FGF7	MBOAT2
FNDC5	SLC1A2	KDELR2	LATS2	SH2D1A
	VAV3	CREG1	ATL2	PIK3C2A
	RGS4	ZSWIM6	CRYBG3	KANK1
	TBXA2R	CCNC	INSC	NUMB
	DCBLD2		HOMER1	SYDE2
	PPP3CA		PEX5	PPP2R2A
	RNF144A		DUSP7	LBH
			EGLN3	PRKCE
			CHMP7	NUP153
			PSMB11	PAX9
			CAMK2D	SEPHS1
			KCTD21	
			RASA1	
			CLASP2	
			PC	
			MGAT1	
			GCH1	

Table 5.3: Predicted targets of miR-31

Table showing the names of miR-31 targets as predicted by three or more of the four target prediction algorithms (Diana, miRanda, miRDB and TargetScan). Colour of cells shows corresponding section of the above Venn diagram they represent.
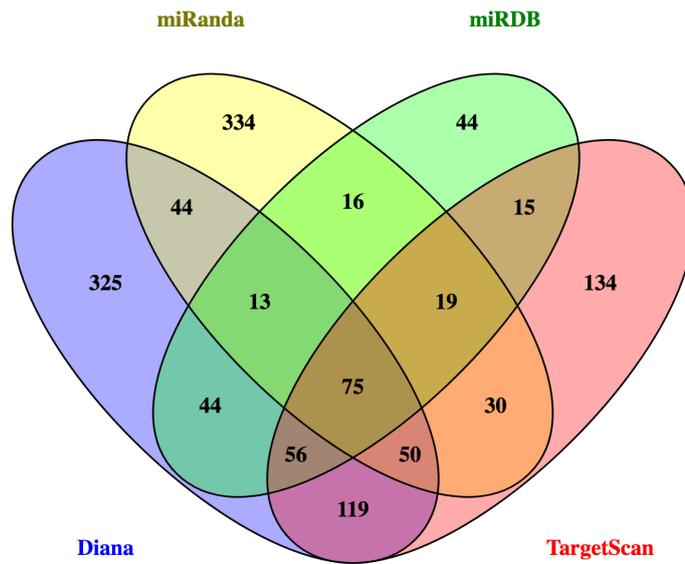
Figure 5.4: Predicted targets of miR-27a
Venn diagram showing the predicted targets of miR-21 generated from four different prediction algorithms (Diana, miRanda, miRDB and TargetScan).

Diana, miRanda and miRDB	Diana, miRDB and TargetScan		Diana, miRanda and TargetScan		miRanda, miRDB and TargetScan	All 4 algorithms	
SFRP1	APPBP2	AFF4	GRB2	THOC1	NDUFS4	FBXW7	NEURL4
UNC13C	TSC1	STX16	KITLG	KCNJ1	ITSN2	PLK2	ADORA2B
TMBIM6	LIN28B	USP46	MAP2K4	INPP1	NPEPPS	KCNK2	SLC6A1
STAG1	SZRD1	PNISR	FAM65B	GDF6	GLRA2	DCUN1D4	DNAJC13
ANKRD40	GXYLT1	GSPT1	TMEM189-UBE2V1	MARCKS	CCNC	GCC2	UNKL
UBR5	PRKX	GRIN2D	ISL1	TOMM40L	NLN	NRK	NCOA7
SBF2	RELN	USP51	KIAA1109	KHSRP	FAM184A	PDIA5	CEP135
GRIA4	ABCA1	DNAJC27	RCAN2		GALNT3	CDS1	PLCL2
SNRNP27	NGFR	SEC22A	FAM193B		PSMA1	AQP11	SNAP25
VIP	CREB1	EDEM3	GFPT2		VAV3	FLRT3	REPS1
FOXP2	SEMA6A	FRS3	HBEGF		NMB	WSB1	NR2F6
CPNE8	SLC35F1	CSRNP1	GABRP		CYP39A1	CKAP4	CDR2
TRIM50	LPAR6	SS18L1	SLC16A10		NRXN1	PPARG	C1orf52
	PELI2		PARD6B		TROVE2	GRM5	YWHAQ
	ROR1		DKK2		RPS6KA5	HOXA10	SLC39A11
	B4GALT3		RGS17		RNF182	CSRP2	C8orf4
	HOXA5		BMI1		KDM3A	PPP1CC	PDHX
	GATC		CCDC149		SFXN2	ZHX1	STK39
	NABP1		HOXB8		FOSB	SPRY2	EYA1
	TRPV3		YWHAB			UBE2V1	MIER3
	CREBRF		CCNG1			RAPGEF2	LIMK1
	ABHD17C		COL21A1			DTNA	NGFRAP1
	SNX12		FASTKD5			RPGRIP1L	SLITRK1
	CCNJ		SFXN4			PKIA	LONRF1
	SHE		MBTD1			RREB1	TRIM23
	ZBTB34		GALNT5			AKIRIN1	RNF139
	TMEM170B		EFNB2			ST6GALNAC3	EYA4
	GAB1		RAD9B			ZNF800	CA10
	GNS		HORMAD2			ZMYM4	GALNT7
	TNPO1		TMUB1			E2F7	PDK4
	SOWAHA		ZIC5			MMD	CCNK
	TLK2		VEGFC			GOLM1	MED14
	GPAM		CNN3			PDE7B	
	ARHGEF26		CNOT7			CDH11	
	TAB3		CACNA2D3			NOVA1	
	TMCC1		TRAF3IP3			FAM133B	
	NXT2		PANK1			MATN3	
	DLL4		STYK1			PSPC1	
	ATXN10		NEDD4			RSBN1L	
	ZCCHC24		HOXA13			PRKCB	
	B3GNT7		PAX9			PAIP2	
	C2CD2		GCA			PDS5B	
	NIPAL4		TFPI			TNRC18	

Table 5.4: Predicted targets of miR-27a
Table showing the names of miR-27a targets as predicted by three or more of the four target prediction algorithms (Diana, miRanda, miRDB and TargetScan). Colour of cells shows corresponding section of the above Venn diagram they represent.
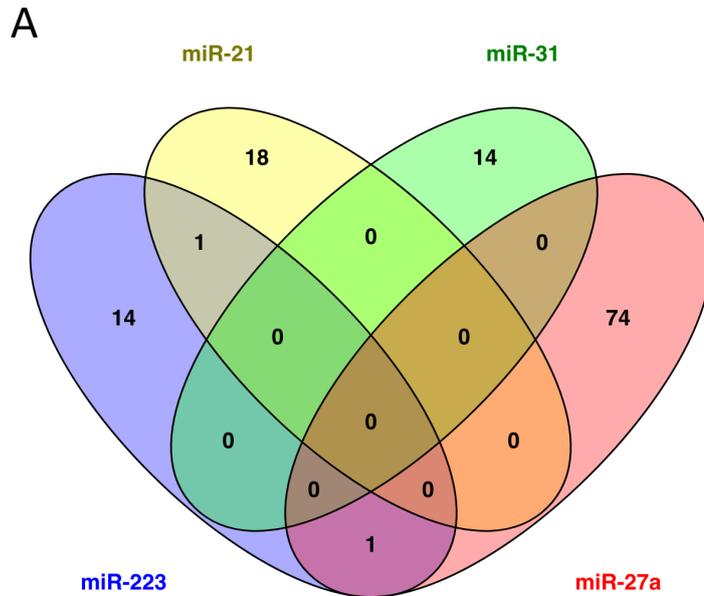
microRNAs	Common predicted targets
miR-223 and miR-21	ARMCX1
miR-223 and miR-27a	FBXW7
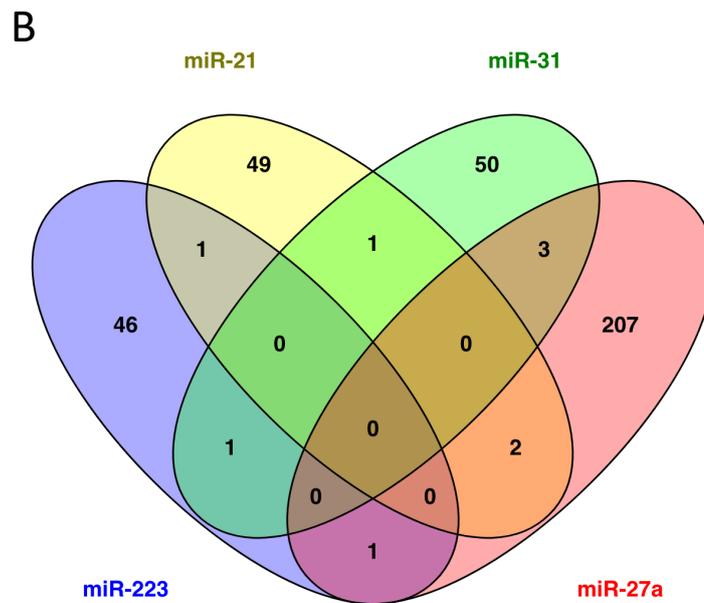
microRNAs	Common predicted targets		
miR-21 and miR-31	ZSWIM6		
miR-223 and miR-21	ARMCX1		
miR-31 and miR-27a	VAV3	CCNC	PAX9
miR-223 and miR-31	RASA1		
miR-21 and miR-27a	SPRY2		SLC16A10
miR-223 and miR-27a	FBXW7		

Figure 5.5: Predicted targets of multiple miRNAs
Showing the common targets from the lists predicted by all four algorithms (A) or three or more algorithms (B) in Venn diagram format, with the named predicted crossover targets in tables below. Colour of cells shows corresponding section of the Venn diagram they represent.

possibility that they regulate different pathways involved in this response. This will be discussed further below.

5.3   Discussion

The present Chapter identified a large number of predicted targets. Given the time constraints of this PhD project, there was not sufficient time to evaluate potential targets experimentally. This experimental validation was also not possible due to the fact that the cells in which the miRs act was not known, as they were identified in cell-free PD effluent. It was possible to characterise the cells which produced and released the miRs, but it has not yet been determined whether the miRs act within the cells that produce them or in distant cells. In the absence of functional validation, selected targets are reviewed here, and current literature was used to discuss whether they might be relevant in patients with acute PD-related peritonitis.

5.3.1 Different algorithms

The four different algorithms used here all utilise different criteria to predict mRNA targets of miRs of interest, as shown in the comparison in Table 2.4. All four algorithms are designed to look for a good seed match, by looking at the first 2-8 nucleotides from the 5' end of the miR and comparing it to sequences in the mRNA for a Watson-Crick match. These seed sequences can comprise all eight nucleotides (8mer), seven of the eight nucleotides (7mer) or only six nucleotides (6mer). A seed match of at least a 6mer is essential for miR targeting of mRNA, and therefore is included in all algorithms used to be able to identify possible targets.[459,602,604,605]

All four algorithms also compare conservation between species, due to the fact that the miR seed region has a generally higher evolutionary conservation than non-seed regions. This provides evidence that a sequence match may be functional due to the observation that it appears to have been evolutionarily selected for. However, data shown earlier (Figure 3.8) suggest that although miRs may be conserved between organisms on the level of the nucleotide sequence, their role may be different, even between relatively closely related organisms such as humans and mice. The use of conservation as a selection tool for predicted targets therefore means some interactions that are exclusive to humans may be missed. However, this tool can be used to look at some of the more conserved pathways

which miRs regulate, as a starting point for functional analysis of miRs in response to infection.[459,602,604–606]

Free energy is another measure of stability of the interaction between an mRNA and the corresponding miR, with a more stable interaction identified by negative free energy. This is only used by three of the four algorithms (Diana, miRanda and miRDB; not TargetScan). Another measure of the likelihood of a reaction being "real" is the accessibility of the target site in the mRNA. If the secondary structure of the mRNA makes it unlikely that the predicted target site is open for miR targeting or requires a large energy input to make it available, then the interaction is not thought to be relevant. This is also used for three of the four algorithms to identify predicted mRNA targets (Diana, miRanda and miRDB; not TargetScan).[459,602,604,605]

These are the four main criteria utilised by the four target prediction algorithms used here, although there are other factors involved in identifying predicted targets. For example, target site abundance is used by Diana, to predict the number of potential miR binding sites that are present in the 3' UTR of an mRNA. Although only one target site is needed for miR-dependent regulation of a target gene, this interaction is thought to be more functionally relevant if multiple target sites are present in the 3' UTR.[459,602,605] Machine learning approaches, often using support vector machines, are also being used more widely, where the prediction model is trained on a training dataset to help to predict real interactions more accurately. This allows determination of the predictive power of each of the criteria, to allow better ranking of predicted targets.[459,604,605]

5.3.1.1 Diana

The full name for Diana is Diana-microT-CDS. This software uses two training datasets, one from PAR-CLIP (photoactivatable-ribonuclease-enhanced crosslinking and immunoprecipitation) data and the other from microarray data (to learn the specific contribution of multiple sites in a target). This algorithm is different to the others used in this analysis, in that it looks in both the 3' UTR and the protein coding sequence (CDS), whilst the others only look for target sites in the 3' UTR. The most important features in ranking predicted targets are the efficiency of binding (based on an extended seed sequence), the distance to the nearest end of the 3' UTR or CDS, distance to adjacent binding site(s), free energy, conservation and Adenylate-uridylate (AU) content (AU-rich

elements are markers of increased mRNA instability and thought to be involved in miR targeting of mRNAs). The accessibility of the 3' UTR is another feature taken into consideration but not a major factor. The 3' UTR and CDS are analysed individually, then a combined score is presented for each target. The results are detailed including the scores and information on the predicted target gene location, as well as links to relevant publications and whether the interaction is predicted by miRanda or TargetScan, or if it has been experimentally validated. This algorithm can give different results to the others due to the inclusion of the CDS, although the stringent conditions used for selection meant that the present study did not result in excessive numbers of Diana-predicted mRNA targets for each of the four miRs. The reference to previous experimental validation is a useful feature, to observe if this is worth pursuing and if it may indeed be a functional interaction. The targets identified here will be discussed in more detail below, including whether they have been experimentally validated and in which models.[451,452,459,602,604,605]

5.3.1.2 miRanda

The miRanda-miRSVR algorithm uses the miRanda method to identify target sites and then a support vector regression to rank them. miRanda has three steps: (i) identification of sequence matches; (ii) free energy prediction; and (iii) conservation of the binding site and its position. The sequence comparisons use the whole miR, not just the seed region, but matches within the seed region are weighted more highly. To rank the predicted targets, the support vector regression was trained on experiments using HeLa cells. Matches are ranked by the predicted effect a miR will have on the mRNA expression by taking into account site accessibility, AU content of flanking regions, target site position in the 3' UTR and the length of the 3' UTR. This algorithm is thought to be effective, although it does have some problems as it may not always use the most up to date miRBase database version. However, with the miRs this PhD thesis focuses on being among the most well studied and earliest discovered miRs, this was deemed not to be a major concern for the predictions in this Chapter.[453,454,459,602,604,605]

5.3.1.3 miRDB

miRDB is also called MirTarget2, and this algorithm is based on a support vector machine trained on a large microarray dataset. The features in this model include seed conservation, seed match (in the seed region only), base composition in the flanking regions, secondary structure (includes site accessibility and free energy), and location within the 3' UTR. One

major limitation with this algorithm is that the training dataset only contains mRNAs with one target site, and that there are none with multiple binding sites. This was done by the software developers to limit potential confounding factors and reduce the complexity involved with multiple sites, however this limitation may skew the dataset somewhat, as an increased number of potential target sites in a 3' UTR may suggest an increased possibility of a "real" miR:mRNA interaction.[455,456,459,604]

5.3.1.4 TargetScan

This prediction software can either rank candidates on their predicted efficiency of targeting or conservation. For this analysis, predicted efficiency of targeting was used, where the score is derived from experimental results with factors including 3' compensatory pairing, local AU content and position contribution. The conservation rating takes into account how strongly conserved each k-mer is (8mer, 7mer, etc.). This software contains links to the 3' UTR and previous publications where this interaction has been published. To be able to clearly see the number of predicted target sites for a specific miR, as well as the different miRs predicted to bind to a specific mRNA 3' UTR, allows ease of use and simple interpretation of results. The ability to see all predicted target sites, ignoring conservation, is also an option to remove the bias explained above for other algorithms that rely on conservation as a selection criterion. However, as this is a preliminary screen for potentially well conserved targets and pathways, this option was not used and the target list presented in this Chapter contains only well conserved sites.[457–459,602,604–606]

The sensitivity and specificity of target prediction algorithms can often be quite low, with false positive rates a problem.[602] For example, one study showed that TargetScan had a sensitivity of only 20%.[604,612] This is another reason for using all four algorithms in parallel, as any target predicted by multiple algorithms is more likely to represent a real interaction, therefore suggests most promise for experimental validation.

5.3.2 Selected genes predicted to be targeted by only one miR

A selection of genes that were only predicted to be targeted by one miR and their potential biological relevance in acute infection is discussed below. Those selected were thought to be most relevant due to their reported roles in either infection or the immune system, specifically innate immune responses or inflammation.

5.3.2.1 Predicted miR-223 targets:

5.3.2.1.1 PR/SET domain 1 (PRDM1)

PRDM1 is a repressor of beta-interferon (IFN-β) gene expression, with its gene located on chromosome 6, and it is predicted to be targeted by miR-223 as its mRNA is predicted to contain a 7mer in its 3' UTR. This protein is expressed across many different types of tissues, including the oesophagus, endometrium and colon.[613] PRDM1 is an attractive candidate for future investigations due to its known role in the immune response. It controls expression of effector cytokines in natural killer cells, both IFNγ (interferon gamma) and TNF-α and TNF-β (tumour necrosis factor alpha and beta).[614] PRDM1 is induced during dendritic cell maturation[615] and is important in antigen presentation.[616] There are many links between PRDM1 and autoimmunity. For example, PRDM1 missense mutations increased T cell proliferation, cytokine secretion and L-selectin expression in Crohn's disease.[617] The mechanism of autoimmune pathogenesis is thought to be through expression in thymic epithelial cells,[618] or through interaction with human parvovirus B19 in autoimmune thyroiditis.[619] These studies propose that PRDM1 can affect many different elements of the immune response, suggesting it may be important in the acute response to PD-related peritonitis.

5.3.2.1.2 Interleukin 6 signal transducer (IL6ST)

IL6ST (also known as gp130) is not only a signal transducer for IL-6 (interleukin 6), but a range of other cytokines in the IL-6 family, as part of a receptor complex. When a member of the IL-6 cytokine family binds to IL6ST, the receptor dimerises and intracellular signalling cascades are initiated.[620] The *IL6ST* gene is located on chromosome 5, and the IL6ST mRNA has two predicted binding sites for miR-223 in its 3' UTR; one 8mer and one 7mer. It is expressed across a wide range of tissues, including in the placenta, liver and spleen.[613] IL-6 is one of the most important cytokines in the acute immune response, and was first identified as a B-cell differentiation factor and required for antibody production.[621] IL-6 and IL6ST are important in immunity against infections, for example IL-6 can be used as a specific biomarker for infection over other inflammatory conditions,[622] and this has also been shown in PD-related peritonitis.[222] IL6ST has a key role to play in anti-viral immunity through both CD4+ and CD8+ T cells.[623] IL-6/IL6ST are also important in many other parts of the immune response including T cell differentiation,[624] neutrophil trafficking[625] and acute-phase protein expression.[621,626] IL-6, and IL6ST, have been implicated in a number of pathological conditions, most notably autoimmune disorders due to their roles in the

immune response,[627] as well as inflammatory conditions including atherosclerosis,[628] sarcoidosis[629] and myocardial infarction.[630] All of this suggests that IL-6 and IL6ST may be important in the acute response to PD-related peritonitis, therefore it is conceivable that targeting by miR-223 may be important to regulate this inflammatory reaction. The interaction between IL6ST and miR-223 has not been experimentally validated, therefore further work would need to confirm this, however it has been shown that miR-142 and miR-143 can regulate IL6ST in a fever-induced immune response.[631] It will be interesting to investigate whether miR-223 also regulates IL6ST and if this has a role in the acute immune response to infection.

5.3.2.2   Predicted miR-21 targets:

5.3.2.2.1   Transforming growth factor beta 1 (TGFB1)

TGF-β1 is the secreted ligand of the TGF-beta superfamily of proteins, which binds to TGF receptors and activates transcriptional pathways via Smad proteins, among others.[632,633] The *TGFB1* gene is expressed on chromosome 19, and the corresponding mRNA contains one highly conserved 8mer predicted miR-21 binding site in its 3' UTR. TGF-β1 is expressed across a wide range of tissues, including the spleen, bone marrow and lung.[613] It is a well-studied protein with roles across many different biological pathways, including embryonic development and tissue homeostasis, proliferation, apoptosis, differentiation and inflammation. Most interesting for the present study is its function in inflammatory pathways and the immune response. TGF-β1 can be both pro- and anti-inflammatory, dependent on the local microenvironment, co-stimulatory factors and the specific cell type.[632,634,635] TGF-β1 can act on naïve T cells, controlling their differentiation towards the pro-inflammatory Th17 phenotype when IL-6 is present, whilst Treg cells are generated when there is no IL-6 present. This suggests that TGF-β1 may help regulate the T cell response dependent on the state of the innate immune reaction.[636] TGFB1 knockout animals have confirmed the regulatory role for this gene in inflammatory processes, when there is no stimulus of the innate immune system. These animals have high rates of death *in utero*, and those that do survive to birth develop inflammatory disease in many tissues, especially the heart and lungs.[213,637] The presence of IL-6 (and other pro-inflammatory acute response proteins) in the peritoneum of peritonitis patients suggests that TGF-β1 would have a pro-inflammatory role in the peritoneum of a peritonitis patient. The proposed suppression by miR-21 may have a protective effect to reduce the inflammatory reaction, however there is a lower level of miR-21 in infected PD effluent. All this means

that it is possible that reduced miR-21 may mean increased TGFB1, therefore a stronger inflammatory reaction, which may influence the resolution of infection. However, this would be dependent on confirmation of a functional interaction between miR-21 and TGFB1. It has been suggested that miR-21 may be regulated by Smad proteins in renal fibrosis, which are controlled by TGFB1,[632] therefore the reverse method of control may be important. It is yet to be proven experimentally whether miR-21 regulates TGFB1, and how this may modulate the acute immune response to infection.

5.3.2.2.2 Recombination signal binding protein for immunoglobulin kappa J region (RBPJ)

RBPJ is a transcriptional regulator involved in the Notch signalling pathway, which regulates multiple cellular processes, including differentiation, proliferation and survival.[638] The *RBPJ* gene is located on chromosome 4 and its mRNA contains one predicted 7mer binding site for miR-21 in its 3' UTR. RBPJ is expressed in a variety of different human tissues, including the placenta, endometrium and bone marrow.[613] RBPJ and Notch signalling are involved in many different aspects of the immune response including T cell cytotoxicity,[639] CD4^{+} memory T cell function,[640,641] B cell development[642] and Th17 cell heterogeneity.[643,644] However, most relevant to acute PD-related peritonitis is the role of RBPJ in the innate, inflammatory acute response. RBPJ has many roles in dendritic cells, including in their homeostasis, tissue-specific development, maturation and control of T cell activation (in both infection and tumour immunity).[638,645,646] RBPJ also affects macrophage activation and TLR-induced gene expression.[638,647] RBPJ is involved in the pathogenesis of inflammatory diseases in skin and the intestinal epithelium,[638,648] as well as in the recruitment of inflammatory leukocytes in vascular inflammation.[649] In this analysis, it is predicted that miR-21 targets RBPJ, but this has not been evaluated experimentally to date. However, it has been shown that miR-133a can regulate RBPJ in dendritic cells in osteosarcoma.[645] The large number of functions of RBPJ in immune responses, both innate and adaptive, suggest that it may be important in acute PD-related peritonitis. It is yet to be evaluated whether targeting by miR-21 is functional, and whether this is important in the acute immune responses.

5.3.2.3 Predicted miR-31 targets:

5.3.2.3.1 Limb bud and heart development (LBH)

LBH, as its name suggests, was first identified in development of limbs and the heart,[650] but it is now known to be a conserved transcriptional regulator with roles in autoimmune

pathologies, cancers and cardiac diseases, as well as development. The gene is located on chromosome 2 and its mRNA has a 7mer predicted to be a binding site for miR-31 in its 3' UTR. LBH is expressed across multiple different human tissues, with high levels in the lung and lymph nodes, among others.[613] LBH has potential roles in cardiac pathologies, due to its association with heart development.[650–652] It has also been identified as a biomarker and potential therapeutic target in breast, lung and nasopharyngeal cancer.[653–655] The mechanistic role of LBH in autoimmune disorders is not clearly defined, but it is a genetic factor associated with rheumatoid arthritis, celiac disease and systemic lupus erythematosus through genome-wide association studies (GWAS), mechanistic and association studies.[656–660] Due to the limited current knowledge on the function of LBH in the immune system, one can only speculate whether LBH has a role in regulation of the immune response, especially during infection. The functional relevance of the proposed interaction between miR-31 and LBH awaits experimental confirmation.

5.3.2.3.2 Protein kinase C epsilon (PRKCE)

PKCε is a serine- and threonine-specific protein kinase that phosphorylates multiple proteins in a range of cellular processes,[661] with expression across a wide range of tissues, including the brain, lung, kidney and many others.[613] The *PRKCE* gene is located on chromosome 2 and its mRNA contains one 8mer predicted binding site for miR-31 in its 3' UTR. Due to its role in multiple cellular processes, PKCε has been identified as a risk factor in cancers,[661] including lung cancer,[662] small B-cell lymphoma[663] and gallbladder cancer.[664] PKCε is involved in inflammatory pathways and a range of inflammatory pathologies. For example, PKCε was identified as altered in asthma and important in its pathogenesis.[665] Also, PRKCE is a risk factor for diabetic nephropathy, with links to the inflammatory pathogenesis.[666] Here, the PRKCE mRNA was identified as a potential target for miR-31, but this has not been experimentally validated. However, PRKCE has been shown to be targeted by other miRs including by miR-146a (an inflammatory miR) in anterior uveitis, a rare inflammatory eye condition, and papillary thyroid tumorigenesis;[667,668] by miR-218 in gallbladder cancer;[664] by miR-182 in lung cancer;[669] and by miR-129 in cell cycle arrest.[670] What is already known about the many roles of PKCε in inflammation and immune responses suggest that this protein may be important in the acute response to PD-related peritonitis, although this has not yet been investigated. It is also potentially regulated by miR-31, which would need to be experimentally validated to see if this is a functional interaction and if it has a role in peritonitis.

5.3.2.4 Predicted miR-27a targets:

5.3.2.4.1 Monocyte to macrophage differentiation associated (MMD)

MMD is not a well-studied gene, and not much is known about its function. It is expressed in *in vitro* differentiated macrophages, but not freshly isolated monocytes.[671] It is also expressed highly in fat, and in a range of other tissues including the brain, placenta and lymph node. The gene is located on chromosome 17 and contains one highly conserved 8mer in the 3' UTR of its mRNA that is predicted to be a miR-27a binding site. This protein has seven transmembrane regions and is part of the progesterone and adipoQ receptor family.[672] MMD has a role in nitric oxide (NO) and TNF-α production in macrophages via the ERK pathway,[673] as well as in macrophage differentiation.[671] MMD has been localised to organelles within the cell such as the Golgi apparatus,[673,674] where it functions via the ERK pathway to sense Ras,[675] and may have a role in EMT in tumour cells.[676] In the present study, MMD is predicted to be a target of miR-27a, this has not been experimentally validated. However, it has been shown that MMD is functionally regulated by miR-140, and the knock-on effects on the ERK pathway have effects on the growth of lung cancer cells.[677] Due to the role that MMD plays in the differentiation and function of macrophages, which are important cells in the acute immune response to peritonitis, it is likely that changes in MMD may be functionally relevant. The regulation of this protein by miR-27a is yet to be proved, and it would be interesting to investigate this in acute PD-related peritonitis.

5.3.2.4.2 Transcriptional and immune response regulator (TCIM or C8orf4)

TCIM, also known as *C8orf4* and *TC-1*, encodes a positive regulator of the Wnt/β-catenin pathway expressed across a wide range of tissues, although there are low levels in leukocytes, the brain and thymus.[678] The gene is located on chromosome 8 and its mRNA contains a conserved 8mer predicted binding site for miR-27a in its 3' UTR. TCIM has been associated with worse prognosis in multiple types of cancer due to positive regulation of β-catenin genes which are associated with invasiveness and aggressiveness.[679] These pathologies include lung cancer,[680,681] ovarian cancer[682] and breast cancer,[683] as well as thyroid cancer, from which it got its original name of TC-1 (thyroid cancer-1).[678,684] TCIM is an endothelial inflammatory factor, which both induces, and is induced by, inflammatory stimuli to form a positive feedback mechanism.[685] TCIM is involved in mediating responses to cellular stress in models of both heat shock responses and lung fibrosis.[686,687] Although the levels of TCIM detected in leukocytes are low, this is due to the fact that it is a

haematopoietic regulator determining lineage differentiation from progenitor cells, meaning that levels in differentiated cells are generally much lower.[688] TCIM appears to be involved in the reaction of non-myeloid cells to inflammatory stimuli, suggesting that this might be important in the mesothelial cells and fibroblasts in the peritoneum. While there are no published studies showing miR targeting of TCIM, it can speculated that, if the interaction between miR-27a and TCIM is shown to be functional, this may be important to control the positive feedback loop initiated in mesothelial cells and fibroblasts by inflammatory conditions, which may exacerbate the damage caused by a peritonitis episode. This theory would need substantial experimental evidence to support it.

5.3.3 Selected genes predicted to be targeted by multiple miRs

These targets discussed below were evaluated, in terms of what is already known about them, due to the fact that they were predicted by multiple algorithms to be targeted by multiple miRs.

5.3.3.1 F-box and WD repeat domain containing 7 (FBXW7)

The *FBXW7* gene encodes an E3 ubiquitin ligase and is located on chromosome 4. The mRNA it encodes is predicted to have three 8mer binding sites for miR-223 in its 3' UTR. This protein is expressed across a wide range of tissues, with highest expression in the skin and brain.[613] FBXW7 has a major role in many pathways including tumorigenesis (as a potent tumour suppressor), lipid metabolism, cell proliferation, stemness and differentiation.[689–691] Recent studies have also shown its importance in both infection, including antiviral and anti-prion innate immune responses,[690,692,693] and inflammation. For example, FBXW7 regulates C/EBPδ which then attenuates inflammatory signalling via TLR4,[694] and it has a role in the inflammatory component of cancers.[695] FBXW7 is important in autophagy in both antiviral immunity[692] and inflammation in acute kidney injury,[696] whilst it also has a role in regulating the NF-κB pathway.[697] The miR-223/FBXW7 axis is important in the macrophage activation state and response to inflammatory stimulus, via TLR4.[489] miR-223 can directly target FBXW7, shown by a luciferase reporter assay in gastric cancer cells.[698,699] All of this evidence, along with studies showing an inverse correlation between miR-223 levels and FBXW7 mRNA levels,[700–702] suggest that this is a real target of miR-223. Also, it may be relevant in acute peritonitis infections due to the relevance in other models of innate immunity.[690,692,693] FBXW7 has also been suggested to be a target of miR-27a, with direct targeting shown via a luciferase reporter assay in oesophageal

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squamous cell carcinoma cells, with this being an important pathway for tumorigenesis.[703] The direct targeting of FBXW7 via miR-27a is also implicated in clear cell renal cell carcinoma migration and invasion.[704] There is less indication of the role of this interaction/regulation in infection or inflammation, but there is a likelihood that the interaction between FBXW7 and both miR-223 and miR-27a may play a role in the acute immune response to infection. This needs further work to investigate its relevance in PD-related peritonitis.

5.3.3.2 Armadillo repeat containing X-linked 1 (ARMCX1)

ARMCX1, also known as *ALEX1* (Arm protein lost in epithelial cancers chromosome X protein 1), is a gene located on the X chromosome[705,706] and is a tumour suppressor, therefore is lost in epithelial cancers.[707–711] However, it is proposed that ARMCX1 can also act as an oncogene, for example in cervical cancer.[707] ARMCX1 is expressed by all cells except peripheral blood leukocytes,[711] with highest levels in the ovary.[613] This mitochondrial protein is specific to eukaryotes and is highly expressed in neuronal tissues.[712] There is limited research on this protein, with no implications so far for a role in either infection or inflammation. Although it is a predicted target of both miR-223 and miR-21 (with one 8mer predicted for both miRs), no functional interaction between miR-223 and ARMCX1 has been shown. There is one study that looked into the relationship between ARMCX1 and miR-21 in rats, showing a negative correlation between miR-21 expression and ARMCX1 expression, although there is no indication of whether this is a direct or indirect effect.[713] The role of this protein in acute infection is unknown, but its identification as a potentially important target for miR-223 and miR-21 during PD-related peritonitis suggests it may be important in inflammation. This would need substantial further investigation to confirm if this protein and the interaction with miR-223 and miR-21 is relevant for acute inflammatory processes.

5.3.3.3 Sprouty RTK signalling antagonist 2 (SPRY2)

SPRY2 is a member of the sprouty family of genes, which encode RTK signalling agonists. SPRY2 is expressed across a wide range of tissues, including fat, gall bladder, spleen and brain.[613] This gene is on chromosome 13 and its mRNA predicted to have an 8mer binding site for both miR-21 and miR-27a in its 3' UTR. In fact, it has been experimentally validated that miR-21 represses SPRY2 expression in a range of cancer cell types, in liver fibrosis and in T cells activated by space flight.[714–721] It is also regulated by miR-27b (which has the same

recognition sequence as miR-27a) in glioma cells,[722] with functional effects on the cancer progression, mostly due to the effect of SPRY2 on EMT or on the extracellular-signal-regulated kinase (ERK) pathway, as SPRY2 is an antagonist of ERK.[719,720] A decrease in SPRY2 promotes EMT in multiple cancer models,[719,720] as well as in lens epithelial cells in cataracts,[723] whilst an anti-inflammatory role for SPRY2 via ERK signalling in both rheumatoid arthritis and IgA nephropathy has also been reported.[724–726] The role of SPRY2 is varied; it can control proliferation or phenotypic variation in a range of cell types and conditions, including T cells, B cells and macrophages in cancers and inflammatory conditions.[727–734] SPRY2 may have a role in endotoxin tolerance in monocytes,[735] and inhibition of SPRY2 polarises macrophages towards the M2 phenotype,[729] suggesting it has a role in the innate immune response. SPRY2 has many roles in the immune response (both acute and adaptive) and is a validated target of two of the miRs identified in this Chapter as important in PD-related peritonitis (miR-21 and miR-27a). It is therefore conceivable that decreased levels of miR-21 and miR-27a may lead to an increase in SPRY2 expression which might enhance EMT. This could have a serious effect on the consequences of a peritonitis episode, accelerating the progression to technique failure. This hypothesis would need to be tested further by evaluating which cell types are taking up, and responding to, miR-21 and miR-27a, and by investigating the effects of this on SPRY2 protein levels.

5.3.3.4 Ras p21 protein activator 1 (RASA1)

RASA1 is located on chromosome 5 and encodes a cytoplasmic GTPase activating protein that suppresses RAS function by enhancing its weak GTPase activity. It is abundantly expressed in the placenta, with lower levels across many adult human tissues.[613] In the 3' UTR of its mRNA it is predicted to have one 8mer for both miR-223 and miR-31, as well as a 7mer-A1 for miR-21. In the analysis conducted here, RASA1 was only predicted to be targeted by miR-223 and miR-31, although there is some experimental evidence that miR-21 may functionally target RASA1, too.[736–738] RASA1 is an important protein in vascular development, as RASA1 knockout animals are not viable and mutations in humans are strongly linked to a vascular condition called capillary malformation–arteriovenous malformation.[739–741] RASA1 has been well studied in different cancers including ovarian, lung and breast cancers.[742–744] miR-223 has been shown to functionally target RASA1 in colorectal cancer and macrophage activation, showing roles in cell proliferation and activation.[745,746] RASA1 is essential for immune cell function, as demonstrated by its role in the positive selection of thymocytes.[747] The link between miR-223 and RASA1 has also been

demonstrated functionally in cardiac fibroblasts after myocardial infarction, where miR-223 regulates cardiac fibrosis through the direct targeting of RASA1.[748] miR-31 can directly target RASA1 in the oncogenesis of intrahepatic cholangiocarcinoma,[749] colorectal cancer[750] and pancreatic cancer,[751] as well as in the re-epithelialisation stage of wound healing[752]. These important roles in many different cellular processes in both epithelial cells and macrophages suggest that RASA1 may be important in the acute immune response to infection, given its roles in thymocyte selection and macrophage polarisation,[746,747] which may be important in the response to PD-related peritonitis.

5.3.3.5 Vav guanine nucleotide exchange factor 3 (VAV3)

VAV3 is a member of the guanine nucleotide exchange factors for Rho GTPases, with involvement in actin cytoskeleton rearrangements and transcriptional alterations. Its gene is located on chromosome 1 and is expressed in a wide variety of tissues, with high levels in kidney, skin, colon and oesophagus, amongst others.[613] The 3' UTR of VAV3 mRNA has many predicted binding sites for different miRs, including a 7mer for both miR-31 and miR-27a. VAV3 knockout animals have a wide range of pathologies, including cardiovascular disorders and impaired cerebellum development.[753] VAV3 has been implicated in different human pathologies, with roles in cardiovascular disorders,[754] glaucoma[755] and osteoporosis,[756] as well as acting as an oncogene in multiple cancers, including gastric and breast cancers.[757,758] The role for VAV3 in immune cell function has been well studied, with roles in B and T cell signalling,[759,760] neutrophil function,[761,762] macrophage phagocytosis[762–764] and natural killer cell cytotoxicity.[765] VAV3 is also essential for the innate immune response to fungal infections,[766] and confers a risk factor for inflammatory diseases such as colitis, IgA nephropathy and type 1 diabetes.[767–769] VAV3 is a direct target of other miRs including miR-489 and miR-499,[770,771] but it is unknown whether the predicted interaction with miR-31 or miR-27a is a functional one. The relevance of a possible regulation by the candidate miRs identified here is unknown, although this protein is important in neutrophil recruitment in response to inflammatory stimuli[772] and is altered in lymphocytes stimulated with LPS or infected with EBV.[773,774] This confirmed role of VAV3 in the immune system identifies it as a strong candidate for further research into acute responses to PD-related peritonitis.

5.3.3.6 Cyclin C (CCNC)

CCNC controls cell cycle progression by interacting with cyclin dependent kinase 8 (CDK8). This growth-promoting role means it is associated with some types of cancer.[775–778] The gene is located on chromosome 6 and is expressed in a wide range of tissues, including the thyroid, gall bladder and kidney.[613] The 3' UTR of its mRNA contains one predicted conserved 8mer for both miR-31 and miR-27a and is therefore predicted to be a target of both miRs. CCNC has been implicated in viral infections, with roles in the innate response including the initiation of innate immunity genes in *Drosophila*[779] and plants (specifically upon infection with *Fusarium graminearum*).[780] The CCNC and CDK8 complex is important in regulation of innate immune activation in myeloid cells, especially upon TLR9 activation.[781,782] The role of CCNC in maintaining cell growth is also important in maintaining PBMC viability in culture after exposure to hypoxic conditions, when other cyclins are decreased by hypoxia.[783] The main focus of research on this protein has been on its primary role in the cell cycle, whilst there is some evidence that it has a potential role within the immune system. Although there is no experimental evidence suggesting CCNC may be targeted by miR-31 or miR-27a, it has been shown to be directly regulated by miR-206 in melanoma.[784] The potential for CCNC to be an important regulator of the acute immune response will need to be analysed further.

5.3.3.7 Paired box 9 (PAX9)

PAX9 is a member of the paired box family of transcription factors encoded by genes on chromosome 14. The mRNA is predicted to have an 8mer binding site for miR-31 and an 8mer for miR-27a in its 3' UTR. The expression of this gene is very high in the oesophagus, with high levels also present in the salivary gland and prostate.[613] This protein is one of the most widely studied and most important genes in odontogenesis (tooth development), with mutations being involved in tooth developmental conditions[785–788] including oligodontia,[789–791] hypodontia[792,793] and anodontia.[794] PAX9 has been studied in cancers, including oral squamous cell carcinoma, chronic lymphocytic lymphoma and lung cancer. This tumorigenic protein maintains proliferation and inhibits apoptosis in cancer.[795,796] The role in craniofacial development is also described, with an association between PAX9 polymorphisms and the formation of a cleft lip or palate.[797,798] PAX9 has also been shown to be involved in epithelial differentiation.[796] There is a potential role for single nucleotide polymorphisms (SNPs) in PAX9 in aggravating the effect of human cytomegalovirus (HCMV) on reducing the birth weight of new-borns.[799] PAX9, as a transcription factor, is known to

regulate expression of genes involved in many different roles, including cell proliferation, resistance to apoptosis and migration. The conservation of PAX9 across all vertebrates allowed for further studies in mouse models,[796] although there are no reported roles for PAX9 in immunity and inflammation, with most studies focusing on developmental associations. With regard to its potential role during PD-related peritonitis, its strong expression in many different epithelial cells suggest it could be present in the resident cells lining the peritoneal cavity, and therefore may be targeted by miRs present in the PD fluid. However, this would need further investigation to verify if this is the case.

5.3.3.8 Zinc finger SWIM-type containing 6 (ZSWIM6)

ZSWIM6 is located on chromosome 5 with one predicted 8mer binding site for miR-21 and a 7mer site for miR-31 in the 3' UTR of its mRNA. This conserved protein is currently of unknown function, but it has been associated with neurodevelopment and neurocognitive disorders.[800–805] GWAS suggest a possible link to schizophrenia,[804] as well as an association with severe intellectual disability and acromelic frontonasal dysostosis (a neurodevelopmental disorder).[800,801,803,805] It is thought to have a role in the epigenetic regulation of gene transcription, through interacting with chromatin remodelling complexes, as well as possibly in the ubiquitination pathway.[800] A knockout mouse for ZSWIM6 is viable, although it has severe neurocognitive problems, similar to the intellectual and neurodevelopmental deficiencies shown in humans with mutations in ZSWIM6.[800,805] Current work has focused on the role in neurological disorders, while its role in response to infection or inflammation is unknown. A range of tissues and cell types express ZSWIM6, including the brain, gall bladder and colon.[613,800–805] Targeting by miR-21 and miR-31, if validated, may be relevant in PD-related peritonitis, but this has not been investigated yet and may require considerable work to examine.

5.3.3.9 Solute carrier family 16 member 10 (SLC16A10)

SLC16A10 (also called *TAT1* and *MCT10*) is located on chromosome 6 and encodes an aromatic amino acid transporter located at the plasma membrane and expressed across a range of tissues including the placenta, skin and kidney.[613,806] In the 3' UTR of its mRNA it is predicted to have an 8mer binding site for both miR-21 and miR-27a. There is a knockout model for SLC16A10, and these SLC16A10 deficient animals develops normally but have higher blood plasma and urine concentrations of the aromatic amino acids (phenylalanine, tryptophan and tyrosine). This transporter also plays a role in the uptake and efflux of

thyroid hormone.[807–811] The role in tryptophan transport indicates that this protein may be important in the pathogenesis of a rare autosomal recessive metabolic disorder called blue diaper syndrome.[806,811,812] SLC16A10 is involved in a range of cancers, both as a biomarker and in the pathogenesis, including endometrial, ovarian and bladder cancers, with increased levels detected in the blood of patients with malignant tumours, and a negative correlation with prognosis, as well as a biomarker for benign tumours in some disorders.[813–816] There have been no published studies relating SLC16A10 to either the immune response, inflammation or infection. However, it is known that tryptophan can modulate immune responses, especially in the generation of tolerance.[817] With regard to PD-related peritonitis one could therefore speculate that alterations in SLC16A10 may play a role in differing tryptophan levels, which may contribute to the immune response during acute infection. This would require further investigation, as well as validation whether SLC16A10 is indeed a direct target of miR-21 and/or miR-27a.

5.4   Future prospects

This chapter investigates potential targets of the four candidate miRs and briefly evaluates their potential role in the response to acute PD-related peritonitis. Due to the nature of target prediction, and the large number of potential targets that have been identified through this analysis, it is essential to further this work through experimental investigations. For example, it would be prudent to investigate the levels of these target proteins in cell-free PD effluent samples and levels of the mRNAs in cells from patients, to attempt to correlate with the levels of the candidate miRs. It would also be important to construct reporter assays to ascertain if the predicted interactions between miR and mRNA are functional. Until these investigations are conducted, this analysis is merely theoretical, and the importance of the proposed interactions can only be speculated upon.

6 Stabilisation of extracellular microRNAs in PD effluent

6.1 Introduction

miRNAs are useful biomarkers in biological fluids due to their stability outside cells,[389] either by association with protein complexes[431,435,436] or inside extracellular vesicles (EVs).[389] There are a range of different types of EVs, as defined by both size and method of generation. The smallest form are exosomes, which are 30-120 nm in diameter and are released from cells when multivesicular bodies, which enclose them inside the cell, fuse with the cell membrane, expelling the contents.[389,401,412] Microvesicles are larger EVs, of approximately 100-1000 nm diameter. These are released by direct budding off from protrusions of the plasma membrane.[401,412,415] The final type of EV currently classified are apoptotic bodies. These are larger (1-5 μm) and are formed by membrane blebbing during the later stages of apoptosis[424]. All three types of EVs contain miRNAs, with the miRNA composition different to the cells from which they originate, suggesting they are specifically packaged into EVs for functions including intercellular communication.[406,415,416,424] It has been shown that miRNAs released in EVs can be taken up and are functionally active in distant target cells.[403–405]

miRNAs can also be stabilised by association with protein complexes. For example, the Argonaute (AGO) proteins form part of the RNA-induced silencing complex (RISC), which allow miRNAs to target and repress mRNA targets.[399,436] miRNAs bound to AGO proteins are highly abundant in biological fluids and are protected from natural RNases.[436] This is thought to be an active and functional form of intercellular communication.[399,437,438] miRNAs are also often found bound to lipoprotein complexes.[431] This is an active process as miRNAs are specifically selected for extracellular secretion, meaning they have a functional role in communicating between cells.[431–435]

Although it is known that miRNAs are stable in PD effluent,[327] it has not been demonstrated how this stability is achieved, either through EVs, protein complexes, or a combination of both. To investigate this, PD effluent from infected and stable patients, as well as cell culture supernatant from cultured neutrophils, was analysed for the presence of miR-223 in fractions representative of EVs generated by differential centrifugation. The samples both before and after EV-depletion were tested to determine the susceptibility of miR-223 to RNase treatment before and after proteinase treatment. Size-based chromatography

and subsequent plate-based immunological assays determined the particle characteristics and cellular origin of miR-223-containing fractions from PD effluent. A reporter assay was then set up to identify functional uptake of miR-223 from PD effluent, both with and without EVs present.

6.1.1 Aims:

The aims of this chapter were to:

1. Identify how miR-223 is stabilised in PD effluent and medium supernatant from cultured neutrophils
2. Investigate the cellular origin of extracellular miR-223 in PD effluent
3. Set up a luciferase-based reporter assay to determine functional miR-223 uptake

6.2 Results

6.2.1 Identification of miR-223 in extracellular vesicles in PD effluent

To identify how miR-223 is stabilised in PD effluent and media supernatant from cultured neutrophils, differential centrifugation was used to isolate fractions from cell-free PD effluent and media supernatant. These were representative of cell debris (P2), larger microvesicles (P3) and smaller exosomes (P4), with miR-223 measured in the supernatants and pellets after each step (Figure 6.1). This was conducted in PD effluent from patients with PD-related peritonitis (Figure 6.1A) and from stable PD patients (Figure 6.1B). Culture supernatants of neutrophils from healthy donors stimulated with LPS were processed in the same manner and served as experimental controls (Figure 6.1C). miR-223 was abundantly present in samples after all centrifugation steps in infected PD effluent, but there was a substantial amount of miR-223 present in each of the pellets. This was especially important in the pellets representing the different forms of EVs (microvesicles, P3, and exosomes, P4) (Figure 6.1A). In the PD effluent from stable patients, the amount of miR-223 present in the starting material was low, therefore the amount in each of the supernatants and pellets from the differential centrifugation steps was variable. This does however indicate that the small amount of miR-223 present was not abundantly protected by EVs, as P3 and P4 did not contain a large proportion of the remaining miR-223 (Figure 6.1B). In the media supernatant from cultured, stimulated neutrophils there was a very small amount of miR-223 present in the pellets representative of the EVs (P3 and P4), with
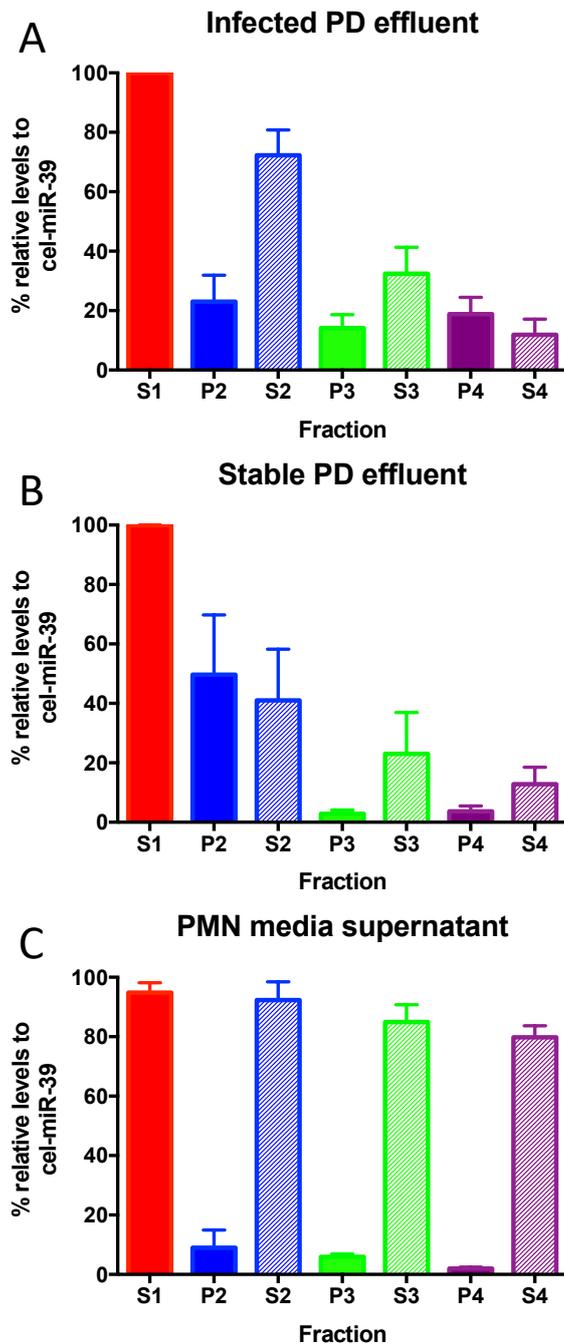
Figure 6.1: miR-223 is present in fractions representative of extracellular vesicles in infected PD effluent

miR-223 levels detected in differentially spun cell-free PD effluent from infected (A) and stable (B) patients, compared to media supernatant from cultured, LPS-stimulated neutrophils (C). S1 represents the cell-free supernatant before centrifugation, S2 represents removal of dead cells and large cellular debris (present in P2), S3 represents removal of larger extracellular vesicles or microvesicles (present in P3) and S4 represents supernatant after removal of smaller extracellular vesicles or exosomes (present in S4). Data presented as mean $\pm$ SEM of 5 separate patients (A and B) or 4 donors (C), and as a percentage of amount of miR-223 present in the uncentrifuged supernatant (S1).

the majority still in the supernatants after high speed ultracentrifugation (Figure 6.1C). This suggests that miR-223 is present in EVs in infected PD effluent but not in medium supernatant from cultured neutrophils, implying that the culture conditions used here for neutrophils were dissimilar to those experienced in an infected peritoneum.

6.2.2 Stability of miR-223 to RNase and Proteinase treatment

To understand how miR-223 is stabilised in biological samples, selective treatment with RNase A and proteinase K was employed, alone and in combination. To do this, PD effluent from infected and stable patients, and culture supernatant from stimulated neutrophils, was treated with RNase A and Proteinase K, before and after EV depletion (Figure 6.2). As a positive control for this experiment, cel-miR-39 was spiked into the samples before treatment. This miRNA is not protected from RNase activity therefore acts as a control to show the RNase is functional, as demonstrated in Figure 6.2.

Before EV-depletion, the miR-223 in infected PD effluent was resistant to both RNase A and proteinase K treatment, both alone and in combination (Figure 6.2A). This suggests that miR-223 is mostly present inside EVs, not associated with protein complexes. When EVs were depleted by ultracentrifugation, the remainder of miR-223 was more susceptible to RNase A treatment and addition of proteinase K also increased degradation (Figure 6.2B). This suggested that the miR-223 that was outside EVs was likely to be bound to protein complexes to protect it from RNases. There was, however, still a proportion of miR-223 that was not protected from RNase activity.

Stable PD effluent contained low levels of miR-223 to start with, so the variability of miR-223 detection in these samples was high. A proportion of the miR-223 present was susceptible to degradation after both RNase A and proteinase K treatment, alone and in combination. This was true for effluent before and after EV depletion (Figures 6.2C and D). This suggested that a proportion of the total miR-223 might have been protected by both EVs and protein complexes. However, the low amount of miR-223 present meant it was difficult to understand the biological relevance of these mechanisms of stabilisation.

Culture supernatant from stimulated neutrophils (Figures 6.2E and F) was treated similarly as control. Of note, the miR-223 present before EV-depletion was more susceptible to degradation by RNases than in PD effluent (Figure 6.2E compared to Figure 6.2A),

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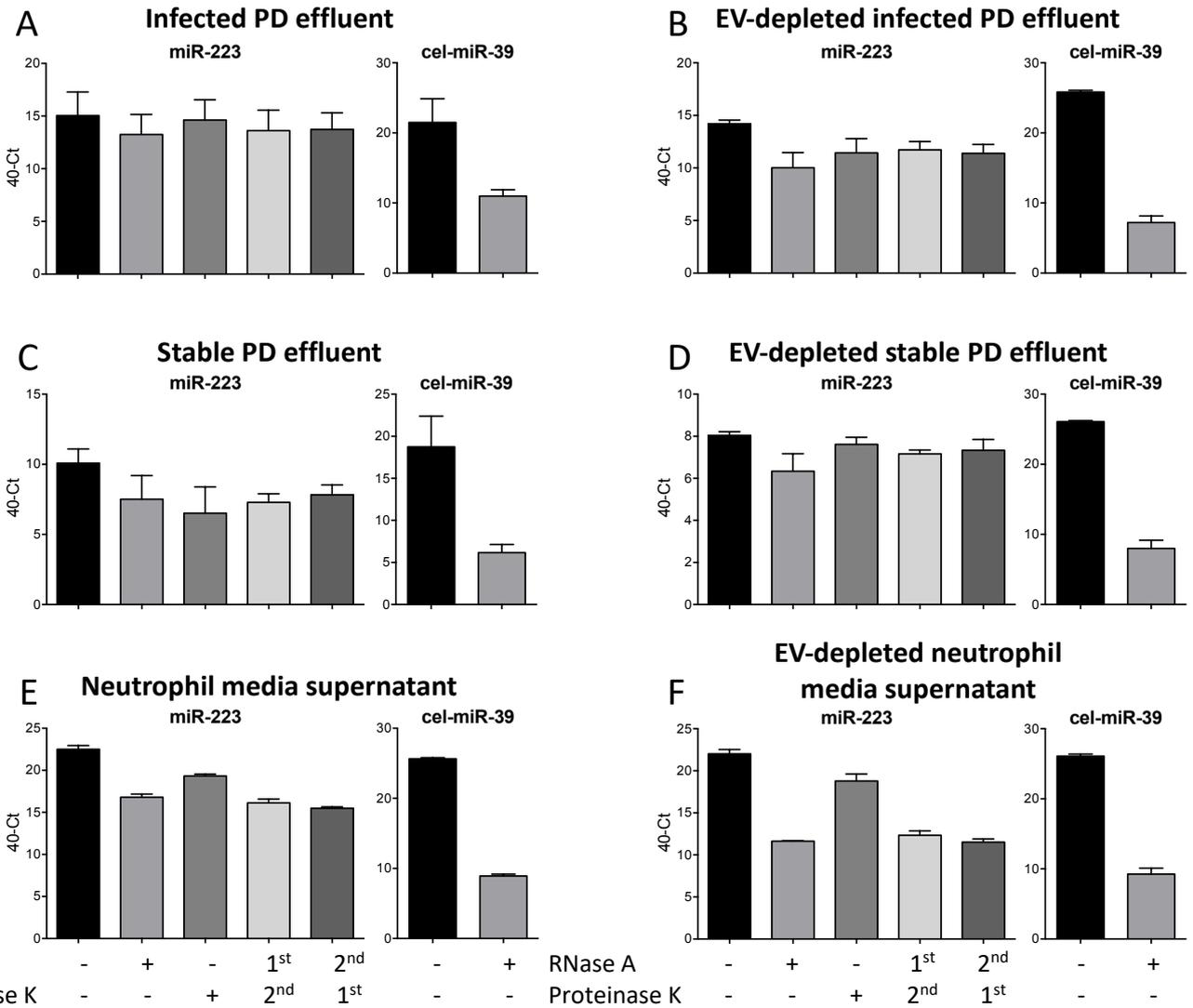
Figure 6.2: miR-223 is protected from RNase A and Proteinase K treatment in PD effluent

PD effluent from infected patients (A and B), from uninfected patients (C and D), and media supernatants from cultured, LPS-treated neutrophils (E and F) was treated with RNase A, proteinase K or a combination of both. Showing both cell-free supernatant (A, C and E) and supernatant that has been depleted of small extracellular vesicles (B, D and F). Data represented as mean ± SEM for 5 patients (A and B), 4 patients (C and D), or 3 donors (E and F), with raw 40-Ct data showing levels of miR-223 and cel-miR-39.

suggesting a proportion of the extracellular miR-223 was not protected. Proteinase K treatment also decreased the amount of miR-223 detected, suggesting a proportion was stabilised by association with protein complexes. When RNase treatment followed proteinase treatment, a similar amount of miR-223 was present as when RNase A was used alone, confirming that miR-223 was bound to protein complexes to prevent degradation (Figure 6.2E). When EVs were depleted from medium supernatant from cultured neutrophils (Figure 6.2F), a larger proportion of miR-223 was susceptible to RNase treatment, suggesting it was not protected by either EVs or protein complexes. Proteinase treatment alone also decreased the level of miR-223 slightly, although the difference between RNase treatment alone and RNase following proteinase treatment was minimal, suggesting that only a small proportion of miR-223 was protected in this way.

Taken together, there was a difference between how cells at the site of infection release miR-223 and how neutrophils under controlled culture conditions release miR-223 in reaction to inflammatory stimuli. It is likely that a combination of both EVs and protein complexes are used to stabilise miR-223 in both PD effluent and culture supernatant, but these proportions are different between the two types of samples, with miR-223 present in a higher proportion of EVs in PD effluent whilst it is protected by protein complexes in media supernatants.

6.2.3 miR-223 is present in exosomes

To confirm whether miR-223 was indeed present in EVs, specifically in exosomes as shown in an unrelated study before,[410] PD effluent samples from 5 infected patients were passed through a size exclusion chromatography column to separate different sized particles. These fractions were then analysed for markers of exosomes (CD9 and CD81), neutrophil surface markers (CD15), soluble protein complexes (represented by human serum albumin) and miR-223. This was to detect whether miR-223 was present in the fractions which represented exosomes, and co-localised with neutrophil markers or with soluble protein complexes. Neutrophils were thought to be the main source of extracellular miR-223 as they produce and release the most miR-223 of the cell types investigated previously in the *in vitro* models (Figure 4.3). They are also the most abundant cell type in infected PD effluent when there is a considerable increase in miR-223 (Figure 3.5), and it is known that they produce functional miR-223 in other experimental models.[410]

PD effluents from 5 patients were each separated into 25 fractions by size-exclusion chromatography. Each fraction was analysed on a plate-based immunological assay for CD9 and CD81 to identify which fractions represented exosomes. As seen in Figure 6.3A and B, CD9 was present in high levels only in fractions 4-7, with a peak at fraction 6 suggesting the majority of exosomes were present in this fraction. CD81 was presenting two peaks, one at fraction 6 and another at fraction 11 (Figure 6.3C and D). This suggested that there might have been two populations of EVs of different sizes, possible exosomes and microvesicles. The reason for this second peak is not known at this time. Co-separation of the EVs labelled with CD9 and/or CD81 with the neutrophil marker CD15 (Figure 6.3E and F) indicated that a proportion of the EVs in the infected effluent samples was derived from neutrophils.

As control, the samples were also probed for human serum albumin, as a representative of total soluble protein. Here, the largest amounts of this marker were observed in later fractions, from 7 to 15 (Figure 6.3G and H), demonstrating that fractions representing soluble protein complexes could be clearly separated from those containing EVs.

Finally, selected fractions were subjected to RT-qPCR to detect miR-223. To this end, fractions 1, 6, 8, 11 and 20 were selected as they represented the different types of particles present; void column volume (1), CD9$^+$ exosomes (6), particles that were not tetraspanin-positive (i.e. CD9$^-$ and CD81$^-$) (8), free protein (11), and negative for all markers (20) (Figure 6.3). miR-223 was not detectable in the void volume or the negative fractions (1 and 20, Figure 6.3I and J). The largest amount of miR-223 was present in fraction 6 (Figure 6.3J), which represented the exosome fraction, a substantial proportion of which was likely derived from neutrophils. This suggested that the majority of miR-223 in patient effluent was stabilised by incorporation into exosomes. There was still some miR-223 present in the later fraction 11, which was representative of free protein (Figure 6.3J). This confirmed that some miR-223 was indeed bound to protein complexes, as predicted by the previous experiments.

The main outcome of these experiments was that a large proportion of miR-223 was stabilised extracellularly in infected PD effluent by incorporation into exosomes, most likely neutrophil-derived exosomes, suggesting that miR-223 may be actively released by neutrophils in response to infection.
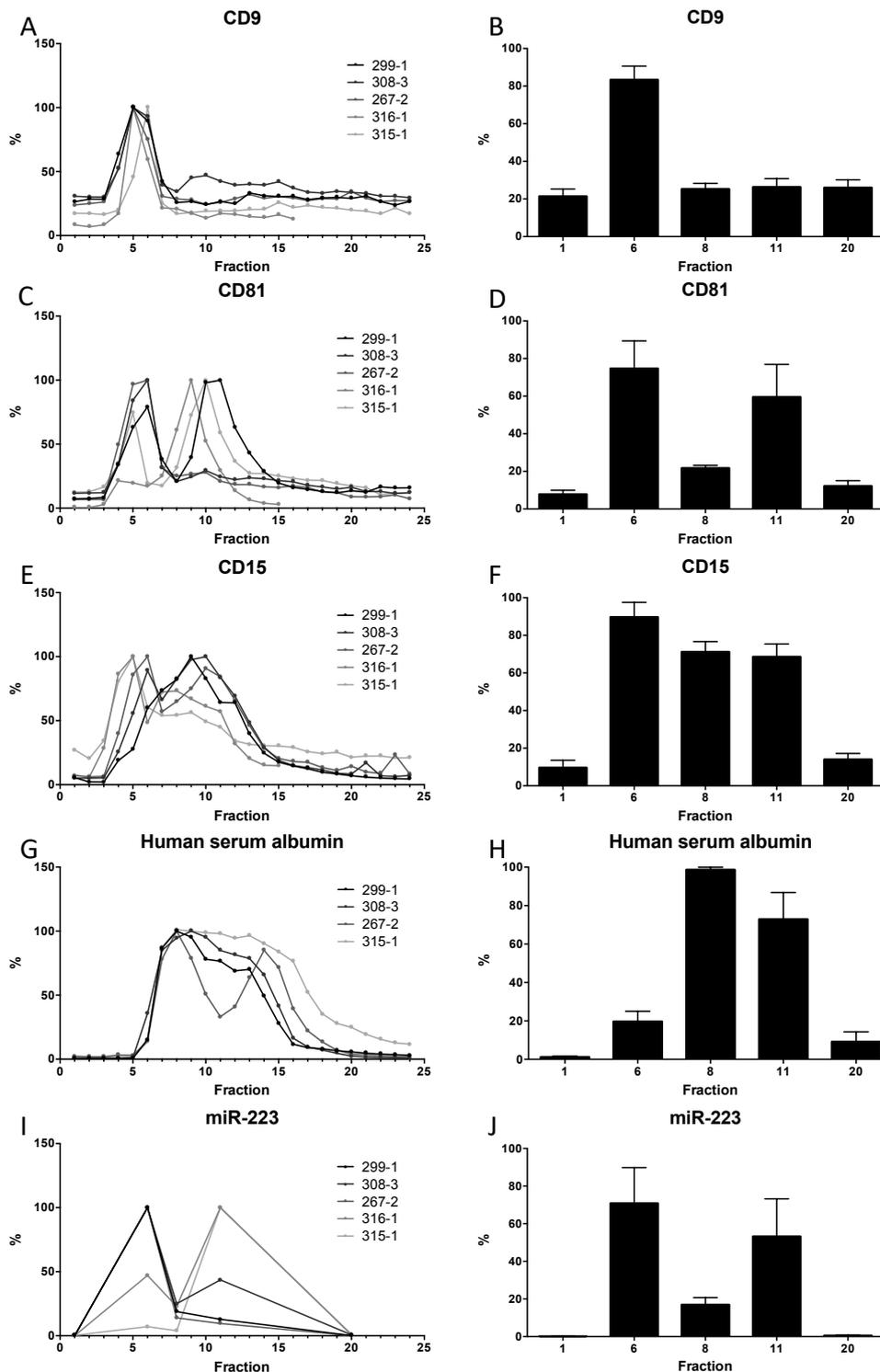
Figure 6.3: Levels of CD9, CD81, CD15, human serum albumin and miR-223 in different fractions representing different sized particles from infected PD effluent
Levels of CD9 and CD81 (EV markers), CD15 (neutrophil surface marker), human serum albumin (representative of soluble protein) and miR-223 in fractionated PD effluent from 5 PD patients on day 1 of a peritonitis episode. A, C, E, G and I show all fractions tested across 5 patient samples (25 for all protein markers, 5 for miR-223) as percentage level compared to the fraction containing the maximum amount of each marker. B, D, F, H and J show the 5 fractions measured for all markers, and the percentage level compared to the fraction containing the maximum amount of each marker, shown as mean ± SEM for 5 patients.

6.2.4 miR-223 reporter assay

To determine whether miR-223 is functional when released into PD effluent, and specifically whether it is taken up by recipient cells, a luciferase-based reporter assay was developed to allow the observation of changes in miR-223 activity in a cell line that does not express miR-223. To achieve this, a miR-223 reporter plasmid was generated by using the pGL3 control plasmid and inserting five perfect miR-223 binding sites to generate an artificial 3' UTR attached to the luciferase gene. Using this construct, any miR-223 that was functional and taken up by the reporter cell was expected to result in a decrease in luciferase activity.

The reporter plasmid was generated through cloning of an artificial 3' UTR into the pGL3 control plasmid as detailed in the Materials and Methods (Figure 2.1). Briefly, it involved linearization of the pGL3 control plasmid and cloning in the synthetic 3' UTR, which was formed by two overlapping oligonucleotides. Stellar competent *E. coli* were transformed with the newly generated miR-223 reporter plasmid and positive colonies were selected by positive growth on agar supplemented with ampicillin (Figure 6.4A), and colony PCR confirmed the cloning was successful (Figure 6.4B). Positive bacteria were grown up and plasmid DNA was extracted by Miniprep. Sequencing results confirmed the plasmid contained the artificial 3' UTR as designed (sequence and alignment in appendix 9.3).

A previous study showed that miR-223 released in exosomes by neutrophils can be taken up and be functional in lung epithelial cells as recipient cells.[410] In the case of PD-related peritonitis, it was speculated that resident peritoneal cells may take up extracellular miRNAs. Hence, MeT-5A cells, a mesothelial-like cell line, were selected for transfection of the reporter assay plasmid. Importantly, MeT-5A cells only express very low levels of miR-223 under normal culture conditions, as shown in Figure 6.5A.

To confirm that the plasmid was working as desired, both the reporter plasmid and the pGL3 control plasmid (without the artificial 3' UTR) were transfected into MeT-5A cells, and 24 hr later miRNA mimics were co-transfected into the cells, either miR-223 or a control miRNA (miR-C). Luciferase activity was determined 48 hr after this second transfection. As seen in Figure 6.5B, there was an approximately 70% reduction in luciferase activity when the reporter plasmid and the miR-223 mimic were co-transfected into the cells, whilst the
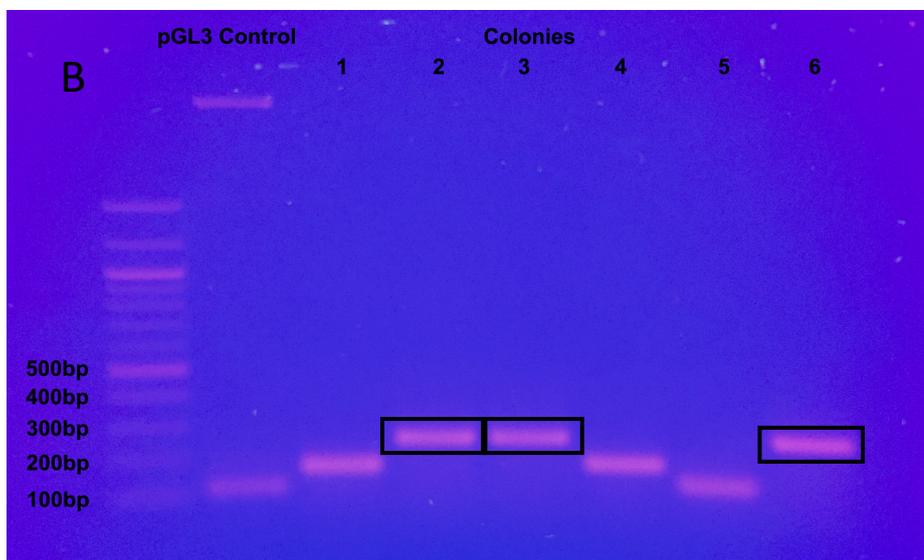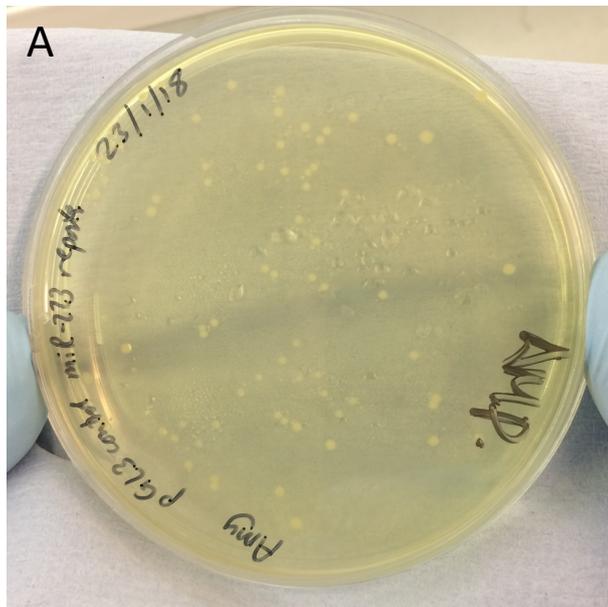
Figure 6.4: Positive cloning for generation of miR-223 reporter plasmid
A shows positive colonies for transformation of miR-223 reporter plasmid into Stellar competent *E. coli*, represented by white colonies on Amp+ agar plate conferring resistance to ampicillin. B shows colony PCR results from six selected colonies run alongside pGL3 control plasmid. pGL3 control plasmid without insertion is represented by band of approximately 115bp, whilst pGL3 control plasmid with inserted artificial 3' UTR is represented by band of approximately 283bp, as seen in colonies 2, 3 and 6.

A MeT-5A miR-223 expression

B Reporter assay controls
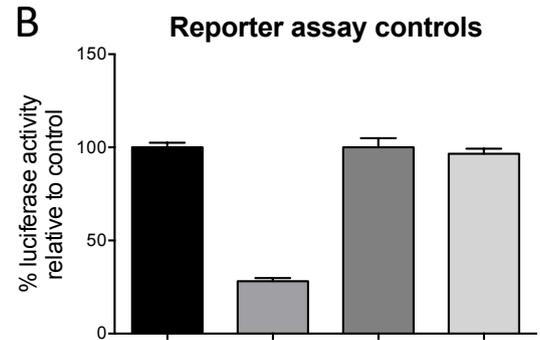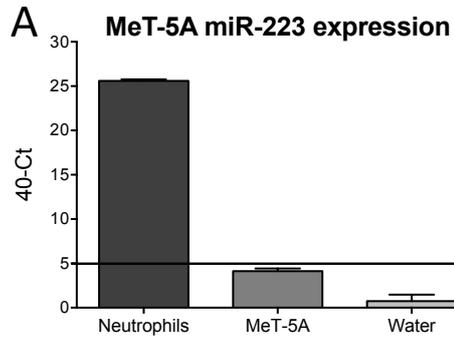
miR-233 reporter plasmid	+	+	-	-
pGL3 control plasmid	-	-	+	+
miR-C mimic	+	-	+	-
miR-223 mimic	-	+	-	+

C Reporter assay - Patient 1

miR-223 reporter plasmid	+	-	+	-	+	-
pGL3 control plasmid	-	+	-	+	-	+
PD effluent	+	+	-	-	-	-
EV-depleted PD effluent	-	-	+	+	-	-
Concentrated EVs from PD effluent	-	-	-	-	+	+

D Reporter assay - Patient 2
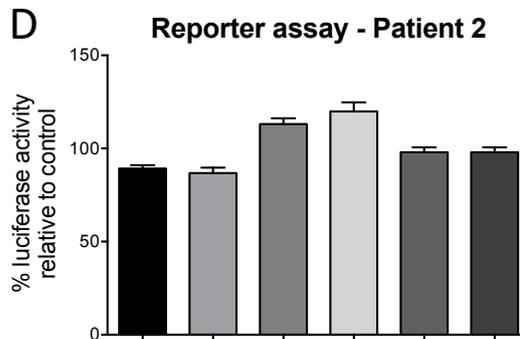
miR-223 reporter plasmid	+	-	+	-	+	-
pGL3 control plasmid	-	+	-	+	-	+
PD effluent	+	+	-	-	-	-
EV-depleted PD effluent	-	-	+	+	-	-
Concentrated EVs from PD effluent	-	-	-	-	+	+

Figure 6.5: Reporter assay showing functional uptake of miR-223 alters luciferase activity

A shows MeT-5A cells have no endogenous miR-223 expression, compared to neutrophils and water, with horizontal line drawn at 40-Ct = 5 to represent the limit of detection. B demonstrates reporter assay controls with transfection of miR-223 reporter or pGL3 control plasmids, and then transfection with miRNA mimics (control (miR-C) or miR-223). C and D show cells transfected with either miR-223 reporter or pGL3 control plasmid, and then treated with either cell-free PD effluent, PD effluent depleted of extracellular vesicles (EVs, by ultracentrifugation) or concentrated EVs isolated from PD effluent (by ultracentrifugation), from 2 separate patients. Data represented as mean $\pm$ SEM for triplicate wells and plotted as % luciferase activity relative to cells transfected with relevant plasmid and control miRNA mimic (miR-C).

control plasmid or the control miRNA mimic did not affect the luciferase activity, showing this effect was specific for miR-223.

Next, we wanted to determine whether the miR-223 observed in neutrophil-derived EVs in infected PD effluent was functionally taken up by recipient cells. To do this, a preliminary experiment was conducted where MeT-5A cells were transfected with the reporter plasmid (or control plasmid), then treated with either PD effluent, EV-depleted PD effluent or concentrated EVs from PD effluent from two separate patients (Figure 6.5C-D). Although there were differences between the treatments, there was no specific downregulation of luciferase activity detectable, i.e. reporter plasmid transfection and PD effluent treatment was not different from control plasmid transfection and PD effluent treatment. The altered luciferase levels observed by addition of the different forms of PD effluent was likely a non-specific effect due to the complex nature of this biological fluid.

Taken together, while a robust miR-223 reporter assay could be established in this chapter, preliminary experiments applying this reporter assay to infected PD effluent did so far not reveal a specific functional role of extracellular miR-223 in patient samples. This assay would need further optimisation to determine whether the miR-223 released into the PD effluent in EVs is functionally taken up by a specific recipient cell type.

6.3   Discussion

In this Chapter, I show that miR-223 is stabilised extracellularly in PD effluent partly by incorporation into EVs, it is likely that these include neutrophil-derived exosomes, as evidenced by co-segregation of miR-223 and the exosome and neutrophil markers CD9, CD81 and CD15 by size exclusion chromatography, and by resistance to degradation by RNase A. This Chapter also shows that miR-223 is likely to be stabilised in a different way when released from neutrophils in culture, with a larger proportion likely to be stabilised in the soluble phase by protein complexes rather than being released within EVs. A reporter assay was set up to determine whether miR-223 released into PD effluent can be taken up and functional in a recipient cell line. This was provisionally tested using PD effluent from two infected patients but requires further optimisation.

6.3.1 miR-223 is stabilised by EVs and protein complexes

This Chapter provides evidence that miR-223 is stabilised in PD effluent, by both a combination of incorporation into EVs and association with protein complexes. This was shown by ultracentrifugation isolating fractions containing EVs with high amounts of miR-223 present, the stabilisation of miR-223 to both RNase and proteinase activity, and association of miR-223 with fractions from size-exclusion chromatography that co-segregates with exosome markers. It is likely that some of these EVs are released from neutrophils, as shown by EV-containing fractions showing CD15+ staining. These experiments also showed that some miR-223 in infected PD effluent is associated with protein complexes as demonstrated by its presence in supernatants after removal of EVs, susceptibility to proteinase activity, and association of miR-223 with fractions representative of soluble protein. These data demonstrate how multiple biological pathways are likely used to release stable extracellular miR-223, with this increasing the likelihood that release of miR-223 is an active process.

The isolation of EVs by ultracentrifugation is a standardised procedure that is reliable and reproducible for cell culture supernatants and biological fluids, and is widely used as a first step in EV characterisation.[818] Although no quality control steps were used here to confirm the purity of EV isolation or to characterise the pellets, it could reasonably be assumed that the protocol allowed sufficient preparation of the desired EVs, as described. For the RNase and proteinase treatment of samples, there was a positive control to show susceptibility to RNase A degradation of "spiked-in" cel-miR-39, but no positive control to confirm protease activity. However, the protocol was optimised and published previously by our lab,[438] therefore we can assume that any changes in miR-223 levels here were due to degradation as a result of the treatments.

This is the first time a characterisation of EVs has been conducted in PD effluent to establish extracellular miRNA stability, including the use of size-exclusion chromatography columns. The physical properties of PD effluent, once rendered cell-free, are similar to urine so the protocols followed were adapted from those already optimal for urine. The markers used to detect the presence of exosomes were CD9 and CD81. These tetraspanins, named due to their four transmembrane domains, are almost always found across a lipid membrane and are often used as a marker for exosomes.[819] One novel observation for PD effluent is a double CD81 peak in the size-exclusion chromatography characterisation of particle sizes

(Figure 6.3C). This methodology is effective at separating EVs from protein, as demonstrated in the literature[820] and by separation of the CD9 and human serum albumin peaks in Figures 6.3A and 6.3G. It is not clear why there was a second peak, possibly representing a separate population of CD81^{+} particles or CD81 unbound to a membrane. This could also be due to the complex nature of the infected patient samples which were used. PD effluent is a mixture of many different cells and soluble factors, which are likely to influence the EVs released. This could be a novel finding that a second population of CD81^{+} vesicles are present in PD effluent, or it could be a result of the many different variables present in PD effluent. The deviation between the five samples analysed here demonstrates how variable PD effluent from patients is. For example, Figure 6.3C shows some of the patient samples had a double CD81 peak whilst others only had one or other of these. To further investigate and characterise the particles present in PD effluent would require a larger number of patient samples to attempt to answer some of these questions.

There are many studies detailing the role of miR-223 as a useful biomarker in cell-free biological fluids,[471,474,821–825] but few define how it is stabilised extracellularly. Some show that miR-223 is incorporated into exosomes. For example, increased exosomal miR-223 is an inflammatory marker in lumbar disc herniation, with dysregulation in its release associated with persistent pain.[826] Release of miR-223 in EVs is an inflammatory mediator in a range of different biological situations including platelet activation,[418,827] macrophage differentiation and activation,[588,828,829] different cancers[828,830] and lung epithelial cell inflammation.[410] miR-223 is also stabilised in biological fluids by protein complexes. miR-223 is one of the most abundant miRNAs bound to high-density lipoprotein (HDL) and low-density lipoprotein (LDL) complexes, although the majority of extracellular miRNA is not bound to these complexes.[431,831,832] miR-223 associated with HDL can functionally regulate targets in recipient cells, including intercellular adhesion molecule 1 (ICAM-1) to reduce inflammation in endothelial cells[432] and Ras homolog gene family member B (RhoB) in hepatocytes.[431] What these earlier studies and the work shown here both demonstrate is that miR-223 is an important extracellular biomarker stabilised by incorporation into EVs and association with protein complexes, with the proportion of EVs and protein complexes changing according to specific conditions which the cells encounter (i.e. cultured neutrophils release fewer EVs compared to cells in an infected peritoneum). The confirmation that miR-223 is stabilised in PD effluent from peritonitis patients, likely at least partially, by neutrophil-derived EVs is a novel finding.

6.3.2 miR-223 reporter assay

To confirm if miR-223 can be taken up and is functionally active in a distant recipient cell type, a luciferase-based reporter assay, with an artificial 3' UTR targeted by miR-223, was set up in a MeT-5A cell line which does not itself express miR-223. Transfection with an artificial miR-223 mimic showed that the reporter responded specifically to miR-223 by a reduction in luciferase activity. However, when the reporter cell line was treated with PD effluent, EV-depleted PD effluent or concentrated EVs from PD effluent from two patients, there were alterations in luciferase activity, but none of these were specific to miR-223. This was likely due to the complex nature of the primary patient material tested, with many confounding factors present in infected PD effluent which could not be controlled for in this preliminary experiment.

There are different reasons why this assay failed to detect a functional role for miR-223 in PD effluent. The primary problem may have been the complex nature of PD effluent and how the other components of the mixture may alter the expression of luciferase. For example, the cytokines present in the PD effluent may stimulate the MeT-5A cells to alter expression of the miR-223 reporter plasmid, therefore luciferase activity is altered in a non-specific manner. Another is that the actual concentration of miR-223 present in the PD effluent or concentrated EVs was unknown, therefore it could have been too low to have had a specific effect. For comparison, 10 ng of the miRNA mimic was used to generate a positive response of functional miR-223 on the luciferase activity, and is not known whether this amount of miRNA is biologically relevant.

The 3' UTR used in this reporter assay was artificially designed to give a strong effect, as is seen in the positive control. However, the 5 artificial binding sites for miR-223 may not be the most biologically relevant target for miR-223. When other studies looked for positive effects of miRNA regulation in distant recipient cells, they often used the full 3' UTR of a validated target for that specific miRNA, with mutations in the specific binding site to show specificity for that miRNA. For example, FBXW7 is a validated target of miR-223 in many different biological systems through luciferase-based reporter assays,[489,698,699] therefore it could have been more biologically relevant to use such a target to show true effects. However, this was not used in this preliminary experiment as no specific target cell type had been identified. It therefore was not possible to determine a likely mRNA target that

might be biologically relevant in this specific response to PD-related peritonitis. If the target cell type(s) could be identified, then experimental validation of predicted target(s) could be more easily conducted.

Met-5A cells were chosen for these experiments due to the fact that they are a mesothelial cell line and hence an attractive candidate for a possible miR-223 target cell type. In addition, Met-5A cells lack endogenous miR-223 expression, thereby allowing the development of a sensitive miR-223 reporter assay in those cells. However, this cell line may not be representative of a true miR-223 target cell in PD-related peritonitis. The main cell types known to express miR-223, and therefore with proven functional roles in cellular processes, are those of a myeloid lineage, for example, miR-223 is important in neutrophil activation and recruitment via the inflammasome or IL-6/STAT3 pathways.[488,833,834] However, miR-223 can also be released by myeloid cells to act in distant recipient target cells. These include lung epithelial cells responding to an inflammatory stimulus[410] and basal keratinocytes in response to injury.[835] It might have been more relevant to look at primary cells due to the changes gained during the immortalisation process of cell line generation.[836] However, a cell line was chosen for this proof-of-principle experiment due to the ability to generate a positive starting point for further investigations. To further optimise this reporter assay, it might now be necessary to test a range of different cell types, both primary cells and established cell lines, to observe which cell type(s) may respond best to miR-223 in EVs released in response to PD-related peritonitis.

A lack of a miR-223-specific effect on luciferase activity may be due to either a lack of biological effect, as discussed above, or as a result of miR-223 being unable to get or function inside the cell. These could be biological reasons or problems with the experimental protocol. Further optimisation of the reporter assay is now required, using different cell types, attempting to assist EV entry into the cell, and determining the optimum miR-223 concentration to cause an effect.

6.4 Conclusions

In summary, this Chapter shows that miR-223 is stabilised in PD effluent from peritonitis patients. It is likely that this, at least partially, is by incorporation into neutrophil-derived exosomes, which protect the miRNA from RNase activity. A reporter assay was successfully set up to measure uptake and functional activity of miR-223 in a mesothelial cell line. This

assay now requires further optimisation to confirm whether the miR-223 in PD effluent is functional in specific distant target cells.

7   General Discussion and Future Work

7.1   General discussion

Peritonitis is a serious complication of PD and is a major cause of catheter removal and transition to an alternative RRT, as well as a main or contributing cause of mortality in 2-6% of PD patient deaths.[86] This serious clinical problem is exacerbated by inappropriate treatments, usually broad-spectrum antibiotics used to cover the most likely causes, before the specific pathogen can be identified through lengthy culture techniques.[88] Distinguishing between the different causative pathogens is essential to be able to classify patients into those that might need more intensive treatment and hospitalisation (for example, polymicrobial infections) from those with more minor infections that should recover with standard treatments (for example, CNS).[223] The immune response to pathogens is specific enough to distinguish between different causative organisms, as shown through previous work on the "immune fingerprint" model. This is where a range of soluble and cellular factors can be used to classify patients on both causative organism and clinical outcome (i.e. technique failure).[222]

It is known that miRNAs are important in the majority of cellular processes, including immune responses.[250] The role of extracellular miRNAs as biomarkers has already been explored in many different biological fluids, including in PD effluent as markers of fibrosis or peritoneal membrane function.[388,442] In this thesis it was shown that four miRNAs (miR-223, miR-21, miR-27a and miR-31) are altered in peritonitis at the site of infection, in PD effluent, and that miR-21 can be used to distinguish between different pathogens (classified into Gram positive or Gram negative organisms). The specificity and sensitivity of miRNAs in so many different cellular processes[228] suggests they could potentially contribute to the immune fingerprint model to distinguish between different pathogens.

These miRNAs were thought to have different roles in the acute immune response due to their cell-specific expression patterns. miR-223 is predominantly expressed by the immune cells which enter the peritoneum during an infection, as it is a myeloid-restricted miRNA.[473] miR-21 and miR-27a are expressed by both resident and immune cells at a similar level. miR-21 is one of the most well-studied miRNAs, with a range of roles across many different cell types including in inflammatory diseases such as CVD and psoriasis.[508,522] miR-27a is not known to be restricted to any specific cell type, however its link to inflammatory

conditions, such as relapsing MS, suggests it may be important in acute immune responses.[501] miR-31 was only expressed and released by resident peritoneal mesothelial cells and fibroblasts. This restriction to epithelial cells is in agreement with previous studies that suggest it plays a pleiotropic role in regulating a range of responses to different stimuli, including in inflammatory and infectious diseases.[535,548]

These four miRNAs are potentially important in pathways responsible for immune responses due to some of their potential mRNA targets being involved in cytokine signalling, production and sensing, inflammatory pathways, and macrophage differentiation. For example, miR-223 is predicted to regulate IL6ST, an IL-6 signal transducer, whilst miR-21 is predicted to target the cytokine TGF-β1. miR-31 is predicted to regulate PKCϵ, which is implicated in immune and inflammatory diseases, and miR-27a is predicted to target MMD, which controls differentiation of macrophages from monocytes. These predictions would need experimental validation to prove if they are functional interactions and if they are important in the acute response to PD-related peritonitis.

The latter sections of this thesis have focused on miR-223, which is an important regulator of inflammation.[473,474] The release of miR-223 was shown to be an active process due to its extracellular stability, which is crucial to suggest that it is used for intercellular communication. The role of miRNAs in communication between immune and non-immune cells is important in other inflammatory situations, for example in lung injury and heart disease.[409,410] miR-223 has been utilised as a biomarker for diagnosis or progression of sepsis, RA and some infections in animals.[474,478,486] It is thought that miR-223 may suppress inflammation due to spontaneous inflammation and more severe inflammatory reactions in KO animals.[410] miR-223 is also important in neutrophil and macrophage differentiation and maturation, therefore it is predicted to be important in the acute response to infection.[359,490] miR-223 has not previously been investigated in PD effluent, although it has been used in peritoneal ascites as a biomarker of spontaneous bacterial peritonitis, compared to peritoneal carcinomatosis. This suggests the specific inflammatory reaction induced by infection is different to that induced by cancer, and miR-223 can be used to distinguish between them.[449]

Nucleic acids that are release from cells are normally degraded by endonucleases that are present in biological fluids. However, miRNAs are often stable extracellularly and are protected from natural RNases. This stability is due to active release from cells, either within EVs or associated with protein complexes.[389] miR-223 is abundantly released from myeloid cells in culture (particularly neutrophils), as well as by myeloid cells at the site of infection (i.e. in PD effluent from peritonitis patients). This thesis provides evidence that cells in culture and those at the site of infection have different mechanisms of miR-223 release and stabilisation, with a larger proportion of miR-223 present in EVs in PD effluent compared to culture supernatant. It was proposed that a proportion of these EVs in PD effluent are derived from neutrophils, as has been demonstrated previously in other inflammatory conditions.[410] A reporter assay to observe functional miR-223 uptake was developed but required further optimisation. This assay was developed using the same principles utilised for confirmation of predicted mRNA targets of miRNAs, where an artificial 3' UTR was inserted after a luciferase reporter gene in a plasmid, which then reduced the luciferase activity in the presence of functional miR-223. The controls for this assay show a functional uptake of miR-223 causes a reduction in luciferase activity, however the molecular mechanisms of this process are not known. It would be prudent to investigate how EVs that contain miRNAs are released by cells, with a focus on how this is regulated particularly in response to inflammatory stimuli, as well as how they are taken up by target cells.

In summary, the findings in this thesis show the important role miRNAs play in the acute response to infection. miRNAs can help diagnose peritonitis in PD patients and help distinguish between different infectious organisms. They are functionally expressed and released by specific cell types, and are potentially important in communication between different cell types which may not express specific miRNAs but can respond to exogenously produced miRNA.

7.2   Future work

The aim of my research was to investigate how local miRNAs are involved in the acute immune response to PD-related peritonitis, with a specific focus on use of miRNAs as biomarkers and any potential roles in communication between different cell types present in an infected peritoneum. However, further work is necessary to expand this work to investigate in more detail the functional role of the miRNAs identified here as potential

biomarkers and their role in the resolution of infection. miR-223 was identified as a myeloid cell-derived miRNA but it may be important in signalling pathways involving resident peritoneal cells. To investigate this further, the reporter assay developed here would need optimisation, possibly in a range of potential target cell types. Identification of the cell type(s) which take up extracellular miR-223 would also help elucidate the specific pathways regulated, by observing differences in mRNA levels of miR-223 targets. This thesis shows a list of potential mRNA targets for the four miRNAs of interest, without any experimental validation. To observe whether these interactions are functional would be important to help determine the role of these four miRNAs in the acute response to infection.

The role of miRNAs as biomarkers in PD-related peritonitis has been shown here using a small, single-centre patient cohort with well-defined infections. It would be interesting to see if these observations could be replicated in a larger multi-centre patient cohort consisting of patients with a range of different infections. Work is ongoing to expand the immune fingerprint model to other acute infections, and detection of the specific miRNAs identified here would help understand the specificity of these as biomarkers and their transferability to other important clinical questions.

Detection of miRNAs is currently reliant on RT-qPCR, which is a rapid detection method but requires specialist sample processing techniques. To advance this research towards a point-of-care test that could be used by clinicians would require novel miRNA detection methods, for example electrochemical detection.[837] Preliminary work in this area is ongoing to discover whether this is viable in complex biological fluids, using a variety of novel methodologies. Electrochemical detection of miRNAs in biological fluids is the most prominent method that does not require an amplification step, whilst others include the use of nanoparticles and magnetic beads. New techniques using novel amplification methods, different to traditional PCR, have also been developed including rolling circle and isothermal amplification.[838–840]

The functional work conducted here preliminarily investigates into *in vitro* models. Although this is most relevant to patient outcomes, further *in vivo* models could be developed to observe systemic roles for the miRNAs identified here. As observed in the preliminary *in vivo* work conducted here, there are differences in miRNA functions between closely related organisms, but miR-223 has been investigated in mice and shows similar

alterations in clinically relevant models.[410] Use of a more appropriate model of PD may allow further functional investigation into the role of miR-223 in acute PD-related peritonitis.

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9 Appendix

9.1 Patient information

Patient ID	Date	Type	Culture result	Notes
G00-088-02	25/05/2011	Stable	N/A	Never infected
G00-098-06	16/11/2012	Stable	N/A	Never infected
G00-113-01	17/11/2011	Stable	N/A	Never infected
G00-114-01	24/11/2011	Stable	N/A	Never infected
G00-128-06	04/04/2013	Stable	N/A	Never infected
G00-132-04	26/06/2013	Stable	N/A	Never infected
G00-135-02	23/04/2014	Stable	N/A	Never infected
G00-140-01	10/05/2013	Stable	N/A	Never infected
G00-147-01	13/11/2013	Stable	N/A	Never infected
G00-219-02	28/10/2015	Stable	N/A	Never infected
G00-132-15	04/09/2015	Stable	N/A	9 months after infection
G00-201-07	08/10/2015	Stable	N/A	Never infected
G00-203-04	07/10/2015	Stable	N/A	Never infected
G00-215-02	18/03/2015	Stable	N/A	Never infected
G00-219-02	28/10/2015	Stable	N/A	Never infected
G00-191-02	05/03/2015	Stable	N/A	Never infected
G00-221-02	04/11/2015	Stable	N/A	Never infected
G00-171-02	13/03/2015	Stable	N/A	>2 years after infection
G00-246-02	25/11/2015	Stable	N/A	3 months after infection
G00-218-03	20/01/2016	Stable	N/A	Never infected
G00-181-21	26/10/2016	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-114-07	21/11/2016	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-012-02	03/01/2012	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-080-02	12/01/2012	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-184-02	02/05/2014	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-188-04	06/03/2015	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-136-01	06/02/2015	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-009-01	19/12/2008	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-025-01	03/08/2009	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-035-01	08/03/2010	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-045-01	11/10/2010	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-152-04	27/11/2013	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive

G00-184-03	23/06/2014	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-181-14	09/01/2016	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-244-02	29/02/2016	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-107-01	20/11/2011	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-143-02	27/10/2014	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-152-08	26/03/2015	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-154-01	14/10/2014	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-179-07	24/02/2015	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-215-04	30/10/2015	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-167-01	03/10/2013	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-126-02	19/03/2013	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-150-01	19/03/2013	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-184-01	07/03/2014	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-181-01	24/02/2014	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-256-01	16/03/2016	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-244-01	01/02/2016	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-098-17	11/07/2015	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-197-01	04/06/2014	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-188-08	03/02/2016	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-154-02	23/02/2015	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-154-04	10/05/2016	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-082-04	19/04/2013	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-115-03	05/09/2012	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-080-01	07/03/2011	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-068-04	29/10/2012	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-077-01	24/02/2011	Peritonitis	Coagulase negative *Staphylococcus*	Gram positive
G00-002-01	10/09/2008	Peritonitis	Coagulase negative *Staphylococcus epidermis*	Gram positive

G00-188-05	07/04/2015	Peritonitis	Coagulase negative *Staphylococcus epidermis*	Gram positive
G00-039-02	08/09/2010	Peritonitis	*Staphylococcus aureus*	Gram positive
G00-070-01	10/02/2012	Peritonitis	*Staphylococcus aureus*	Gram positive
G00-068-08	30/03/2014	Peritonitis	*Staphylococcus aureus*	Gram positive
G00-098-16	11/07/2015	Peritonitis	*Staphylococcus aureus*	Gram positive
G00-188-07	08/08/2015	Peritonitis	*Staphylococcus aureus*	Gram positive
G00-098-25	27/07/2016	Peritonitis	*Staphylococcus aureus*	Gram positive
G00-061-02	05/08/2016	Peritonitis	*Staphylococcus aureus*	Gram positive
G00-098-12	09/06/2014	Peritonitis	*Staphylococcus aureus*	Gram positive
G00-056-03	14/03/2011	Peritonitis	*Staphylococcus aureus* (MRSA)	Gram positive
G00-027-01	16/09/2009	Peritonitis	*Corynebacterium amycolatum*	Gram positive
G00-037-01	24/03/2010	Peritonitis	*Coryneform*	Gram positive
G00-040-02	21/10/2010	Peritonitis	*Enterococcus faecalis*	Gram positive
G00-150-02	19/04/2013	Peritonitis	Vancomycin resistant *Enterococcus* (VRE)	Gram positive
G00-150-03	09/05/2013	Peritonitis	Vancomycin resistant *Enterococcus* & *Enterococcus faecium*	Gram positive
G00-023-01	12/07/2009	Peritonitis	Microaerophilic *Streptococcus*	Gram positive
G00-018-02	12/08/2008	Peritonitis	Microaerophilic *Streptococcus*	Gram positive
G00-004-01	21/10/2008	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-019-02	02/11/2010	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-039-01	28/06/2010	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-070-10	08/03/2013	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-116-06	03/10/2013	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-156-01	08/11/2013	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-219-03	02/03/2016	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-023-03	23/11/2011	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-019-01	29/06/2009	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-294-01	10/06/2017	Peritonitis	Alpha haemolytic *Streptococcus*	Gram positive
G00-135-04	17/09/2014	Peritonitis	*Streptococcus sanguinis*	Gram positive
G00-237-01	25/04/2015	Peritonitis	*Streptococcus sanguinis*	Gram positive
G00-227-01	08/12/2014	Peritonitis	*Streptococcus B*	Gram positive
G00-013-01	05/03/2009	Peritonitis	Alpha haemolytic *Streptococcus*, Non-haemolytic *Streptococcus* & Coliform *Streptococcus*	Gram positive
G00-179-06	30/01/2015	Peritonitis	Coagulase negative *Staphylococcus*, Alpha haemolytic *Streptococcus* & *Propionibacterium*	Gram positive
G00-021-01	30/06/2009	Peritonitis	Gram +ve *Bacillus* & *Corynebacterium/Diphtheroids*	Gram positive

G00-012-01	10/02/2009	Peritonitis	Coagulase Negative *Staphylococcus* & *Corynebacterium*	Gram positive
G00-040-01	03/09/2010	Peritonitis	Coagulase negative *Staphylococcus* & *Enterococcus*	Gram positive
G00-159-01	28/03/2013	Peritonitis	*Enterococcus faecalis* & *Streptococcus bovis*	Gram positive
G00-181-07	25/04/2015	Peritonitis	*Corynebacterium jeikeium*	Gram positive
G00-011-02	03/02/2009	Peritonitis	No growth	
G00-003-03	02/07/2009	Peritonitis	No growth	
G00-026-01	03/08/2009	Peritonitis	No growth	
G00-024-02	20/08/2008	Peritonitis	No growth	
G00-003-05	07/09/2009	Peritonitis	No growth	
G00-030-01	11/12/2009	Peritonitis	No growth	
G00-038-01	22/04/2010	Peritonitis	No growth	
G00-163-01	03/06/2013	Peritonitis	No growth	
G00-177-01	04/02/2014	Peritonitis	No growth	
G00-051-11	24/03/2014	Peritonitis	No growth	
G00-181-02	09/06/2014	Peritonitis	No growth	
G00-147-08	19/03/2015	Peritonitis	No growth	
G00-257-01	20/03/2016	Peritonitis	No growth	
G00-260-01	19/12/2016	Peritonitis	No growth	
G00-003-01	20/10/2008	Peritonitis	No growth	
G00-003-02	27/11/2008	Peritonitis	No growth	
G00-011-01	19/01/2009	Peritonitis	No growth	
G00-069-01	08/02/2011	Peritonitis	No growth	
G00-115-01	29/11/2011	Peritonitis	No growth	
G00-189-02	30/05/2014	Peritonitis	No growth	
G00-016-01	21/05/2009	Peritonitis	*Acinetobacter*	Gram negative
G00-189-01	26/05/2014	Peritonitis	*Acinetobacter baumannii*	Gram negative
G00-238-09	12/08/2016	Peritonitis	*Acinetobacter ursingii*	Gram negative
G00-175-01	08/01/2014	Peritonitis	*Enterobacter*	Gram negative
G00-196-01	04/06/2014	Peritonitis	Gram negative *Enterobacter*	Gram negative
G00-147-07	10/03/2015	Peritonitis	*Escherichia coli*	Gram negative
G00-136-04	22/05/2015	Peritonitis	*Escherichia coli*	Gram negative
G00-132-09	01/12/2014	Peritonitis	*Escherichia coli*	Gram negative
G00-143-03	12/03/2015	Peritonitis	*Escherichia coli*	Gram negative
G00-156-03	30/03/2014	Peritonitis	*Escherichia coli*	Gram negative
G00-180-02	20/02/2015	Peritonitis	*Escherichia coli*	Gram negative
G00-020-01	29/06/2009	Peritonitis	*Escherichia coli*	Gram negative
G00-031-01	23/12/2009	Peritonitis	*Escherichia coli*	Gram negative
G00-136-03	05/05/2015	Peritonitis	*Escherichia coli*	Gram negative
G00-117-06	07/09/2012	Peritonitis	*Escherichia coli*	Gram negative
G00-164-02	25/10/2013	Peritonitis	*Escherichia coli*	Gram negative
G00-238-02	09/08/2015	Peritonitis	*Escherichia coli*	Gram negative
G00-136-02	18/04/2015	Peritonitis	*Escherichia coli*	Gram negative
G00-266-01	11/06/2016	Peritonitis	*Escherichia coli*	Gram negative
G00-015-01	22/04/2009	Peritonitis	*Escherichia coli*	Gram negative
G00-210-12	31/07/2017	Peritonitis	*Escherichia coli*	Gram negative
G00-250-01	23/03/2016	Peritonitis	*Escherichia coli* & Gram -ve *Bacillus*	Gram negative
G00-179-08	16/03/2015	Peritonitis	*Klebsiella*	Gram negative

G00-227-05	13/05/2015	Peritonitis	*Neisseria*	Gram negative
G00-054-03	19/03/2011	Peritonitis	*Morganella morganii*	Gram negative
G00-022-01	01/07/2009	Peritonitis	*Pseudomonas aeruginosa*	Gram negative
G00-029-01	12/10/2009	Peritonitis	*Pseudomonas aeruginosa*	Gram negative
G00-022-02	01/07/2009	Peritonitis	*Pseudomonas aeruginosa*	Gram negative
G00-084-01	02/04/2011	Peritonitis	Gram negative *Bacillus*	Gram negative
G00-248-01	16/08/2016	Peritonitis	*Acenetobactor* & Gram +ve *Bacillus*	Gram negative
G00-174-01	06/12/2013	Peritonitis	*Pseudomonas aeruginosa* & Anaerobic Gram -ve *Bacillus*	Gram negative

9.2 miRNAs on TLDA cards

RNU44	hsa-miR-139-5p	hsa-miR-195	hsa-miR-224	hsa-miR-335
RNU48	hsa-miR-140-3p	hsa-miR-196b	hsa-miR-23a	hsa-miR-337-5p
U6 snRNA	hsa-miR-141	hsa-miR-197	hsa-miR-23b	hsa-miR-338-3p
ath-miR159a	hsa-miR-142-3p	hsa-miR-198	hsa-miR-24	hsa-miR-339-3p
hsa-let-7a	hsa-miR-142-5p	hsa-miR-199a	hsa-miR-25	hsa-miR-339-5p
hsa-let-7b	hsa-miR-143	hsa-miR-199a-3p	hsa-miR-26a	hsa-miR-33b
hsa-let-7c	hsa-miR-145	hsa-miR-199b	hsa-miR-26b	hsa-miR-340
hsa-let-7d	hsa-miR-146a	hsa-miR-19a	hsa-miR-27a	hsa-miR-342-3p
hsa-let-7e	hsa-miR-146b	hsa-miR-19b	hsa-miR-27b	hsa-miR-342-5p
hsa-let-7f	hsa-miR-146b-3p	hsa-miR-200a	hsa-miR-28	hsa-miR-345
hsa-let-7g	hsa-miR-147	hsa-miR-200b	hsa-miR-28-3p	hsa-miR-346
hsa-miR-1	hsa-miR-147b	hsa-miR-200c	hsa-miR-296	hsa-miR-34a
hsa-miR-100	hsa-miR-148a	hsa-miR-202	hsa-miR-296-3p	hsa-miR-34c
hsa-miR-101	hsa-miR-148b	hsa-miR-203	hsa-miR-298	hsa-miR-361
hsa-miR-103	hsa-miR-149	hsa-miR-204	hsa-miR-299-3p	hsa-miR-362
hsa-miR-105	hsa-miR-150	hsa-miR-205	hsa-miR-299-5p	hsa-miR-362-3p
hsa-miR-106a	hsa-miR-152	hsa-miR-208	hsa-miR-29a	hsa-miR-363
hsa-miR-106b	hsa-miR-154	hsa-miR-208b	hsa-miR-29b	hsa-miR-365
hsa-miR-107	hsa-miR-155	hsa-miR-20a	hsa-miR-29c	hsa-miR-367
hsa-miR-10a	hsa-miR-15a	hsa-miR-20b	hsa-miR-301	hsa-miR-369-3p
hsa-miR-10b	hsa-miR-15b	hsa-miR-21	hsa-miR-301b	hsa-miR-369-5p
hsa-miR-122	hsa-miR-16	hsa-miR-210	hsa-miR-302a	hsa-miR-370
hsa-miR-125a-3p	hsa-miR-17	hsa-miR-211	hsa-miR-302b	hsa-miR-371-3p
hsa-miR-125a-5p	hsa-miR-181a	hsa-miR-212	hsa-miR-302c	hsa-miR-372
hsa-miR-125b	hsa-miR-181c	hsa-miR-214	hsa-miR-30b	hsa-miR-373
hsa-miR-126	hsa-miR-182	hsa-miR-215	hsa-miR-30c	hsa-miR-374
hsa-miR-127	hsa-miR-183	hsa-miR-216a	hsa-miR-31	hsa-miR-375
hsa-miR-127-5p	hsa-miR-184	hsa-miR-216b	hsa-miR-32	hsa-miR-376a
hsa-miR-128a	hsa-miR-185	hsa-miR-217	hsa-miR-320	hsa-miR-376b
hsa-miR-129	hsa-miR-186	hsa-miR-218	hsa-miR-323-3p	hsa-miR-376c
hsa-miR-130a	hsa-miR-188-3p	hsa-miR-219	hsa-miR-324-3p	hsa-miR-377
hsa-miR-130b	hsa-miR-18a	hsa-miR-219-1-3p	hsa-miR-324-5p	hsa-miR-380-3p
hsa-miR-132	hsa-miR-18b	hsa-miR-219-2-3p	hsa-miR-325	hsa-miR-381
hsa-miR-133a	hsa-miR-190	hsa-miR-22	hsa-miR-326	hsa-miR-382
hsa-miR-133b	hsa-miR-191	hsa-miR-220	hsa-miR-328	hsa-miR-383
hsa-miR-135a	hsa-miR-192	hsa-miR-220b	hsa-miR-329	hsa-miR-384
hsa-miR-135b	hsa-miR-193a	hsa-miR-220c	hsa-miR-330	hsa-miR-409-5p
hsa-miR-136	hsa-miR-193a-5p	hsa-miR-221	hsa-miR-330-5p	hsa-miR-410
hsa-miR-138	hsa-miR-193b	hsa-miR-222	hsa-miR-331	hsa-miR-411
hsa-miR-139-3p	hsa-miR-194	hsa-miR-223	hsa-miR-331-5p	hsa-miR-412
hsa-miR-422a	hsa-miR-505	hsa-miR-524-5p	hsa-miR-628-5p	mmu-miR-124a
hsa-miR-423-5p	hsa-miR-506	hsa-miR-525	hsa-miR-629	mmu-miR-129-3p
hsa-miR-424	hsa-miR-507	hsa-miR-525-3p	hsa-miR-636	mmu-miR-134

hsa-miR-425-5p	hsa-miR-508	hsa-miR-526b	hsa-miR-642	mmu-miR-137
hsa-miR-429	hsa-miR-508-5p	hsa-miR-532	hsa-miR-651	mmu-miR-140
hsa-miR-431	hsa-miR-509-3-5p	hsa-miR-532-3p	hsa-miR-652	mmu-miR-153
hsa-miR-433	hsa-miR-509-5p	hsa-miR-539	hsa-miR-653	mmu-miR-187
hsa-miR-448	hsa-miR-510	hsa-miR-541	hsa-miR-654	mmu-miR-374-5p
hsa-miR-449	hsa-miR-511	hsa-miR-542-3p	hsa-miR-654-3p	mmu-miR-379
hsa-miR-449b	hsa-miR-512-3p	hsa-miR-542-5p	hsa-miR-655	mmu-miR-451
hsa-miR-450a	hsa-miR-512-5p	hsa-miR-544	hsa-miR-660	mmu-miR-491
hsa-miR-450b-3p	hsa-miR-513-5p	hsa-miR-545	hsa-miR-671-3p	mmu-miR-495
hsa-miR-450b-5p	hsa-miR-515-3p	hsa-miR-548a	hsa-miR-672	mmu-miR-496
hsa-miR-452	hsa-miR-515-5p	hsa-miR-548a-5p	hsa-miR-674	mmu-miR-499
hsa-miR-453	hsa-miR-516a-5p	hsa-miR-548b	hsa-miR-708	mmu-miR-615
hsa-miR-454	hsa-miR-516b	hsa-miR-548b-5p	hsa-miR-744	mmu-miR-93
hsa-miR-455	hsa-miR-517a	hsa-miR-548c	hsa-miR-758	mmu-miR-96
hsa-miR-455-3p	hsa-miR-517b	hsa-miR-548c-5p	hsa-miR-871	
hsa-miR-483-5p	hsa-miR-517c	hsa-miR-548d	hsa-miR-872	
hsa-miR-484	hsa-miR-518a-3p	hsa-miR-548d-5p	hsa-miR-873	
hsa-miR-485-3p	hsa-miR-518a-5p	hsa-miR-551b	hsa-miR-874	
hsa-miR-485-5p	hsa-miR-518b	hsa-miR-556-3p	hsa-miR-875-3p	
hsa-miR-486	hsa-miR-518c	hsa-miR-556-5p	hsa-miR-876-3p	
hsa-miR-486-3p	hsa-miR-518d	hsa-miR-561	hsa-miR-876-5p	
hsa-miR-487a	hsa-miR-518d-5p	hsa-miR-570	hsa-miR-885-3p	
hsa-miR-487b	hsa-miR-518e	hsa-miR-574-3p	hsa-miR-885-5p	
hsa-miR-488	hsa-miR-518f	hsa-miR-576-3p	hsa-miR-886-3p	
hsa-miR-489	hsa-miR-519a	hsa-miR-576-5p	hsa-miR-886-5p	
hsa-miR-490	hsa-miR-519c	hsa-miR-579	hsa-miR-887	
hsa-miR-491-3p	hsa-miR-519d	hsa-miR-582-3p	hsa-miR-888	
hsa-miR-492	hsa-miR-519e	hsa-miR-582-5p	hsa-miR-889	
hsa-miR-493	hsa-miR-520a#	hsa-miR-589	hsa-miR-890	
hsa-miR-494	hsa-miR-520a	hsa-miR-590-5p	hsa-miR-891a	
hsa-miR-499-3p	hsa-miR-520b	hsa-miR-597	hsa-miR-891b	
hsa-miR-500	hsa-miR-520d-5p	hsa-miR-598	hsa-miR-892a	
hsa-miR-501	hsa-miR-520e	hsa-miR-615-5p	hsa-miR-9	
hsa-miR-501-3p	hsa-miR-520f	hsa-miR-616	hsa-miR-92a	
hsa-miR-502	hsa-miR-520g	hsa-miR-618	hsa-miR-95	
hsa-miR-502-3p	hsa-miR-521	hsa-miR-624	hsa-miR-98	
hsa-miR-503	hsa-miR-522	hsa-miR-625	hsa-miR-99a	
hsa-miR-504	hsa-miR-523	hsa-miR-627	hsa-miR-99b	

9.3 Plasmid generation

9.3.1 Overlapping oligos designed

Fragment 1

5'GCCGTGTAATTCTAGATCGtggggtatttgacaaactgacaATCGtggggtatttgacaaactgacaATCGtggggtatttgacaaactgacaCCGGTAATtggg3'

3'CGGCACATTAAGATCTAGCaccccataaactgtttgactgtTAGCaccccataaactgtttgactgtTAGCaccccataaactgtttgactgtGGCCATTAaccc5'

Fragment 2

5'acaCCGGTAATtggggtatttgacaaactgacaATCGtggggtatttgacaaactgacaATCGCTAGAGTCGGGGCGG3'

3'tgtGGCCATTAaccccataaactgtttgactgtTAGCaccccataaactgtttgactgtTAGCGATCTCAGCCCCGCC5'

9.3.2 3' UTR designed

GCCGTGTAATTCTAGATCGtggggtatttgacaaactgacaATCGtggggtatttgacaaactgacaATCGtggggtatttgacaaactgacaCCGGTAATtggggtatttgacaaactgacaATCGtggggtatttgacaaactgacaATCGCTAGAGTCGGGGCGG

AAA = 15 bp overhang

AAA = miR-223-3p perfect binding site

AAA = linker sequence

9.3.3 Insert sequence

```
<Serial Cloner V2.5> -- <12 Oct 2018   11:17>
Restriction map of Clone 6
Showing restriction enzymes cutting maximum 1 time [using RELibrary as a Restriction Enzyme Library]
###
```



```
                                                                      >miR-223 binding site
                                      >miR-223 binding site                    |
                                               |
TGTCTGCTCGAAGCGGCCGGCCGCCCCGACTCTAGCGATTGTCAGTTTGTCAAATACCCCACGATTGTCAGTTTGTCAAATACCCCAATTACCGGTGTCA  < 100
ACAGACGAGCTTCGCCGGCCGGCGGGGCTGAGATCGCTAACAGTCAAACAGTTTATGGGGTGCTAACAGTCAAACAGTTTATGGGGTTAATGGCCACAGT
           10        20        30        40        50        60        70        80        90
```

```
                                        >miR-223 binding site
   >miR-223 binding site                                          >miR-223 binding site
        |                                    |                            |
GTTTGTCAAATACCCCACGATTGTCAGTTTGTCAAATACCCCACGATTGTCAGTTTGTCAAATACCCCACGATCTAGAATTACACGGCGATCTTTCCGCC  < 200
CAAACAGTTTATGGGGTGCTAACAGTCAAACAGTTTATGGGGTGCTAACAGTCAAACAGTTTATGGGGTGCTAGATCTTAATGTGCCGCTAGAAAGGCGG
       110       120       130       140       150       160       170       180       190
```

```
CTTCTTGGCC   < 210
GAAGAACCGG
```

```
Features :
miR-223 binding site   : [40 : 61]
miR-223 binding site   : [66 : 87]
miR-223 binding site   : [96 : 117]
miR-223 binding site   : [122 : 143]
miR-223 binding site   : [148 : 169]
```

9.3.4 Alignment with designed 3' UTR

```
Alignment of Sequence_1:  [pGL3 miR-223 reporter seq] with  Sequence_2: [Clone 6]

Similarity : 210/300 (70.00 %)

Seq_1  1      GGAAAACTCGACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAG   60
                                                       ||||||||||||||||||||
Seq_2  210    ----------------------------------------GGCCAAGAAGGGCGGAAAG   192


Seq_1  61     ATCGCCGTGTAATTCTAGATCGtggggtatttgacaaactgacaATCGtggggtatttga   120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  191    ATCGCCGTGTAATTCTAGATCGTGGGGTATTTGACAAACTGACAATCGTGGGGTATTTGA   132


Seq_1  121    caaactgacaATCGtggggtatttgacaaactgacaCCGGTAATtggggtatttgacaaa   180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  131    CAAACTGACAATCGTGGGGTATTTGACAAACTGACACCGGTAATTGGGGTATTTGACAAA   72


Seq_1  181    ctgacaATCGtggggtatttgacaaactgacaATCGCTAGAGTCGGGGCGGCCGGCCGCT   240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  71     CTGACAATCGTGGGGTATTTGACAAACTGACAATCGCTAGAGTCGGGGCGGCCGGCCGCT   12


Seq_1  241    TCGAGCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTG   300
              |||||||||||
Seq_2  11     TCGAGCAGACA-------------------------------------------------   1
```

Features [Seq_2]:
miR-223 binding site   : [40 : 61]
miR-223 binding site   : [66 : 87]
miR-223 binding site   : [96 : 117]
miR-223 binding site   : [122 : 143]
miR-223 binding site   : [148 : 169]

9.3.5 Plasmid map

pGL3 Control miR-223 Reporter – 5424 nt

1...48 MCS
48...250 SV40 promoter
280...1932 Luciferase gene
1905...1924 Seq primer F
1934...1939 XbaI
1939...2102 miR-223 Reporter
1964...2102 SV40 late polyA [5']
2167...2187 Seq primer R
2271...2323 SV40 late polyA [3']
ColE1 origin 3537...2909
AmpR 4348...3689
Amp prom 4616...4588
F1 ori 5123...4683
LacZ alpha 5203...5135
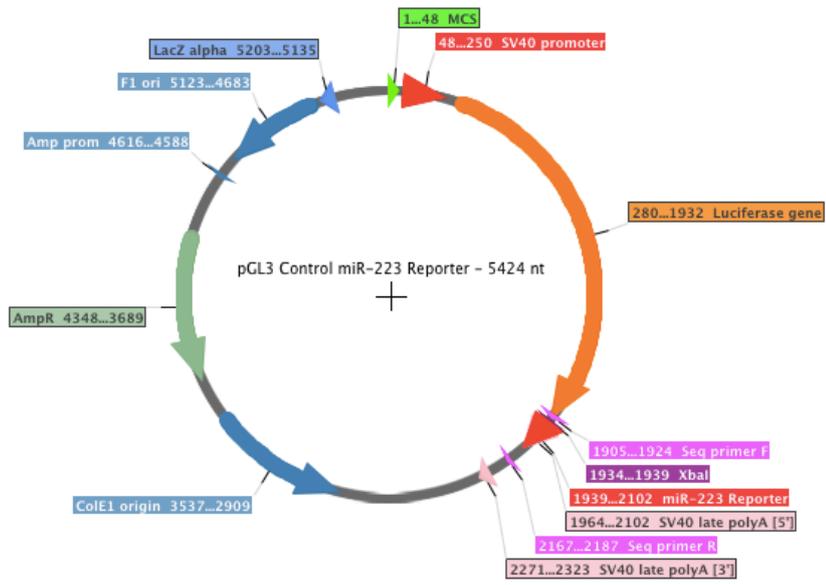
9.4 Presentations during my PhD studies

9.4.1 Poster presentations

- Cardiff University Division of Infection and Immunity Annual Meeting, Cardiff, 2016. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

- British Society of Immunology Annual Congress, Liverpool, 2016. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

- WKRU Annual Meeting, Swansea, 2016. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

- Diabetic Nephropathy Research Symposium, Cardiff, 2016. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

- 30th Cardiff University Annual School of Medicine and Dentistry Postgraduate Research Day, Cardiff, 2017. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

- Cardiff University Division of Infection and Immunity Annual Meeting, Cardiff, 2017. Title 'Pathogen-specific local microRNAs in peritoneal dialysis patients with acute peritonitis'

- 13th European Peritoneal Dialysis Meeting, Dublin, 2017. Title 'Pathogen-specific local microRNAs in peritoneal dialysis patients with acute peritonitis'

- British Society of Immunology Annual Congress, Brighton, 2017. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

- WKRU Annual Meeting, Swansea, 2018. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

9.4.2 Oral presentations

- South West RNA Club, Cardiff, 2017. Title 'Pathogen-specific local microRNAs in peritoneal dialysis patients with acute peritonitis' – best PhD presentation award

- 31st Cardiff University Annual School of Medicine and Dentistry Postgraduate Research Day, Cardiff, 2017. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

- 13th European Peritoneal Dialysis Meeting, Dublin, 2017. Title 'Pathogen-specific local microRNAs in peritoneal dialysis patients with acute peritonitis' – best basic science presentation award

- WKRU Annual Meeting, Cardiff, 2017. Invited speaker. Title 'microRNAs as biomarkers of infection in PD'

- 32nd Cardiff University Annual School of Medicine and Dentistry Postgraduate Research Day, Cardiff, 2018. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

- South West RNA Club, Exeter, 2018. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

- Cardiff University Division of Infection and Immunity Annual Meeting, Cardiff, 2018. Title 'The role of local microRNAs in the pathogen-specific immune response to acute peritonitis in peritoneal dialysis patients'

9.5   Publications during my PhD studies

- Liuzzi AR, Kift-Morgan A, Lopez-Anton M, **Brook AC**, Friberg IM, Zhang J, Roberts GW, Donovan KL, Colmont CS, Toleman MA, Bowen T, Johnson DW, Topley N, Moser B, Fraser D, and Eberl M. Human γδ T-cells and MAIT cells expand at the site of microbial infection, amplify inflammatory responses and induce local tissue remodeling. (J Immunol. 2016 Sep 15; 197(6):2195-207).

- Neha Dhingra Pottanat; **Amy C. Brook**, Maria Bartosova, Hanna Cortado, Sudipti Gupta, Birong Li, Ashley R. Jackson, Martin Vonau, Shira Cohen, Maria Ferrara, Christina B. Ching, John David Spencer, Annelie Brauner, Donald J. Fraser, Claus Peter Schmitt, Matthias Eberl, Rose Ayoob, Brian Becknell. Analysis of the Ribonuclease A Superfamily of Antimicrobial Peptides in Patients Undergoing Chronic Peritoneal Dialysis. Manuscript in preparation.

- **Brook AC**, Jenkins R, Clayton A, Kift-Morgan A, Raby A-C, Shepherd A, Mariotti B, Bazzoni F, Bowen T, Fraser D, Eberl M. Neutrophil-derived miR-223 as local biomarker of bacterial peritonitis. Manuscript in preparation.