The role of microRNAs and Ischaemic Preconditioning in Kidney Ischaemia Reperfusion Injury

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Thesis Summary

Successful kidney transplantation transforms outcome for patients with end stage kidney disease. Delayed graft function (DGF) following Ischaemia Reperfusion Injury (IRI) is a major problem, is hard to predict or monitor, and preventative or therapeutic strategies are lacking. Ischaemic Preconditioning (IPC) may limit IRI, but results are variable and potential mechanisms are not well defined. The aims of this thesis were to study the role of microRNAs, which are post-transcriptional regulators of gene expression vital in many physiological and pathophysiological processes, in the context of IRI, IPC and DGF. An *in vivo* model of IRI and IPC was developed, and histological, biochemical and mRNA kidney injury marker analyses were undertaken. MicroRNAs were then profiled using both Next Generation Sequencing (NGS) and hybridisation arrays, and changes in selected microRNAs confirmed by RT-qPCR. Histology scores, serum creatinine and expression of kidney injury markers were significantly reduced in IPC compared with IRI. Microarray and NGS analysis identified a highly reproducible IRI signature, which was attenuated by IPC. Subsequently, microRNAs were profiled using Taqman Low Density Array (TLDA) and validated by RT-qPCR, from urine samples of kidney transplant patients with and without DGF. A DGF microRNA profile was uncovered, with overlap to the results from the IRI model. These data have identified a microRNA signature of IRI that was attenuated by IPC, which also improved outcome. Urinary microRNAs also showed a promising capability to predict DGF in human kidney transplantation. MicroRNAs thus show significant promise as biomarkers and potential therapeutic targets in this context.

Publications and Presentations arising from this Thesis

Peer-reviewed Publications:

1. ***Khalid U***, Ablorsu E, Szabo L, Jenkins RH, Bowen T, Chavez R, Fraser DJ. MicroRNA-21 (miR-21) expression in hypothermic machine perfusate may be predictive of early outcomes in kidney transplantation. *Clinical Transplantation.* **30**(2): 99-104.

2. ***Khalid U***, Pino-Chavez G, Nesargikar P, Jenkins RH, Bowen T, Fraser DJ, Chavez R. Kidney ischaemia reperfusion injury in the rat: the EGTI scoring system as a valid and reliable tool for histological assessment. *J Histol Histopathol.* 2016, **3** (1). http://dx.doi.org/10.7243/2055-091X-3-1.

3. ***Khalid U***, Jenkins RH, Pino-Chavez G, Bowen T, Fraser DJ, Chavez R. A Localized Ischemic Preconditioning Regimen Increases Tumour Necrosis Factor α expression in a Rat Model of Kidney Ischaemia-Reperfusion Injury. *Experimental and Clinical Transplantation.* **13**(6): 535-42.

4. ***Khalid U***, Bowen T, Fraser DJ, Jenkins RH. Acute Kidney Injury: a paradigm for miRNA regulation of the cell cycle. *Biochemical Society Transactions.* **42**(4): 1219-23.

Oral Presentations at Conferences:

1. ***Khalid U,*** Pino-Chavez G, Nesargikar P, Jenkins RH, Bowen T, Fraser DJ, Chavez R. A new comprehensive scoring system is an accurate and robust tool for histological assessment of rat kidney IRI. 17th Congress of the European Society for Organ Transplantation (Brussels, Belgium), 13th – 16th September 2015.

2. ***Khalid U***, Ablorsu E, Szabo L, Jenkins RH, Bowen T, Fraser DJ, Chavez R. Expression of miR-10a, -21, -29a, -221 and -429 in hypothermic machine perfusate predicts early outcomes in kidney transplantation. 17th Congress of the European Society for Organ Transplantation (Brussels, Belgium), 13th – 16th September 2015.

3. ***Khalid U***, Jenkins RH, Pino-Chavez G, Bowen T, Chavez R, Fraser DJ. What is the role of microRNAs in Kidney Ischaemia Reperfusion Injury (IRI)? Welsh Association of Renal Physicians and Surgeons (WARPS) Annual Meeting, 20th June 2015. [**Winner of The Bloodworth Prize**]

4. ***Khalid U***, Jenkins RH, Pino-Chavez G, Bowen T, Chavez R, Fraser DJ. The role of microRNAs in Ischaemia Reperfusion Injury (IRI) in the context of Kidney Transplantation. Cardiff Medical Society (Cardiff), 16th April 2015. [**Winner of the WT Edwards Medal**]

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1. ***Khalid U***, Jenkins R, Simpson K, Ablorsu E, Bowen T, Fraser DJ, Chavez R. Urinary microRNA expression predicts delayed graft function in kidney transplantation. 17th Congress of the European Society for Organ Transplantation (Brussels, Belgium), 13th – 16th September 2015.

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4. ***Khalid U***, Jenkins R, Pino-Chavez G, Bowen T, Fraser DJ, Chavez R. The effect of localised ischaemic preconditioning (IPC) on histological architecture, expression of AKI markers and microRNA-21 expression in a rodent model of ischaemia-reperfusion injury (IRI). Joint British Transplantation Society and Nederlandse Transplantatie Vereniging Congress (Bournemouth), 11 – 13th March 2015.

5. ***Khalid U***, Jenkins R, Pino-Chavez G, Nesargikar P, Bowen T, Fraser DJ, Chavez R. The effect of localised ischaemic preconditioning (IPC) on mRNA expression of acute kidney injury markers and cytokines in a rodent model of ischaemia-reperfusion injury (IRI). British Transplantation Society Annual Congress (Glasgow), 26th – 28th February 2014.

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Glossary of Abbreviations

AA	Aristolochic acid
AGO	Argonaute
AKI	Acute Kidney Injury
AKt	Protein Kinase B
APKD	Adult polycystic kidney disease
ATP	Adenosine triphosphate
Bax	BCl2-Associated X protein
Bcl-2	B-cell lymphoma 2
BMI	Body Mass Index
C1q	Complement 1q protein
C3	Complement 3
C3a	Complement 3a component
C5a	Complement 5a component
C5b	Complement 5b component
C6	Complement 6
C7	Complement 7
C8	Complement 8
C9	Complement 9
Ca^2	Calcium
CAD	Chronic allograft dysfunction
CAMR	Chronic antibody mediated rejection
CD-DGF	Cadaveric donor kidney transplant with DGF
CD-No DGF	Cadaveric donor kidney transplant without DGF
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
cDNA	Complementary deoxy ribonucleic acid
cGMP	cyclic Guanosine Mono Phosphate
CIT	Cold ischaemic time
CKD	Chronic kidney disease
COX-2	Cyclo-oxygenase-2
CVA	Cerebrovascular accident
DAMPs	Danger-associated molecular patterns
DBD	Donation after brainstem death
DCD	Donation after circulatory death
DGCR8	DiGeorge syndrome critical region 8
DGF	Delayed graft function
DM	Diabetes mellitus
DNA	Deoxy ribonucleic acid
ECD	Extended criteria donor
eGFR	Estimated glomerular filtration rate
EGTI	Endothelial, Glomerular, Tubular, Interstitial
EPO	Erythropoietin
ERK	Extracellular regulated Kinase
ESRF	End-stage renal failure
FSGS	Focal segmental glomerulosclerosis
G2	Growth 2/Mitosis phase of cell cycle

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GN	Glomerulonephritis
GSK-3β	Glycogen Synthase Kinase-3 beta
H&E	Haematoxylin and eosin.
H2O2	Hydrogen peroxide
HBI	Hypoxic brain injury
HD	Haemodialysis
HIF-1α	Hypoxia inducible factor 1
HLA	Human Leucocyte Antigen
HMGB-1	High Mobility Group Box 1
HMP	Hypothermic machine perfusate
HO-1	Heme-oxygenase-1
HSP	Heat shock proteins
HTA	Human Tissue Authority
HTN	Hypertension
ICAM-1	Intracellular adhesion molecule 1
ICH	Intracranial haemorrhage
ICT	Intracranial thrombosis
IF	Interstitial fibrosis
IFN-γ	Interferon-gamma
IgA N	IgA nephropathy
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-18	Interleukin 18 (IL-18),
IL-4	Interleukin 4
IPC	Ischaemic preconditioning
IPC-C 10-20	10mins of ischaemia followed by 20mins of reperfusion
IPC-C 15-20	15mins of ischaemia followed by 20mins of reperfusion
IPC-C 20-20	20mins of ischaemia followed by 20mins of reperfusion
IPC-P 10-5	3 cycles of 10mins ischaemia and 5mins reperfusion
IPC-P 2-5	3 cycles of 2mins ischaemia and 5mins reperfusion
IPC-P 5-5	3 cycles of 5mins ischaemia and 5mins reperfusion
IPOC	Ischaemic Post-conditioning
IRI	Ischaemia Reperfusion Injury
JAK	Janus Kinase
JBIOS	Joint Biological Services Unit
K^+	Potassium
KIM-1	Kidney injury molecule 1
LASA	Laboratory Animal Science Association
LCM	Laser capture micro dissection
LD	Living donor
LD-No DGF	Living donor kidney transplant without DGF
LIPC	localised IPC
MAC	Membrane attack complex
MDRD	Modification of Diet in Renal Disease
MEK/MAP2K	Mitogen-Activated Protein Kinase Kinase
mIR	microRNA
miRISC	microRNA-induced silencing complex

mPTP	Mitochondrial permeability transition pore
mRNA	Messenger ribonucleic acid
Na^+	Sodium
NAWCO	Named Animal Care and Welfare Officer
NC3RS	The National Centre for the Replacement, Refinement and Reduction of Animals in Research
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGAL	Neutrophil gelatinase-associated lipocalin
NGS	Next Generation Sequencing
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2-	superoxide anion
PAMPs	Pathogen-associated molecular patterns
PCA	Principal component analysis
PD	Peritoneal dialysis
PDCD4	Programmed cell death protein 4
PI3K	Phosphatidylinositol-3-OH Kinase
PKC	Protein Kinase C
PKG	Protein Kinase G
PNF	Primary non-function
PTC	Proximal tubular cell
PTEN	Phosphatase and tensin homolog
RIN	RNA integrity number
RIPC	Remote IPC
RISK	Reperfusion Injury Salvage Kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAFE	Survivor Activating Factor Enhancement
STAT-3	Signal Transducer and Activator of Transcription-3
TLDA	Taqman low density array
TLR	Toll-like receptor
TLR-2	Toll-like receptor 2
TLR-4	Toll-like receptor 4
TNF-α	tumour necrosis factor alpha
Tregs	T regulatory cells
TSP-1	Thrombospondin 1
UW	University of Wisconsin
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
WIT 1	1st warm ischaemic time
WIT 2	2nd warm ischaemic time
WKRTB	Wales Kidney Research Tissue Bank

Contents

Chapter 1 – Introduction

The overall purpose of the work carried out in this PhD project was to understand the role of microRNAs in Ischaemia Reperfusion Injury (IRI) in the context of kidney transplantation. For this, I first used an *in vivo* model of acute kidney injury (AKI) and determined the role of microRNAs within this, and then used this knowledge to assess the utility of microRNAs as non-invasive biomarkers of injury in clinical kidney transplantation. In this introduction, I have first described the clinical context of why this study is important, by highlighting the need for kidney transplantation and increased use of donor organs with increased IRI, and then moved onto the broader topic of kidney IRI, its pathophysiology, potential treatments (including ischaemic preconditioning (IPC)) and the need to assess the role of microRNAs within this.

1.1 Kidney Transplantation

1.1.1 Kidney Disease

Kidney disease has become increasingly common over recent years, largely due to the increased frequency in cardiac disease, diabetes and the ageing population. Currently within the UK, chronic kidney disease (CKD) affects approximately 1 in 10 people, with an annual incidence of over 100 new patients per million of population developing end-stage renal failure (ESRF) necessitating renal replacement therapy (dialysis or transplantation) (1). The most common cause of ESRF is diabetes mellitus (DM) (25%), followed by other causes, which include hypertension (HTN), glomerulonephritis (GN), and adult polycystic kidney disease (APKD) (1, 2).

One of the major risk factors for development of CKD is AKI, a clinical syndrome characterised by acute (hours to days) loss of kidney excretory function. The causes can be classified as pre-renal (functional response of kidneys due to lack of perfusion), intrinsic renal (involving structural renal parenchymal damage) and post-renal (secondary to urinary tract outflow obstruction). Clinically, AKI is usually secondary to a combination of factors such as lack of perfusion, sepsis, hypovolaemia and nephrotoxic drugs. AKI is common with an incidence between 295 and 5000 per million per year, and an estimated treatment cost of £434 - £620 million annually in the UK (3).

Patients who develop ESRF essentially have only two choices: Dialysis (peritoneal dialysis (PD) or haemodialysis (HD)), or kidney transplantation.

Dialysis is a time-consuming, often hospital-based treatment requiring 3-4 sessions per week with a substantial impact on the patient's personal and social lifestyle, including dietary and fluid restrictions. These patients are also at an increased risk of morbidity and mortality largely due to accelerated cardiovascular disease (4).

1.1.2 Kidney transplantation

Kidney transplantation is the treatment of choice for suitable patients with ESRF as it significantly improves quality of life, prolongs survival and is more cost-effective when compared with dialysis (5, 6). Patients with ESRF are assessed for transplantation and if deemed suitable and fit for surgery, they usually wait on average for 3 years in the UK (2 years in Wales) for a transplant (7).

In kidney transplantation there are 3 main sources of organ donation: living donor (LD) kidney – related or unrelated to the recipient; donation after brainstem death (DBD), as defined by brainstem death criteria (which confirms loss of capacity of breathing and consciousness) (8); and donation after circulatory death (DCD), as defined by the Maastricht criteria (9). Within the UK, Maastricht category 3 donors are the most common DCD (cardiac arrest after withdrawal of cardiovascular support on the intensive care unit). In Cardiff Transplant Unit, in 2014-2015, 37.5%, 23% and 39.5% of the kidney transplants were from LD, DBD, and DCD donors respectively (10).

Once the kidney is retrieved from the donor, there is cessation of its blood supply rendering it ischaemic, and when implanted into the recipient patient it

undergoes reperfusion. This process, called ischaemia reperfusion injury (IRI), is an inevitable consequence of transplantation, and is known to have significant adverse effect on graft function. The ischaemic period can be divided into warm and cold ischaemic times. The first warm ischaemic time (WIT 1) is the time between cessation of donor kidney blood supply (ligation of renal artery for DBD and LD, or cardiac arrest for DCD) until perfusion and preservation in cold solution. The cold ischaemic time (CIT) is the duration of perfusion and preservation in cold solution. The second warm ischaemic time (WIT 2) is the interval between removing the kidney from the cold preservation solution until implantation into the recipient and perfusion with recipient's blood. DCD kidneys are exposed to the greatest ischaemic injury, followed by DBD and then LD kidneys.

A lot of progress in transplant surgery has been made over the last few decades relating to optimization of surgical techniques and immunosuppression. What is still lacking is the work needed in the field of IRI and its manipulation to increase donor organ quality and long-term success within the recipient.

1.1.3 Expansion of donor pool

Currently in the UK, there are just under 5700 patients active on the kidney transplant waiting list and this number, in spite of recent improvements, far outweighs the number of donors (10).

In order to address the problem of shortage of donors and the increasing number of patients on the kidney transplant waiting list, there has been a

substantial increase in the use of DCD, 'marginal' and 'extended criteria' donors (ECD) (defined as donors over age 60 or donors aged 50–59 with at least two of the following three medical criteria: history of HTN, final pre-procurement creatinine above 1.5 mg/dL and cerebrovascular accident (CVA) as cause of death) (11-13). Such organs were previously considered unsuitable for transplantation, but are increasingly accepted for use by transplant centres. Despite this increase, 429 (17%) of kidneys offered in 2014-15 in UK were not transplanted (15% DBD and 20% DCD), and 256 (11%) of kidneys retrieved were not transplanted (10).

One-third of kidneys actually transplanted are from donors aged 60 years or older, with 22% being 60-69 years old, and 11% being 70 years or older (10). Kidneys from older donors are associated with poorer long-term outcomes, largely due to the age-related reduction in functioning nephron mass and associated co-morbidities (14). Organs from these donors typically have more extensive IRI damage, resulting in increased incidence of delayed graft function (DGF) and primary non-function (PNF), which is associated with poorer long-term outcomes (15).

1.2 Ischaemia Reperfusion Injury - Clinical Consequences

1.2.1 What is Ischaemia Reperfusion Injury (IRI)?

IRI is a pathological process that is characterised by an initial restriction/occlusion of blood supply to the organ (ischaemia) followed by its subsequent reperfusion and re-oxygenation (reperfusion). It is an inevitable consequence of transplantation as the organ undergoes ischaemia upon procurement from the donor and reperfusion upon its implantation into the recipient.

1.2.2 Delayed Graft Function (DGF)

In transplantation, IRI causes DGF and PNF. DGF in kidney transplantation is a form of AKI in the immediate post-transplantation period, and is defined as the need for dialysis within the first 7 days post-transplantation (16, 17). DGF is relatively common and its incidence affected by the type and quality of the donor including the extent of ischaemic damage, recipient risk profile (18), and transplant centre practice (19). DGF is a significant clinical problem as it prolongs hospital stay, affects the patient's quality of life and often requires an invasive biopsy (which has increased risks of bleeding in the initial post-operative period) to differentiate it from other types of AKI in transplantation such as rejection, further increasing patient morbidity, and as a result has cost implications (16, 20-22).

The definition of DGF has caused much debate in the transplant world and several definitions have been evaluated, based on: kidney function; need for

dialysis; and a combination of both (17). A recent article concluded that no definition of DGF was superior and that the easiest and most widely used (the use of dialysis within the first week post-transplantation) should be adopted as the gold standard (16).

1.2.3 Primary Non-Function (PNF)

PNF is defined as the permanent loss of allograft function after transplantation, with reported incidence up to 8% (23). Both DGF and PNF may be direct consequences of IRI and are more common in DCD and ECD organs, reflecting the negative influence of prolonged WIT1 and CIT (24). PNF is an unfortunate consequence of severe IRI with significant implications on patient survival (23, 25).

1.2.4 Long-term consequences of DGF

DGF has been described as 'one of the most important independent variables of graft failure' (26). This is because it leads to increased rejection episodes (27) and chronic allograft nephropathy (28-30), the compounded effect of which leads to increased risk of graft failure. Despite this, some studies have shown that, although DGF is strongly linked to chronic allograft nephropathy, whether this translates to affecting long-term graft survival may remain a topic of debate (24).

1.3 Ischaemia Reperfusion Injury – Pathophysiology

The pathophysiological processes involved in IRI are complex and include interactions between the endothelium, components of the immune system and cell death programs (31). Briefly, ischaemia and resultant tissue hypoxia cause an increase in vascular permeability and impaired endothelial cell barrier function. Cell death programs including apoptosis, phagocytosis, and necrosis are activated. Apoptosis comprises nuclear fragmentation, loss of mitochondrial integrity and cell shrinkage. Restoration of blood flow upon reperfusion exacerbates the tissue injury through a profound inflammatory response, mediated predominantly by activation of the innate and adaptive components of the immune system (31, 32).

1.3.1 Pathological Characteristics

The kidney consists of an outer renal cortex and an inner medulla. Nephrons, the basic structural functional units of the kidney, are mainly located in the cortex and have an initial filtering portion, the renal corpuscle, which consists of the glomerulus - a network of capillaries, surrounded by a cup liked structure called the bowman's capsule. The corpuscle is followed by the renal tubule, consisting of the proximal convoluted tubule, loop of Henle, and the distal convoluted tubule, which then drains into the collecting ducts. The renal tubule is made of tubular cells with surrounding interstitium. The most important cellular components of the renal cortex in which damage occurs are: tubular, glomerular, endothelial, and interstitial.

In renal IRI, endothelial damage is the result of the initial ischaemia and hypoxia leading to impaired cell barrier function. This causes endothelial swelling followed by cell disruption, eventually leading to loss of endothelial integrity and loss of endothelial cells (33, 34). Tubular injury is the most characteristic feature of kidney IRI since the tubules are particularly prone to ischaemic damage, and therefore potentially reversible acute tubular necrosis is a hallmark of kidney injury. Specifically, in IRI, hypoxia results in formation of plasma membrane blebs and loss of the brush border membrane leading to loss of polarity and integrity of the cellular tight junctions. This leads to cell death and sloughing, causing cast formation. As a result, cast formation and other debris lead to tubular obstruction. These changes, along with tubular necrosis, signify extensive tubular damage (35). Interstitial damage is also seen in IRI, but may also result from chronic and progressive damage of varied aetiology, leading to fibrosis and atrophy. Inflammation, haemorrhage and necrosis are the hallmarks of acute ischaemic damage to the interstitium (36). Glomerular damage is also a hallmark of kidney IRI. Thickening of bowman's capsule, retraction of the glomerular tuft and, in severe cases, glomerular fibrosis are the histological features of ischaemic glomerular damage (37)

1.3.2 Pathophysiology – At the Biochemical/Cellular level

1.3.2.1 Ischaemia-induced Injury

Ischaemia results in deprivation of oxygen and nutrients to the tissue, and accumulation of waste products. The cellular changes that occur, result in tissue damage, predominantly from reactive oxygen species (ROS), free radicals and

lytic enzymes. These changes are described in a review of DGF by Percio et al (38) and summarised in Figure 1.1.

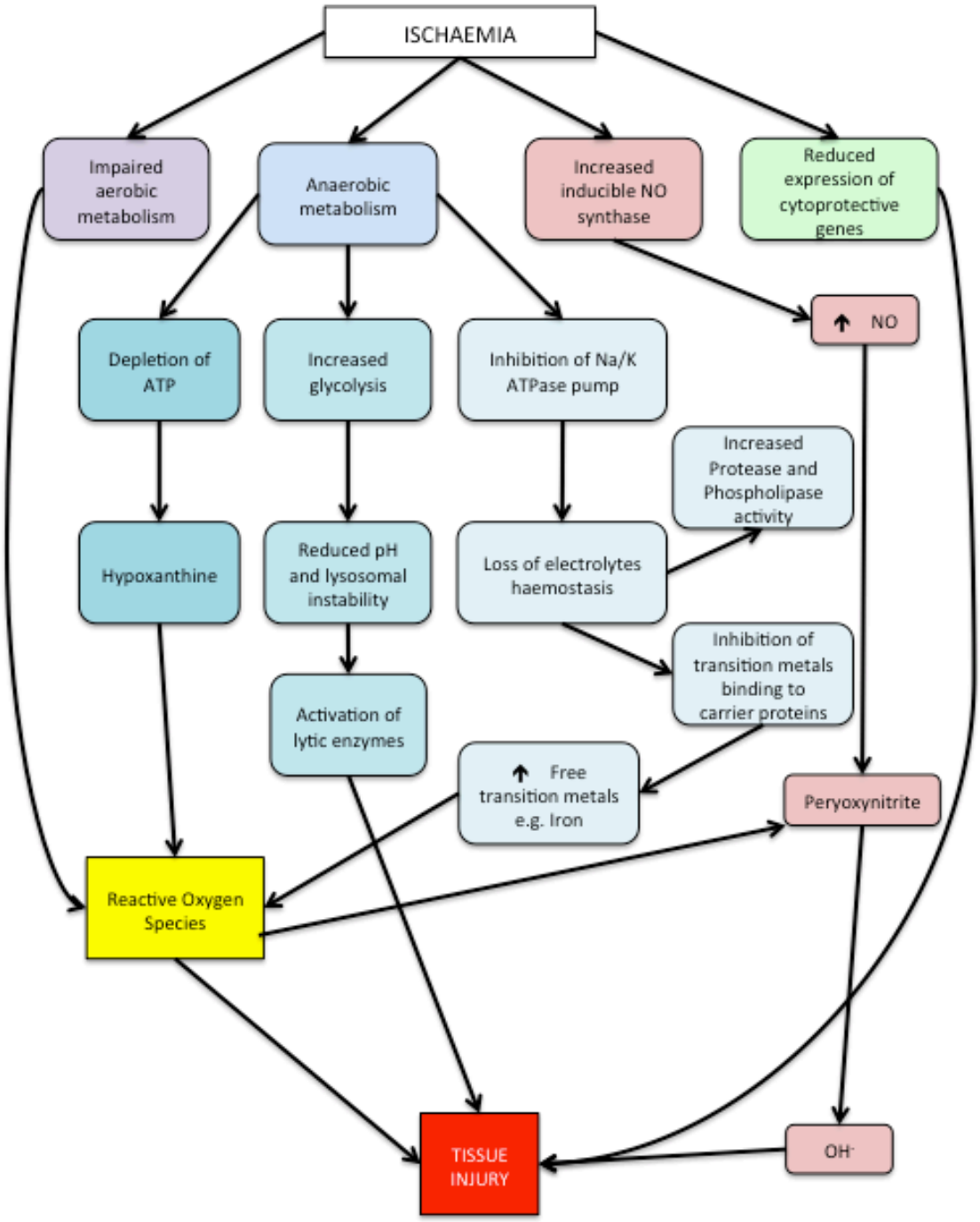

Figure 1.1: Biochemical changes induced by Ischaemia

Ischaemia results in deprivation of oxygen and nutrients to the tissue, causing impaired aerobic metabolism, enhanced anaerobic metabolism, reduced expression of cytoprotective genes, and increased inducible NO synthase. The cellular changes that occur in response to this, result in

tissue damage predominantly from reactive oxygen species, free radicals, and lytic enzymes. Figure adapted from (38).

One of the first changes is that deprivation of oxygen delivery to the cell causes a switch from aerobic to anaerobic metabolism. Anaerobic metabolism is unable to meet the demands of the tissue, and therefore adenosine triphosphate (ATP) levels decrease rapidly breaking down to hypoxanthine, which in anaerobic conditions is metabolised further by xanthine oxidase to ROS (39). Anaerobic metabolism also causes increased anaerobic glycolysis, lactic acid production and resultant reduced intracellular pH, and inhibition of the membrane-bound sodium(Na^+)/potassium(K^+) ATPase pump activity (40). Anaerobic glycolysis and the depletion of ATP cause lysosome membrane destabilisation resulting in leakage of lytic enzymes and consequently cell injury (41).

Inhibition of the Na^+/K^+ ATPase pump causes a disruption in the homeostasis of electrolytes, such as Na^+, Calcium (Ca^{2+}), and Iron. Initially there is a large intracellular increase of Na^+, which causes an accumulation of water resulting in oedema, the degree of which is dependant on the duration of ischaemia (42). As a result of this Na^+ accumulation, the Na^+/Ca^{2+} antiporter starts to work in the reverse direction by stopping its pumping of Ca^{2+} outside of the cell, causing an accumulation of Ca^{2+} (43). Accumulation of Ca^{2+} causes activation of calcium-dependant phospholipases and proteases called calpains and caspases. These remain largely inactive during ischaemia, causing damage upon reperfusion when the intracellular pH becomes neutralised (44). Accumulation of Ca^{2+} also generates more reactive oxygen species. Binding of Iron to its carrier proteins (transferrin and Ferritin) is also inhibited, causing an increase in intracellular

free iron, which acts as a potent catalyst for oxygen radical-generating reactions (45).

Nitric oxide (NO) production is also increased, as Nitric Oxide Synthase (NOS) activity is induced by hypoxia. Nitric oxide reacts with reactive oxygen species to form potent oxidants such as peroxynitrite, which causes tissue damage through oxygen radical induced injury (46).

Finally, ischaemia causes cytoprotective mechanisms to be activated to reduce the cell's metabolic activity, however, reports have suggested that hypoxia can also result in a decreased expression of cytoprotective genes (such as heme-oxygenase-1 (HO-1)), with consequentially increased tissue injury (47).

1.3.2.2 Reperfusion-induced Injury

Reperfusion reinstitutes blood flow to the ischaemic organ/kidney providing rewarming, re-oxygenation, and as a result reconstitution of aerobic metabolism and ATP production, and neutralization of intracellular pH. This activates a generalised and immune-mediated inflammatory response that exacerbates and sustains the renal cell injury, the hallmarks of which involve complex interactions between activated endothelium, leucocytes, and other components such as complement, in addition to damage caused by reactive oxygen species.

Re-oxygenation and normoxia causes a large increase in the production of reactive oxygen species and free radicals. The protective antioxidant enzyme activity is also reduced (39, 45). The high concentration and rapid generation of

reactive oxygen species and reduction of antioxidant activity cause cell death by apoptosis (48). Lipid peroxidation of the cell membrane is also initiated by cytotoxic and highly reactive radicals such as hydroxyl, produced from a reaction between the superoxide anion (O_2-) and hydrogen peroxide (H_2O_2) (45).

Reperfusion also causes a further increase in intracellular Ca^{2+}, which has already accumulated within the cytoplasm and mitochondria, during ischaemic injury. High Ca^{2+} levels within the cytoplasm and mitochondria activate calpains, which in neutral pH conditions of reperfusion, cause direct cell structure damage and death (44). High mitochondrial Ca^{2+} and reactive oxygen species concentration causes opening of the mitochondrial permeability transition pore (mPTP), which leads to cell death (via apoptosis and necrosis) (49, 50).

1.3.3 Endothelial and micro vascular dysfunction

The endothelium is an important component in determining adequate smooth muscle response, vascular tone and leucocyte function. Endothelial and micro vascular dysfunction seen in IRI can be categorised into 3 components: Vasoconstriction, Increased vascular permeability, and Leucocyte recruitment.

Following injury/damage to endothelial cells from IRI, there is an increased release of factors promoting vasoconstriction (such as endothelin-1, thromboxane A2, and platelet derived growth factor-B); and a decreased production of factors promoting vasodilatation including NO. This results in vasoconstriction of the micro vessels, in particular the small arterioles (33, 51, 52).

Injured endothelial cells also express cell adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), and leucocytes (which are already activated in response to the injury) express the counter-receptors (β1-integrin and β2-integrin), leading to augmented endothelial-leucocyte interactions. After the initial rolling of the leucocytes onto the endothelium (mediated by an increased expression of endothelial P-selectin), the interaction between cell adhesion receptors and leucocyte counter-receptors causes immobilisation and adherence of leucocytes to the endothelium. This is followed by transmigration and diapedesis of leucocytes across the endothelium (38, 53). The adherent leucocytes plug the capillaries and enhance the release of vasoactive cytokines, such as tumour necrosis factor alpha (TNF-α), which further augments the vasoconstriction. In addition to this, the coagulation system is activated resulting in further compromise of the micro vascular circulation (33). Furthermore, activated leucocytes within the interstitium release reactive oxygen species and proteolytic enzymes, causing further damage (38).

Damage to the endothelium results in endothelial swelling causing impaired endothelial cell barrier function. There is breakdown of the glycocalyx and disruption of the actin cytoskeleton. These changes cause loss of endothelial cell-cell contacts with a consequential increase in the vascular permeability and loss of fluid into the interstitium. Endothelial swelling also compromises blood flow (54).

In addition to this, there is down regulation of angiogenic factors such as vascular endothelial growth factor (VEGF) and up regulation of antiangiogenic factors, causing a reduction in angiogenesis, reflected in the reduced number of vessels in IRI (54, 55).

1.3.4 Immune response – Innate and Adaptive

IRI is a process that may activate both the innate and adaptive immune response. The innate immune response consists of a non-antigen-specific activation involving predominantly neutrophils, monocytes, macrophages, and natural killer cells, whereas the adaptive immune response consists of antigen-specific activation involving predominantly T-cells.

1.3.4.1 Innate immune response

Activated endothelium causes **neutrophil** adherence and migration into the tissues, in particular the peritubular tissue and interstitium, where they produce reactive oxygen species and proteolytic enzymes, causing significant damage. Neutrophils are amongst the first cells to be recruited at the site of injury. Neutrophils also release pro-inflammatory cytokines including interleukin 4 (IL-4), TNF-α, interferon-gamma (IFN-γ) and interleukin 17 (IL-17). IL-17 itself regulates the IFN-γ-mediated migration of neutrophils to the kidney tissue, and natural killer T-cell activation.

Monocytes migrate to 'healthy non-injured' or 'inflamed/injured' tissue from the bone marrow and differentiate into **macrophages** and dendritic cells. Two broad classes of macrophages have been identified: M1 type (pro-injury) and M2

type (pro-repair). These are determined according to the underlying pathological conditions. In general, M1 macrophages have an increased phagocytic capacity and are seen in high numbers in the first few hours after IRI persisting for up to 1 week, driving a "Th1 immune response" by production of pro-inflammatory cytokines (such as IFN-γ and TNF-α). In contrast, M2 macrophages are present in high numbers at 3-5 days after IRI when tubular cell repair processes have been established, and secrete matrix components involved in tissue repair. They drive a "Th2 immune response, which is predominantly anti-inflammatory and pro-repair, and is mediated by IL-4, interleukin 10 (IL-10) and interleukin 13 (IL-13). Although it remains a useful concept for understanding macrophage function, the M1/M2 macrophage paradigm is increasingly recognized to over-simply the variety of macrophage phenotypes found in the tissue *in vivo (56)*, and important discoveries including tissue-specific resident and other macrophage phenotypes, some with distinct lineage and proliferative control, are currently transforming understanding of macrophage biology (57, 58).

Dendritic cells, also differentiated from monocytes, are activated in IRI, and are involved in the activation of T-cells. Maturation of dendritic cells is induced by danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). They present antigens to T-cells, thus forming an important bridge between the innate and adaptive immune responses. In kidney transplantation, donor dendritic cells are activated in response to brain death, and subsequently activate recipient T-cells.

DAMPs and PAMPs are also recognized by toll-like receptors (**TLRs**), small proteins located on the cell membrane or present within the cytoplasm, which allow the immune system to work more efficiently, by regulating and integrating dendritic cell, T-cell and complement system function. Integration of signal allows greater discrimination between harmful activation (such as infection) and other activation that might not require the same response from the immune system. TLRs detect DAMPs including the nuclear protein 'High Mobility Group Box 1 (HMGB-1)' which binds to the DNA and regulates transcription. In kidney IRI, tubular cells express both TLR-2 and TLR-4. TLR-4 has an important role in kidney IRI and is the subject of an excellent review by Zhao et al (59). Essentially, activation of TLR-4 (induced by endogenous DAMPs) mediates a pro-inflammatory response by facilitating leucocyte infiltration and migration and release of pro-inflammatory cytokines.

The **complement system** is an important component of the immune response to IRI, and is the subject of a review by Sacks et al (60). Complement cascade involves a group of approximately 30 soluble proteins that are activated by 3 overlapping pathways: Classical, Lectin and Alternative. The Classical pathway is activated via binding of complement 1q protein (C1q) to immune complexes, the lectin pathway by binding of mannose-binding lectin to the surface of microorganisms, and the Alternative pathway by direct activation of C3. All 3 pathways lead to C3 deposition onto the surface of the pathogen/damaged cell, marking it for destruction by the membrane attack complex (MAC) (made of complement 5b component (C5b), complement 6 (C6), complement 7 (C7), complement 8 (C8), and complement 9 (C9)) or removal by phagocytosis (60).

Once the complement cascade is activated, there is release of C3a and C5a leading to formation of the MAC, which in turn activates cytokine release, and neutrophil activation and infiltration. This leads to tubular cell injury. In kidney IRI, the alternative pathway is the predominant one (61). Mice deficient in C3 or factor B (an important component of the alternative pathway) are protected from renal IRI (62, 63).

The complement system also activates the adaptive immune response. C3 derived from macrophages binds onto the surface of dendritic cells, promoting their maturation, which as a result activates the T-cell response. In kidney transplantation, brain death in deceased donors activates complement, in particular C5a, which binds to its receptor on dendritic cells, causing activation of T-cells. C3 deposits are also found to be in high concentration in biopsies of deceased donors and may have particular importance in rejection. In addition to this, there appears to be important cross talk between complement and TLRs, adding to the complexity of the innate immune system in IRI (64).

1.3.4.2 Adaptive Immune response

The adaptive immune response is a key component of IRI, and in particular T-cells exhibit both inflammatory and anti-inflammatory roles. T-cells are lymphocytes that play an important role in cell-mediated immunity. There are two predominant types of T-cells: T helper cells, also known as 'cluster of differentiation 4' (CD4+) T-cells because they express CD4 glycoprotein on their surfaces; and cytotoxic T-cells, known as 'cluster of differentiation 8' (CD8+) T-

cells as they express CD8 glycoprotein on their surfaces. Once activated, CD4+ T-cells secrete cytokines that assist other lymphocytes in facilitation of the immune process, whereas CD8+ T-cells are usually involved in destruction of pathogen-infected cells. Both CD4+ and CD8+ T cells have a detrimental role in IRI (65). CD4+ T cells in particular are activated in the presence of co-stimulatory factor 'cluster of differentiation 28' (CD28). T cells are activated by the presentation of an antigen usually expressed on mature dendritic cells although they can also be activated independent of antigen presentation (66). T regulatory cells (Tregs), in contrast are anti-inflammatory, and are found after 3 days following IRI, where they are involved in repair of damaged cells (67).

1.3.5 Cell death programs

IRI activates predominantly 3 different programs of cell death: Necrosis, Apoptosis, and Autophagy-associated cell death, as described by Hotchkiss et al (68).

Necrosis is characterised by swelling of the cell and organelles, followed by rupture of its surface membranes with spillage of intracellular contents. Necrotic cells are potent stimulators of the immune system and cause cytokine release and infiltration of inflammatory cells (68).

Apoptosis involves a self-contained program of cell death, also known as programmed cell death, which is induced by an organized complex 'caspase-signalling cascade'. Apoptosis is characterised by cell and nuclear shrinkage with integrity of the plasma membrane at least until the late stages of the apoptosis

process. Apoptosis is thought to be an important process in kidney IRI, with studies reporting the beneficial effects of inhibiting apoptosis inducers, such as thrombospondin 1 (TSP-1) (69). In addition to this, the expression of apoptosis-related genes was recently investigated in pre-transplantation biopsies of kidney transplant patients, reporting a higher expression of 'BCl2-Associated X protein' (Bax) (pro-apoptotic molecule) and caspase-3 (CASP-3) (apoptosis executor enzyme), and lower expression of 'B-cell lymphoma 2' (Bcl-2) (anti-apoptotic molecule) in deceased donor kidneys compared with LD kidneys, and furthermore lower Bcl-2 levels in patients with DGF, a direct consequence of IRI (70).

Finally, autophagy is a process that is thought to be a protective adaptive response of the cells/tissues to various types of injury including kidney IRI (71). The role of autophagy in renal IRI is complex, and while probably a largely protective response, this may be dependant on factors including the length and extent of ischaemia, with autophagy being protective in shorter ischaemic times and possibly detrimental with more prolonged duration of ischaemia (72, 73).

1.3.6 Therapeutic strategies to ameliorate IRI

Several therapies have been developed to attenuate IRI and the resultant DGF. These have focused on: Management of the donor; Organ preservation; Management of the recipient; and Pharmacological/Experimental therapies.

1.3.6.1 Donor Management

Donor management is essential to achieve reduced IRI. This includes an accurate surgical technique (and relatively fast for DCD donors to reduce the WIT1), optimum cold perfusion of the organ, and reduced cold ischaemic time. There is also an opportunity during donor management for ischaemic or chemical preconditioning of the donor.

1.3.6.2 Organ Preservation

Preservation solutions have been developed and optimised to, reduce osmotic injury to the cell (and reduce cell swelling), maintain electrolyte (especially Ca^{2+}) homeostasis, and reduce acidosis. The current preservation solutions used for static cold storage include Belzer University of Wisconsin (UW) solution and Soltran kidney perfusion fluid (74, 75). Methods to store organs at present include simple cold storage and hypothermic machine perfusion. Hypothermic machine perfusion reduces incidence of DGF when compared with cold storage (76). A multicentre international trial showed that hypothermic machine perfusion was associated with a significantly reduced incidence of DGF, and improved graft survival at 1 and 3 years for DBD and ECD kidneys when compared with static cold storage (77, 78). Over the past few years, developments in the perfusion of donor organs have led to the practice of normothermic machine perfusion in liver transplantation. It has been shown that *ex vivo* normothermic perfusion has many potential advantages over cold perfusion for kidney transplantation, and such practice may allow for the use of increased use of donors with greater ischaemic insult (79).

1.3.6.3 Recipient Management

It is vital that the recipient is in as optimal medical condition as possible. This includes optimum electrolytes and fluid status. Often the recipient may be hypovolaemic following recent haemodialysis, which would routinely aim to bring the patient to their dry weight. It is important to avoid hypovolaemia as this can cause further peri-operative hypo-perfusion and cause a further ischaemic insult. Therefore adequate intravascular volume load should be maintained via central venous pressure monitoring. In addition to this, mannitol is administered before reperfusion, as this may reduce DGF because of its antioxidant properties (80).

1.3.6.4 Pharmacological/Experimental Therapies

Several pharmacological and experimental therapies/agents have been used and proposed to attenuate IRI. These include physical strategies such as ischaemic preconditioning (IPC), and other agents, which have anti-inflammatory, anti-oxidant or vasodilatory properties. These have been described in 2 prominent reviews by Percio (38) and Siedlecki (81) and summarised in in Table 1.1.

Vasodilatory Agents:

- Calcium-channel blockers
- Prostacyclin
- Atrial natriuretic peptide
- Endothelin receptor antagonists

Anti-oxidants:

- Heme-oxygenase-1 (HO-1) induction
- N-acetyl cysteine (NAC)
- Inducible NO synthase inhibitors

Anti-inflammatory Agents:

- Platelet activating factor receptor antagonists
- Inhibitors/Antagonists/Antibodies of cytokines e.g. TNF-α, IL-1
- Immunosuppressants e.g. ATG, MMF
- Complement inhibitors e.g. sCR1, Eculizimab
- Inhibitors of TLR-2 and TLR-4
- Statins

Conditioning strategies:

- Ischaemic preconditioning
- Ischaemic post conditioning
- Chemical conditioning

Table 1.1: Pharmacological/Experimental therapies to prevent Kidney IRI

Some of the proposed pharmacological and experimental agents designed to attenuate IRI and prevent DGF in kidney transplantation, adapted from (38).

IPC and other conditioning strategies are discussed in detail in Section 1.4.

1.4 Ischaemic Preconditioning (IPC)

1.4.1 Definition

IPC is a powerful treatment strategy that involves prior application of short periods of controlled ischaemia, which may confer tolerance and protection to a subsequent episode of IRI.

1.4.2 Background

The phenomenon of IPC was first described in 1986 when Murry et al reported that brief episodes of non-lethal myocardial ischaemia and reperfusion prior to a prolonged episode of myocardial ischaemia reduced the size of the myocardial infarct by 75% in a dog model of acute myocardial infarction (82). This protective strategy has since been supported by many *in vivo* studies in several animal species including small animals (mice (83, 84), rats (85) and rabbits (86)) and large animals (sheep (87) and pigs (88)). Since then IPC has now become the 'gold standard' to which other protective strategies in the heart are compared (89). The benefits of IPC were also seen and reported in other organs, including the kidney.

1.4.3 IPC in the Kidney

Following the 'landmark' studies in the heart, IPC has been shown to be beneficial in ameliorating IRI in the kidney in several animal species, including small animals (mice (90), rats (91) and rabbits (92)) and large animals (pigs (93) and dogs (94)). A systematic review and meta-analysis by Wever et al analysed 58 animal studies that evaluated the effect of IPC in the kidney (95). The overall

conclusion of this review was that IPC effectively reduced renal damage after IRI, in terms of reduced serum creatinine, blood urea nitrogen, and histological renal damage. However, the number of studies included for the meta-analysis was 33, 17, and 15 for serum creatinine, blood urea nitrogen, and histological renal damage respectively. The animal species used within these studies comprised small animals (rat (34), mouse (14), rabbit (4)), and large animals (pig (3) and dog (3)). The review also concluded that there was a huge variation in the type of IPC protocol used and its efficacy, and that this may differ per animal species. Moreover, it identified the need for standardisation in animal experiments evaluating IPC (95).

1.4.4 Variation in IPC Protocols

There is a great deal of variation in the IPC protocols and the IRI model that have been used in experiments, which lead to difficulty in making direct comparisons. IPC stimulus can be applied directly to the organ of interest (localised IPC – LIPC) or remote from it to a different organ or tissue (remote IPC – RIPC). More than half of the studies analysed in Wever's review utilised an IRI animal model, which consisted of a 40 or 45-minute period of index ischaemia, and majority of the studies utilised LIPC as the IPC modality. It found no difference in the efficacy of IPC between LIPC and RIPC (95). The IPC stimulus can be one continuous episode of ischaemia (Continuous) or it may involve 2 or more cycles of ischaemia (Pulsatile). Although many studies have shown benefit from both continuous and pulsatile IPC (96, 97), some show no benefit of IPC at all (98, 99). The interval between the IPC stimulus and ischaemic injury may also vary from 'short' consisting of a few minutes to hours (Early window of protection) to

'longer' consisting of days (Late window of protection). The majority of these studies have evaluated the effects of 'Early' IPC response as compared with 'Late'. However both have shown IPC to be beneficial (96, 100) or not beneficial (99, 101).

1.4.5 Clinical Application of IPC

Based on the majority of animal studies, there is a great potential for IPC to improve outcomes in kidney transplantation. A number of clinical trials in humans have been published reporting the protective effects of IPC in the myocardium, the first one being in 1993 (102). Clinical trials of IPC have now also been shown to have protection in other organs, including liver (103), brain (104), and lung (105). To date, 22 clinical trials have evaluated the effect of IPC on kidney function, all of which have involved application of RIPC to the limbs. These studies show conflicting reports, with some showing that RIPC is beneficial whereas others show that RIPC did not benefit. Most importantly, a very large trial published in NEJM in 2015 showed that RIPC conferred no benefit (106), whereas another large trial published in JAMA showed that RIPC significantly reduced AKI and the need for renal replacement therapy in high-risk patients undergoing cardiac surgery (107). Similarly, trials investigating the effect of RIPC on contrast-induced AKI in patients undergoing coronary angiography have shown protective as well as non-protective effects (108-110). In renal transplantation, Wu et al showed that RIPC enhanced early recovery of renal function following kidney transplantation (111). One study showed that in living donor kidney transplant recipients, RIPC did not improve renal function at

1 and 3 months (112). Chen et al also reported similar findings for RIPC in living donor renal transplantation (113).

1.4.6 Underlying Mechanisms of IPC

The mechanisms underlying IPC remain incompletely defined, although some insight may be gained from cardiac studies. Proposed mechanisms for a protective effect of IPC include trigger factors and intracellular mediators, and are summarised in Figure 1.2. IPC may release local trigger factors, which include adenosine, bradykinin and opioids, and other factors such as natriuretic peptides, and growth factors. Upon binding to their specific cell surface receptors, these trigger factors may propagate the intracellular preconditioning signal. There are three main intracellular pathways implicated in IPC: Reperfusion Injury Salvage Kinase (RISK), Survivor Activating Factor Enhancement (SAFE), and cyclic Guanosine Mono Phosphate/ Protein Kinase C (cGMP/PKC). The common effector for all these pathways is predominantly the modulation of mitochondria, designed to prevent mitochondrial dysfunction, inflammation, apoptosis, and cell death resulting from IRI.

The RISK pathway involves pro-survival anti-apoptotic protein kinases including Phosphatidylinositol-3-OH Kinase (PI3K), Akt (also know as Protein Kinase B), MEK (also known as Mitogen-Activated Protein Kinase Kinase – MAP2K), and Extracellular regulated Kinase (Erk). Activation of this pathway eventually leads to protection as a result of the inhibition of the opening of the mPTP. Induction/opening of mPTP leads to mitochondrial swelling and cell death through apoptosis and necrosis (114).

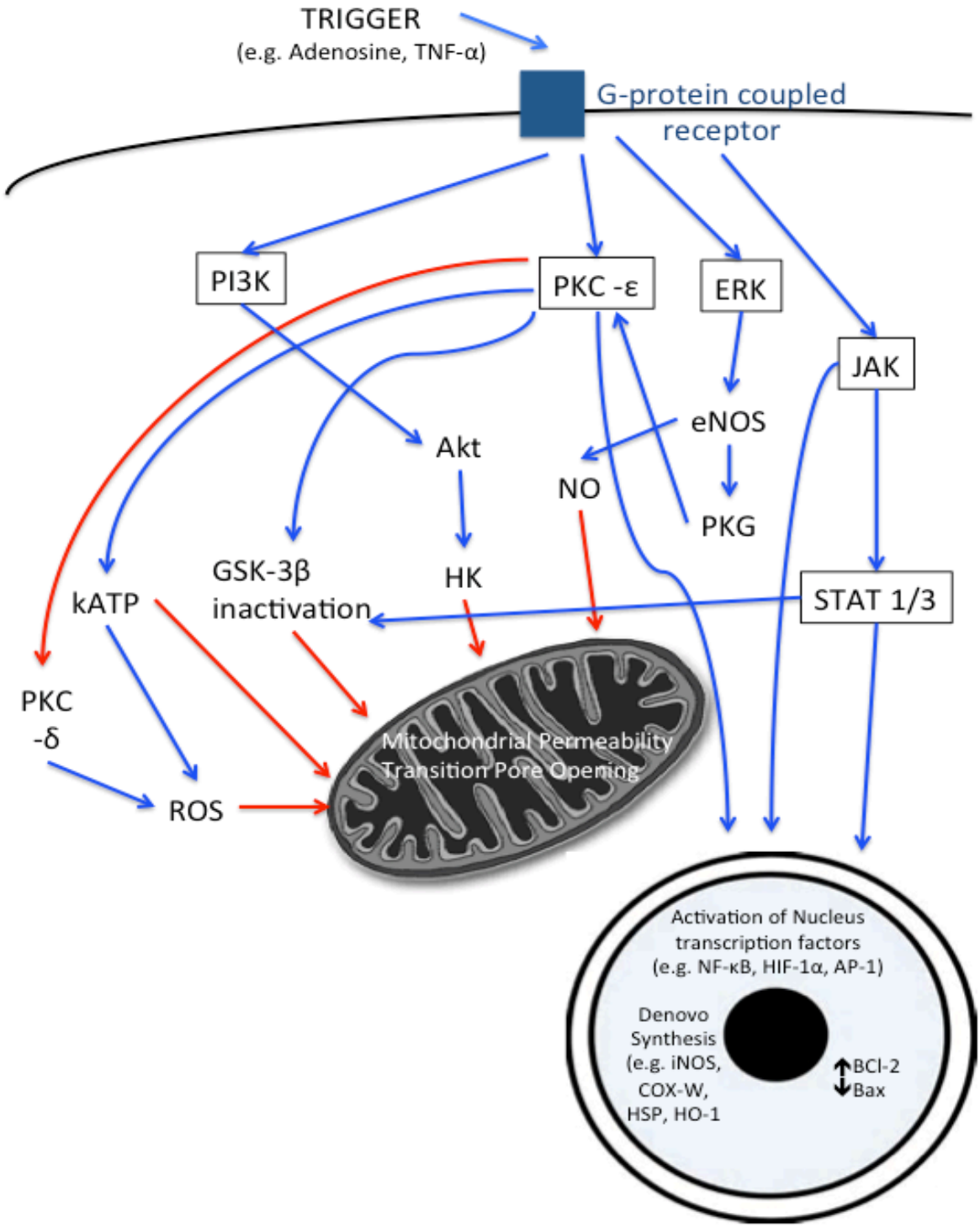

Figure 1.2: Proposed mechanisms underlying Ischaemic Preconditioning (IPC)

IPC stimulus causes the release of local trigger factors, e.g. adenosine, which upon binding to their specific cell surface receptors, cause the propagation of intracellular cascades. The common effector for the intracellular cascades/pathways is predominantly the modulation of mitochondria designed to prevent the damage of mitochondrial dysfunction, inflammation, apoptosis and cell death resulting from IRI. Activation of nucleus transcription factors (e.g. HIF-1α) and denovo synthesis of proteins (e.g. HO-1) is also thought to play an important role in the mechanisms of IPC protection. Blue arrows indicate stimulation, Red arrows indicate inhibition.

The SAFE pathway involves TNF-α, a pro-inflammatory cytokine, as a trigger factor and an intracellular cascade that involves Janus Kinase (JAK) and Signal Transducer and Activator of Transcription-3 (STAT-3). This is independent from the RISK pathway, although there is likelihood of cross-talk between the two pathways (115). The downstream effects of the SAFE pathway include STAT-3 induced increase in the anti-apoptotic protein Bcl-2 and decrease of the pro-apoptotic protein bax (116), and inhibition of mPTP opening via phosphorylation of Glycogen Synthase Kinase-3 beta (GSK-3β) (117). Also important, and mostly attributed to the delayed IPC protection, are the activation of nucleus transcription factors such as 'Nuclear Factor kappa-light-chain-enhancer of activated B cells' (NF-κB) which trigger the de novo synthesis of proteins such as cyclo-oxygenase-2 (COX-2) and heat shock proteins (HSPs). These are activated by both the RISK and SAFE pathways (118).

1.4.7. Other Conditioning Strategies

1.4.7.1 Ischaemic Post-Conditioning

Ischaemic Post-conditioning (IPOC) refers to conditioning the organ of interest after IRI has been induced. This was introduced, as IPC may not always be practical in transplantation (as transplantation is routinely not an elective procedure). IPOC was first shown to limit IRI in the myocardium in 2003 (119) and in the kidney in 2007 (120). Studies that have been conducted since then have largely shown a beneficial effect of IPOC in the kidney. The underlying mechanisms that have been proposed include reduction in reactive oxygen species formation, up regulation of anti-apoptotic factors and down-regulation of

pro-apoptotic factors (121). To date, one human study has evaluated the effect of IPOC in kidney transplantation. This showed no beneficial effects of IPOC in a subset of DCD kidney transplants (122).

1.4.7.2 Pharmacological Preconditioning

Pharmacological or chemical preconditioning is based on the concept of mimicking and inducing the mechanistic protective effects of IPC. Amongst the agents tested, Erythropoietin (EPO) has been most used, and shown to be effective by up regulation of HIF-1α (123), and activation of anti-apoptotic pathways that involve induction of PI3K and STAT-3 intracellular cascades (124). Another agent, glutamine, has also shown protective effects, thought to be due to enhancement of endogenous heat shock protein expression (125). Both of these agents have shown beneficial effects in animal studies. To date, no human study has been conducted to test the effect of chemical or pharmacological preconditioning.

1.5 Control of Gene Expression

The biology of a tissue is largely determined by the genes expressed within it. A gene is essentially the basic physical and functional unit of heredity, and is made up of deoxy ribonucleic acid (DNA). Genes act as instructions for protein synthesis and humans are estimated to have between 20,000 and 25,000 genes. Given the complex patterns of gene regulation and transcription etc., Gerstein et al has provided a more concise and up-to-date definition of a gene as "a union of genomic sequences encoding a coherent set of potentially overlapping functional products" (126).

The regulation of gene expression is essential in normal physiology and its altered expression forms the basis of diseases processes, therefore it is essential that we understand all the steps involved in this. All cells in the human body have the same DNA but the function of each cell may differ depending on its purpose and hence different cell types produce their own unique set of proteins. Proteins are the major functional endpoint of the DNA template and may be involved in many cellular processes for example enzymes, receptors, or signalling molecules. Figure 1.3 shows the steps of the DNA to protein pathway and the points at which it may be regulated. The central paradigm is that DNA is transcribed to messenger ribonucleic acid (mRNA) within the nucleus, which undergoes maturation and is transported to the cytoplasm where it is translated into protein. Each of theses steps are regulated and described as below.

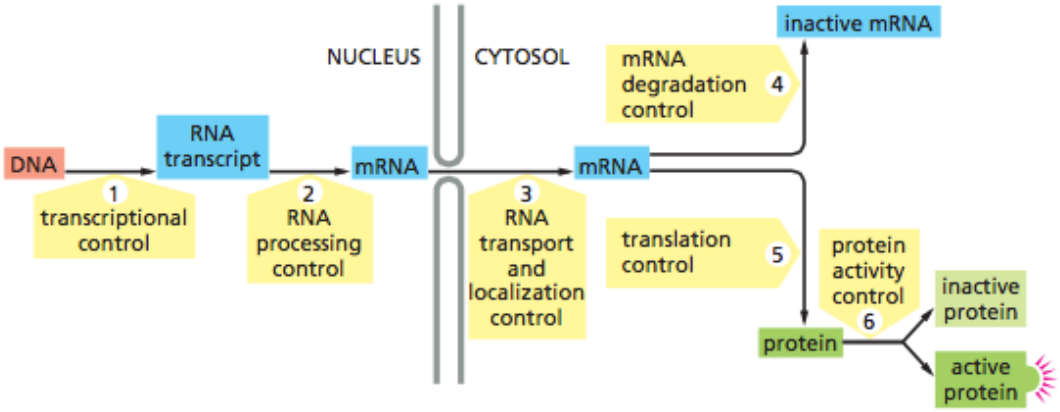

Figure 1.3: Control of Gene Expression

DNA to protein pathway can be regulated at several steps, including transcriptional, post-transcriptional, translational and post-translational levels. This diagram was taken from (127).

1.5.1 Transcriptional Control

The regulation of transcription is orchestrated predominantly by a combination of transcription factors, which act as activators and repressors.

Transcription of DNA to mRNA begins at the *Initiation* site when the *Promoter* region of the gene attracts the enzyme RNA polymerase. This Promoter region also contains *regulatory DNA sequences* that are recognised by proteins known as transcription regulators. Transcription regulators can be activators, which when bound to the regulatory DNA sequence interact with the RNA polymerase to aid initiation of transcription, and repressors which block the access of RNA polymerase to the Promoter region of the DNA sequence (127). Transcription regulators can also control transcription from a distance of even a thousand nucleotide base pairs away from the promoter site. This is aided by DNA looping, which permits contact between the transcription regulator and the transcription complex bound to the promoter region (128, 129). A complex of proteins,

collectively known as the *Mediator*, facilitates this. Transcription activators and repressors promote and prevent the assembly of this *Mediator* respectively. In addition, transcription regulators also attract other proteins that affect the accessibility of the RNA polymerase to the Promoter region, by modulating the chromatin structure. Transcription activators modify the chromatin structure to allow greater access to the underlying DNA, whereas repressors have the opposite effect (130).

In addition to the above, transcription regulators often work as a group controlling the expression of one gene, termed Combinatorial control and it is also evident that one transcription regulator may coordinate the expression of many genes (127).

Furthermore, it is now evident that small RNAs may play an important part in transcriptional control and DNA can itself be modified by methylation to cause gene silencing, adding to the complexity of transcriptional control (131, 132).

1.5.2 Post-transcriptional Control

Post-transcriptional regulation involves controls that start operating once the RNA polymerase has bound to the promoter region of the gene and commenced initiation of the transcription.

Once the mRNA is transcribed, it undergoes modification. One of the modification steps involves processing of the 3' end, including cleavage of the transcript end to finish the mRNA with a polyA tail (133). The site of the cleavage

is subject to control, and may lead to a change in the 3' end of the mRNA and thus a change in the eventual protein structure (134). Another process within the nucleus, which can alter the final mRNA and the resultant protein, is alternative splicing. The splicing machinery causes various permutations of exon inclusion and skipping, resulting in mRNA variations. This is regulated by negative and positive factors that prevent and attract the splicing machinery's access to splicing sites respectively (135). RNA editing is another method of altering the eventual mRNA produced. This involves changes in the nucleotide sequences of the mRNA transcripts, by enzymes that recognise and modify the particular sites required for that particular cell/tissue (136).

Riboswitches, which are present within the mRNA, also play a part in post-transcriptional control by self-regulation. These riboswitches are short RNA sequences that bind to small molecules causing a conformational change in the mRNA (137). Another mechanism of regulation is control of initiation of translation, which will determine the amount of protein produced. There are repressors, which bind to a specific sequence within the 5' cap of the mRNA, an un-translated region of the RNA that guides the ribosome to the first codon of translation, causing the ribosome to miss the first codon and hence repressing the translation process. Cells can inactivate this repression to increase translation into protein when required (138).

In addition to these controls, microRNAs play a key role in post-transcriptional regulation of gene expression. These are discussed in detail in section 1.7.

Only a small proportion of the mRNA transcribed is transported from the nucleus to the cytoplasm for translation. Transcriptional and post-transcriptional regulatory mechanisms ensure that incompletely processed or damaged RNA remains in the nucleus for degradation (139).

1.5.3 Post-translational Control

Once mRNA is transported from the nucleus to the cytoplasm, it undergoes translation into a polypeptide, a chain of amino acids. Once a polypeptide is made it usually requires modification according to the cell requirements. The most common method of modification post-translation is the addition of functional groups, changing the chemical nature of some of the amino acids, and cleavage of the first amino acid methionine (140).

A final regulatory mechanism involves degradation of the protein by proteasomes if the protein is no longer required within the cell (140).

1.6 MicroRNAs

As has been discussed in the previous section, gene expression regulation is a complex process involving combinatorial control of genes by multiple transcription factors at the transcriptional level. At the post-transcriptional level, the emergence of small RNAs, in particular microRNAs has transformed molecular biology over the past decade. It is vital to understand fully their mechanisms of action and expression profiles as this has profound implications for our understanding of disease processes and their treatment.

1.6.1 Background

MicroRNAs are short non-coding RNAs that were first discovered in 1993 by Lee et al who described the first microRNA lin-4, which could regulate the translation of lin-14 mRNA in the nematode *Caenorhabditis elegans* via an antisense RNA-RNA interaction (141). A second group demonstrated that lin-4 regulates (post-transcriptionally) elements in the lin-14 3' un-translated region (3'UTR) to generate a temporal gradient in the lin-14 protein (142). This exciting finding was only properly appreciated in 2000 when it was discovered that let-7 in *C. elegans* is conserved between different animal species (143). This has led to extensive research over the last 15 years and microRNAs have been discovered in all kinds of species including plants and animals.

MicroRNAs are a large family of endogenous, small (approximately 21-22 nucleotides long) non-coding RNAs that are implicated in diverse cellular processes such as cell division, proliferation and apoptosis. Bioinformatic

predictions estimate that the expression of the majority of human protein-coding genes are regulated by microRNAs (144).

1.6.2 MicroRNAs – Biogenesis and Mechanisms of Action

MicroRNA biogenesis (Figure 1.4) begins in the nucleus, where they are transcribed by RNA polymerase II, and sometimes RNA polymerase III, from discrete microRNA loci or excised from intronic sequences, to produce long primary microRNA transcripts. These primary transcripts are made of 1 or 2 stem-loop structures, with a 7-methylguanosine cap and a poly A tail. A micro processor complex, made of an RNAse III, Drosha, and a double-stranded RNA-binding protein, DGCR8 (DiGeorge syndrome critical region 8), cleaves the primary microRNAs into precursor microRNAs, which are hairpin structures of 70 nucleotides in length, called pre-microRNAs. Pre-microRNAs are transported from the nucleus into the cytoplasm via exportin 5, a nuclear export receptor, where another RNAse III, Dicer, further processes them into a >22-nucleotide RNA duplex. A single strand of this duplex, the mature microRNA, is transferred into the microRNA-induced silencing complex (miRISC), whose components include Argonaute (AGO) and GW182 proteins. The other strand, often used as the guide strand for loading onto the RISC complex, is usually degraded.

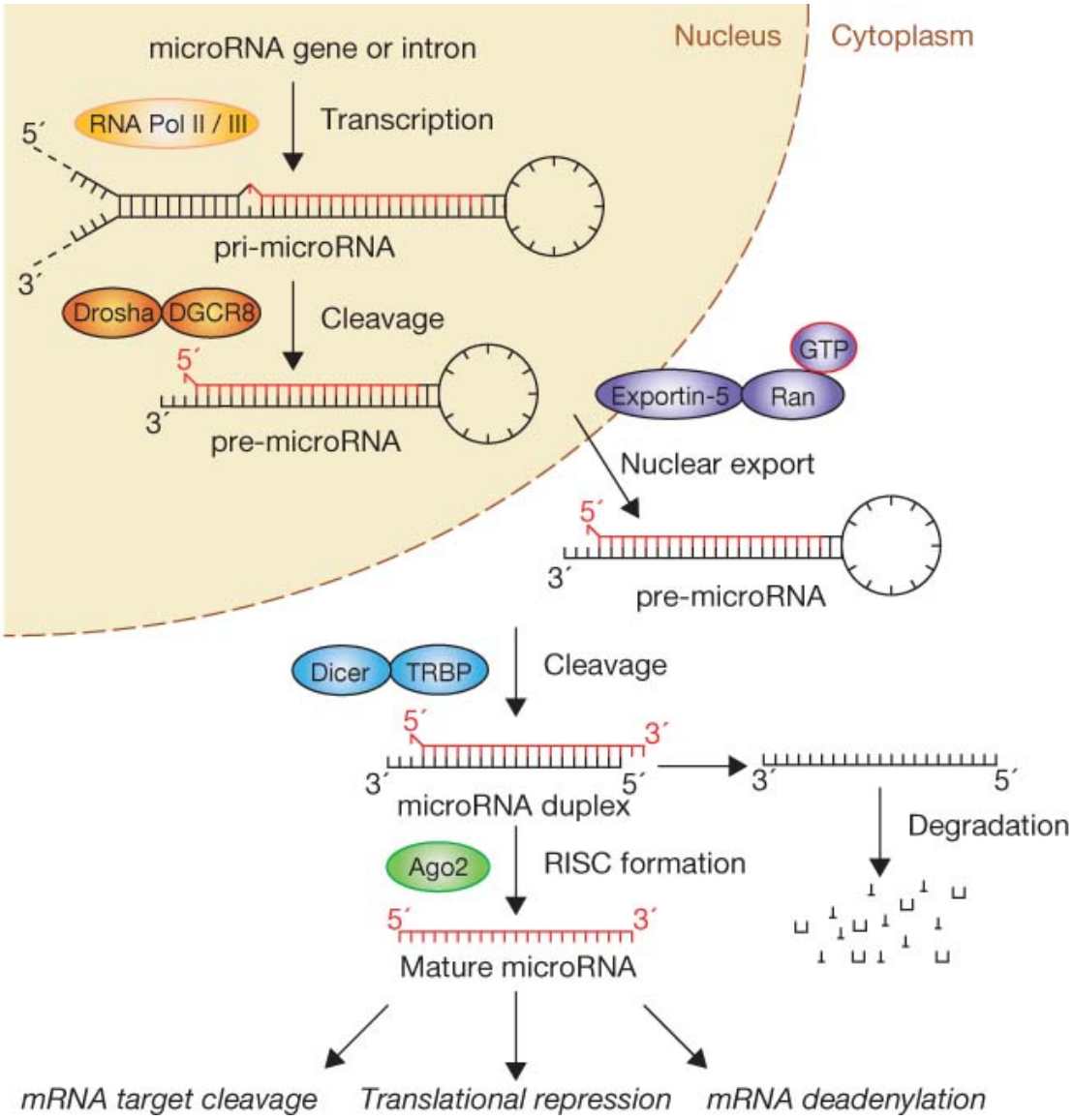

Figure 1.4: Biogenesis of microRNAs

MicroRNAs are transcribed in the nucleus from the microRNA gene or excised from intronic sequences as long primary microRNA transcripts (pri-microRNA) by RNA polymerase II or III. These primary transcripts are cleaved by the micro processor complex (made of Drosha and DGCR8) into precursor microRNAs called pre-microRNAs, which are hairpin structures of 70 nucleotides in length. Pre-microRNAs are exported from the nucleus to the cytoplasm via Exportin 5 (a nuclear export transporter), where the RNase III called Dicer further processes them into a 22-nucleotide RNA duplex. A single strand of this duplex, the mature microRNA, is transferred into the microRNA-induced silencing complex (miRISC), whose components include Argonaute (AGO) and GW182 proteins. The other strand is usually degraded. Once loaded onto the RISC, microRNAs select their mRNA targets via base pairing, causing their cleavage, translational repression or deadenylation, leading to ultimate degradation of the mRNA. Figure taken from (145).

Once loaded onto the RISC complex, microRNAs select their mRNA targets via base pairing between the microRNA and the target sequence of the mRNA script. The target sequence is located predominantly in the 3′UTR of the mRNA transcript, although it can also be located in the 5'UTR region.

The seed sequence, nucleotides in positions 2 to 8 of the microRNA, typically complementarily pairs with the target mRNA sequence. In the case of plants, there is perfect complementarity, leading to cleavage of the mRNA. In mammals, there is partial complementarity and the emerging consensus is that the mRNA transcript is repressed, destabilised and ultimately degraded (146-149). This involves deadenylation (removal or shortening of the poly A tail) which causes decapping of the 5' end of the mRNA transcript, and such uncapped transcripts are degraded by exo-ribonucleases. The precise order and contribution of repression verses degradation is the source of much debate (150), however such target degradation is a major contributor in gene silencing. Importantly, these selection mechanisms enable a single microRNA to potentially target hundreds of transcripts.

Efficient microRNA-mediated translational repression requires correct subcellular localisation. Potential sites for this are the cellular RNA granules known as P-bodies (processing bodies). P-bodies are cytoplasmic foci enriched in miRISC components and enzymes involved in deadenylation, decapping and decay. The function of P-bodies in microRNA-mediated translational repression is controversial, and the majority of AGO proteins, microRNAs and mRNA targets are diffusely distributed in the cytoplasm in sub microscopic particles. However,

there is a dynamic interaction between the sub microscopic particles and P-bodies, with distinct AGO2 kinetics (151). The latter may indicate a defined role for P-bodies in translational repression, potentially storage of specific transcripts or mRNA degradation. Further research will be required to establish the precise role of P-bodies in microRNA-mediated translational repression.

1.6.3 Role in Physiology and Pathology

MicroRNAs have emerged as one of the most important regulatory systems of gene expression, and bioinformatic predictions estimate that the expression of the majority of human protein-coding genes are regulated by microRNAs (144). One microRNA can target many mRNAs and many microRNAs might target one mRNA. This is largely because the microRNA recognition sequence on the target mRNA is very short. Indeed microRNAs are present in every cell and involved in all cellular process (such as growth, metabolism, development and differentiation), in both health and disease.

MicroRNAs have been linked to the pathophysiological processes of many diseases, including cancer, cardiovascular disease and immune disease, subject of a thorough review by Ardekani et al (152). Dysregulation of microRNAs is functionally important in many cancers, with demonstration of unique microRNA expression profiles in cancers including colon, breast, and haematological (153). The expression of microRNAs is linked to many other disease processes, including cardiovascular disease (e.g. myocardial infarction, arrhythmias, cardiac failure) (154), neurodevelopmental disease (e.g. Down's syndrome, Huntington's disease) (155, 156), autoimmune disease (e.g.

rheumatoid arthritis, systemic lupus erythematosus) (157), infection (e.g. viral hepatitis) (158) and other tissue/organ specific diseases including those affecting the kidney.

1.6.4 MicroRNAs in the Kidney

MicroRNAs have been shown to be specific to or enriched within the tissues/organs they originate from. The expression profile of microRNAs in the healthy kidney has been shown to be very important in the normal regulation of the kidney, in processes such as electrolyte and water homeostasis and blood pressure regulation. MicroRNAs that have been shown to be unique and enriched within the kidney (when compared with other organs/tissues) include miR-192, -194, -204, -215, and -216 (159). Moreover, the expression levels of microRNAs within the kidney itself vary according to the type of cells, for example the expression of miR-192 is reported 20-fold higher in the cortex compared with the medulla (160). Regulation of body water takes place via several 'water channels' called aquaporins and it is thought that many microRNAs regulate the expression of these channels, such as miR-320a and its mRNA targets aquaporins 1 and 4 (161). Electrolyte homeostasis is also an important function of the kidney controlled by ion channels and transporters, and microRNAs have been implicated in this context with miR-200b specifically shown to target the expression of the Na/H exchange regulatory factor-1, a key regulator for ion channel apical trafficking (162). Another important role of the kidney is blood pressure control via the renin angiotensin system, in which it has been shown that the expression of renin within the juxtaglomerular apparatus is controlled by microRNAs (163).

1.6.5 MicroRNAs in Kidney disease

In addition to the important role that microRNAs play in normal kidney physiology and homeostasis, they have been also implicated in various kidney diseases, including diabetic nephropathy, APKD, renal carcinoma and AKI (3, 164). AKI is an important clinical syndrome with many causes including IRI, discussed in detail in section 1.7.6. The role of microRNAs in AKI with particular effect on the cell cycle has been published recently in a review article written by us (3).

Yang et al studied severe AKI and subsequent development of fibrosis in various animal models including IRI, unilateral ureteral obstruction and aristolochic acid nephrotoxicity. They demonstrated a causal link between AKI and subsequent development of fibrosis with the 'Gap 2'/'Mitosis' (G2/M) cell cycle arrest of proximal tubular cells (PTCs). Modulating the degree of G2/M cell cycle arrest of PTCs profoundly reduced fibrosis (165).

The mechanism of aristolochic acid induced G2/M cell cycle arrest of PTCs was studied in an *in vitro* model recently in our laboratory (166). Aristolochic acid, in these experiments, led to inhibition and dissociation of the maturation-promoting complex composed of CDK1–cyclin-B1, preventing G2 to M transition. The G2/M cell cycle arrest was associated with the formation of numerous P-bodies, similar to that observed with other nephrotoxins including sodium arsenite (167). Microarray analysis identified differential expression of numerous microRNAs in response to aristolochic acid, including miR-192, miR-194, miR-450a and miR-542-3p. The predominant effect on cell cycle appeared

to be mediated via the induction of miR-192 (166).

Several microRNAs have been identified as protective in the context of AKI including miR-34 (168), miR-125b (169) and miR-127 (170). MicroRNA-34 had a protective effect against PTC apoptosis in a mouse model of cisplatin induced nephrotoxic injury, but the precise mechanism was not defined (168). MicroRNA-125b was identified as protective in cisplatin induced nephrotoxic injury via targeting of the aryl hydrocarbon receptor repressor and indirect induction of MDM2 (169). MicroRNA-127 was induced in a rat model of IRI and *in vitro* in PTCs via hypoxia inducible factor-1α stabilization. MicroRNA-127 has been linked to regulation of focal adhesion complexes and tight junctions, highlighted as important to PTC polarity, and demonstrated to target kinesin family member 3B, which is involved in cell trafficking (170).

1.6.6 MicroRNAs in Kidney Transplantation

1.6.6.1 MicroRNAs in Kidney IRI

One of the major causes of AKI in kidney transplantation is IRI. Upon doing a literature search with the MeSH-terms "MicroRNA" and "Kidney or Renal ischaemia reperfusion injury", 46 studies (in English) were identified, of which 18 were *In Vivo*, as summarized in Table 1.2.

Author	Animal Species	Type of IRI		Tissue	Focus	Findings	MicroRNAs Up regulated	MicroRNAs down regulated
		Ischaemia	Reperfusion					
Wei et al. JASN 2010 (171)	Mouse	30 minutes Bilateral	12h and 48h	Kidney Cortex	Dicer deletion in proximal tubules	Dicer deletion protective against IRI	miR-7 miR-17-3p miR-132 miR-207 miR-362 miR-467 miR-486 miR-489 miR-495 miR-668 miR-685 miR-687 miR-694	miR-18 miR-127 miR-135b miR-296 miR-322 mir-324-3p miR-379 miR-455-3p miR-487b miR-491
Godwin et al. PNAS 2010 (32)	Mouse	30 minutes Unilateral	1d, 3d, 5d, 7d, 14d, 21d, and 30d	Kidney	Immuno-deficient mice (lacking NK, B and T cells)	MicroRNA changes lymphocyte infiltration independent	miR-20a miR-21 miR-146a miR-199a-3p miR-214	miR-187 miR-192 miR-194 miR-805
Saikumar et al. Toxicol Sci 2012 (172)	Rat	30 minutes Bilateral	24h	Kidney Cortex	AKI biomarkers	miR-21 and miR-155 are potential useful biomarkers of AKI	miR-21 miR-155 miR-18a	
Liu et al. Kidney Blood Press Res 2012 (173)	Mouse	45 minutes Bilateral	4h, 24h, 48h	Kidney	Regulation of renal angiogenesis	miR-210 regulates renal angiogenesis after IRI via targeting the VEGF signalling pathway	miR-210	
Wang et al. PloS One 2012 (174)	Mouse	40 minutes Unilateral & Bilateral	24h, 36h	Urine Serum	Biomarkers of AKI	Urinary miR-10a and miR-30d are useful biomarkers of AKI	miR-10a miR-30d	
Xu et al. Kidney Int 2012 (100)	Mouse	30 minutes Bilateral	4h, 24h, 4d	Kidney	Effect of delayed IPC	Delayed IPC protective against IRI	miR-21	
Jia et al. Anesthesiology 2013 (175)	Mouse	30 minutes Bilateral	24h	Kidney	Xenon preconditioning	Xenon preconditioning beneficial	miR-21	
Kaucsar et al. Nucleic Acid Ther 2013 (176)	Mouse	20minutes 30 minutes Unilateral (Contralateral nephrectomy)	24h, 3d, 4d	Kidney	Profiling	miR-21, miR-17-5p and miR-106a are activated during maintenance and recovery phase of IRI	miR-17-5 miR-21 miR-106	
Lorenzen et al. JASN 2014 (177)	Mouse	27 minutes Unilateral & Bilateral	24h, 168h	Kidney	Apoptosis	miR-24 promotes injury by stimulating apoptosis	miR-24	
Bijkerk et al. JASN 2014	Mouse	15 minutes Bilateral	72h, 3 weeks	Kidney	Vascular integrity	miR-126 in haemato-	miR-126	

(178)						poeitic compartment increases neovascularization		
Hu et al. Am J Nephrol 2014 (179)	Mouse	45 minutes Bilateral	24h	Kidney	Apoptosis	miR-21 suppresses expression of PDCD4 and exerts functional protection	miR-21	
Wang et al. Med Sci Monit 2014 (180)	Rat	45 minutes Bilateral	12h	Plasma	Biomarkers of AKI	miR-10a, miR-192, and miR-194 are promising biomarkers of AKI	miR-10a miR-192 miR-194	
Bellinger et al. PloS One 2014 (181)	Mouse	27 minutes Bilateral	3h, 6h, 24h	Kidney Plasma	Profiling	5 miRS elevated during IRI	miR-714 miR-1188 miR-1897-3p miR-877 miR-1224	
Bhatt et al. JASN 2015 (182)	Mouse	30 minutes Bilateral	12-48h	Kidney	Mechanisms of IRI	miR-687 induced by HIF-1 targets Phosphatase and Tensin	miR-687	
Liang et al. Mol Immunol 2015 (183)	Mouse	28 minutes Bilateral	24h	Kidney	Effect of miR-26a on renal IRI	miR-26a attenuates IRI and modulates regulatory T-cells	miR-26a	
Wang et al. Life Sci 2015 (184)	Mouse	60 minutes Bilateral	6h	Kidney	Autophagy	HIF-1α, miR-20a-5p and ATG16l1 linked in autophagy during IRI	miR-20a-5p	
Liu et al. Exp Cell Res 2015 (185)	Rat	30 minutes Bilateral	24h	Kidney	Autophagy	miR-21 inhibits autophagy via targeting Rab11a	miR-21	
Liu et al. Am J Nephrol 2015 (186)	Mouse	35 minutes Unilateral	1d, 3d, 7d	Kidney	Autophagy	miR-34a suppresses autophagy in IRI	miR-34a	

Table 1.2: Summary of Kidney IRI microRNA *In Vivo* studies

Summary of all 18 *In Vivo* studies investigating microRNAs in Kidney IRI

The first of these studies by Wei et al (171) involved the use of Dicer, an essential enzyme that cleaves precursor microRNAs to mature and functional microRNAs. They used a mouse model of 30 minutes bilateral renal IRI in which Dicer was knocked out within the PTCs. They reported that these Dicer knockout mice

were protected from IRI (improved renal function and reduced tissue damage) compared with controls. A microarray analysis of 371 microRNAs identified 173 microRNAs, which were detectable in the renal cortex tissues. Of these, 13 microRNAs were up regulated and 10 down regulated (171).

Godwin et al showed that 30 minutes unilateral renal IRI in a mouse has a unique microRNA expression profile, with 9 differentially expressed microRNAs. Moreover, this expression pattern did not change in immuno-deficient mice (lacking natural killer (NK) cells, B cells, and T cells) signifying that these changes in microRNA expression in kidney IRI were lymphocyte infiltration independent (32). Further mathematical and statistical analysis using principal component analyses by the same group revealed that the pattern of microRNA expression had a "distinct direction based on the trajectory of the first three principal components" (187). *In vitro* experiments revealed that knockdown of miR-21 in tubular epithelial cells increased cell death and apoptosis, suggesting a protective role of miR-21 in preventing tubular injury (32). Saikumar et al showed that in a rat model of 30 minutes bilateral IRI (and gentamicin-induced AKI), 3 microRNAs were significantly up regulated and from downstream target analysis identified their mRNA targets to include proteins involved in apoptosis and cell proliferation (172). Lorenzen et al demonstrated both *in vitro* and *in vivo* that miR-24 caused kidney injury via stimulation of apoptosis, and that its inhibition significantly improved survival and function (177).

One study investigated the role of microRNAs in renal angiogenesis induced by IRI in mice, and showed that 76 microRNAs were differentially expressed in IRI

compared with sham (36 up regulated and 40 down regulated), of which miR-210 up regulation was confirmed by RT-qPCR with *in vitro* demonstration of its role in regulation of angiogenesis via targeting the VEGF pathway (173). Another study showed that miR-126 within the haemopoetic compartment was protective against renal IRI via preservation of components of micro vascular integrity (178).

Xu et al demonstrated that a 15-minute localised IPC attenuated renal IRI induced 4 days later in mice, known as delayed IPC. Moreover an anti-miR-21 caused tubular cell apoptosis by a significant up regulation of pro-apoptotic gene programmed cell death protein 4 (PDCD4), concluding that up regulation of miR-21 contributed to the protective effects of this delayed IPC (100). The importance of miR-21 up regulation in preconditioning was supported by another study, which investigated the effect of xenon preconditioning in a mouse model of renal IRI. Xenon preconditioning stimulus given 24 hours before the onset of IRI significantly reduced injury (morphologically and functionally) through up regulation of miR-21 (175). MiR-21 was found to be protective in another model of murine renal IRI, through its suppression of PDCD4 gene expression and caspase signalling components (179).

Wang et al showed that urinary miR-10a and miR-30d were highly sensitive markers of AKI in a mouse model of renal IRI (174). Kaucsar et al used a Luminex microRNA panel (consisting of 46 microRNAs) in a mouse model of 30 minutes renal IRI, and showed that 5 microRNAs were differentially expressed in IRI, and

in particular miR-21, -17-5p, and -106 were all activated during the maintenance and recovery phases of IRI (176).

One study used a bilateral renal IRI rat model and performed a microarray analysis, identifying 36 aberrantly expressed microRNAs in IRI, of which miR-10a, -192, and -194 were detected in plasma and deemed as potentially useful biomarkers of AKI in this setting (180). Bellinger et al identified 5 microRNAs within the kidney and plasma, that were progressively and concordantly elevated in mice that underwent precisely 27 minutes renal IRI (181).

Bhatt et al showed that miR-687 was important in renal IRI, and in the context of mouse renal IRI its induction was mediated via HIF-1. Further *in vitro* experiments revealed a signalling pathway involving HIF-1, miR-687 and phosphatase and tensin homolog (PTEN) (182). Liang et al used a lentivirus-pre-miR-26a vector to demonstrate that miR-26a attenuated renal IRI in mice via modulation of T regulatory cells (183). Recent studies have focused on autophagy within renal IRI, and have identified miR-20a-5p (184), miR-21 (185), and miR-34a (186) as important in the mechanisms of autophagy in the context of renal IRI.

In summary, several microRNAs have been implicated in IRI, with some overlap between studies. In particular miR-21 seems to be functionally important in kidney IRI, with its particular focus on apoptosis.

1.6.6.2 MicroRNAs in Acute Rejection

Monitoring allograft function post-operatively is important, since failure to treat a potentially reversible and treatable cause such as acute rejection can result in graft loss. Several studies have evaluated the microRNA signature within acute and chronic rejection.

Sui et al (188) identified unique expression patterns of 20 different microRNAs in acute rejection biopsies (8 up-regulated and 12 down-regulated), but this was limited due to a total number of only three patients with acute rejection. Anglicheau et al (189) identified several miRNAs associated with acute rejection, and that the levels of miR-142-5p, miR-155, miR-233, miR-10b, miR-30a-3p and let-7c were highly sensitive and predictive of rejection (>90% sensitivity and specificity). Lorenzen et al (190) analysed urinary miRNAs as non-invasive biomarkers for predicting rejection and reported that miR-10a was up regulated, with miR-10b and miR-210 down-regulated, in patients with acute rejection. Moreover, the expression patterns of miR-210 were able to discriminate between non-treated and treated acute rejection (190). Wilflingseder et al demonstrated a unique microRNA expression profile discriminating between acute cellular rejection, antibody mediated rejection, DGF and controls with no graft injury (191).

Danger et al investigated the microRNA expression profile of patients with chronic antibody mediated rejection (CAMR) compared with those with stable graft function, and found that 10 microRNAs were associated with CAMR, in particular miR-142-5p (192). Another study reported elevated expression levels

of miR-142-5p, miR-142-3p, miR-155, and miR-223 in biopsies of patients with acute T-cell mediated rejection compared with normal biopsies. Moreover, they showed that miR-142-3p and miR-223 expression levels within peripheral blood mononuclear cells were discriminative between patients with acute cellular rejection and those with no rejection (193). Tao et al investigated the microRNA expression profile within serum of patients with rejection and concluded that miR-99a was predictive of rejection (194). A recent study reported that the combined measurement of 5 microRNAs (miR-15b, -16, -103a, -106a, and -107) in blood was able to discriminate between patients with severe T-cell mediated vascular rejection and those with stable grafts (195).

In summary, several studies have shown differential expression of different microRNAs implicated in rejection of the kidney allograft, with the differences reflective of the variability of patients and techniques used for profiling.

1.6.6.3 MicroRNAs in Chronic allograft dysfunction and Fibrosis

Predicting long-term function or diagnosing chronic allograft dysfunction (CAD) with interstitial fibrosis (IF) is important in the management of a kidney transplant patient. Several studies have looked at microRNAs as biomarkers of CAD and IF. The first of these by Scian et al confirmed the differential expression of 5 microRNAs from tissue biopsies of patients with CAD/IF compared to those with normal allografts. They also confirmed differential expression of 3 of these microRNAs (miR-142-3p, miR-204, and miR-211) within urine samples (196). The same group performed a much larger study evaluating 191 samples from 125 kidney transplant patients identifying 22 differentially expressed

microRNAs in patients with CAD/IF (197). Another study also identified a differential expression of microRNAs between patients with tubulointerstitial fibrosis, including up regulation of miR-21 and miR-142-3p, and down regulation of miR-30b and mIR-30c (198). Increased miR-21 has been shown to be important in kidney fibrosis including allograft fibrosis (199, 200).

1.7 Summary and Aims of Thesis

In summary, IRI is a complex pathological process that involves multiple interactions between the endothelium, components of the immune system, cell death programs, and genetic reprogramming. IRI has serious clinical consequences for kidney transplantation and methods to reduce IRI are a hot topic of research. One such method, IPC, has shown variable benefit but its underlying mechanisms are poorly understood. MicroRNAs are essential post-transcriptional regulators of gene expression that are involved in multiple disease processes including IRI. With this background in mind, the objectives of my thesis were:

1. To test the efficacy of IPC in IRI protection *In Vivo*
2. To study the potential role of microRNAs in IPC
3. To investigate the utility of microRNAs as IRI biomarkers in the context of kidney transplantation

Chapter 2 - Methodology

2.1 Animal Experiments

In Vivo studies have been extensively used to investigate the mechanisms of IRI. Various different animal models have been well documented in the literature. I used a well-established animal model of IRI, which had also been successfully used by a recent MD student, Mr Prabhu Nesargikar.

2.1.1 Lewis Rats

Adult (8 to 12 week old) male Lewis rats weighing 180 to 220 g were used in this project. They were inbred and supplied by Harlan Laboratories Ltd. (Derby, UK). Lewis rats possess various features that make them ideal for this work. They are well-established *in vivo* models for biological studies, including the studies carried out by a recent MD student. Lewis rats are 'commercially viable' for such projects and protocols for maintaining their lifestyle requirements are well established and easy to meet by the Joint Biological Services Unit (JBIOS) staff at the Heath campus, Cardiff University. Lewis rats are also very friendly animals, inquisitive in nature, easy to handle, and quite playful. These characteristics are a useful aid in determining their state of health pre and post-operatively.

2.1.2 Preoperative procedure

The rats were delivered 7 days before the experiments, allowing for acclimatisation to their new surroundings. Upon arrival, the rats were checked by the JBIOS staff (Heath Campus) and housed in the conventional holding room in cages (maximum 4 rats per cage). The rats were provided with drinking water, rat chow and sunflower seeds. Twenty-four hours before surgery, the rats were housed in individual cages and provided with analgesia (200μg of

buprenorphine crushed and dissolved in 500ml of drinking water), as recommended by the Named Animal Care and Welfare Officer (NAWCO). Buprenorphine was recommended as it had been shown to provide effective analgesia with minimal side effects (201, 202). They continued to be housed individually with access to analgesia (within their drinking water) until kidney retrieval at 48 hours post surgery.

2.1.3 Operative procedure

Housing, handling, and experimental procedures were carried out in accordance with the local institutional policies and procedures of JBIOS at Cardiff University, licensed by the UK Home Office under the Animals (Scientific Procedures) Act (1986). The study was carried out under the Home Office Project Licences PPL 30/2506 (Chapter 3 experiments) and PPL 30/3098 (Chapter 4 experiments), held by Mr R Chavez, Supervisor for this project. He and I both also held personal licences for this project. The methodology to carry out the procedures was adapted from previous literature, experience of the supervisor (Mr Chavez), and the previous MD student.

2.1.3.1 Operative Theatre and Setup

The operative theatre setup was based on advice received from the NAWCO with input from the Veterinary Officer and the Home Office Laboratory Animal Science Association (LASA) guidelines. The emphasis was on aseptic technique. Surgery was carried out in the operative procedures room, located next to the conventional holding room. The operating surgeons (myself and Mr Chavez) used sterile gloves and gowns. The rat skin was shaved using a hair clipper

(Contura Chrome AC 220-240V, UK) followed by clean scrub of the skin with chlorhexidine spray prior to the skin incision. An overhead lamp was used for lighting and providing adequate heat to maintain body temperature of the rat. The anaesthetic induction chamber, attached to the ventilator machine, was on the main operating table, reducing transfer times for the rats to the operating area. The operating board was made of a corkboard covered in a sterile drape, enabling the use of pins attached to rubber bands for retraction of the limbs. Standard surgical instruments (including a 10' disposable scalpel, mcindoe scissors, non-toothed and toothed forceps and needle holder) were used for the procedure, in addition to micro vascular clips to clamp the renal pedicles. Normal saline was used to keep the peritoneal cavity hydrated. Vicryl sutures (4-0 size) were used to suture the abdominal cavity and skin close. Prior to each procedure, the instruments were deep cleaned with standard foam soap and water followed by autoclave for optimum sterility.

2.1.3.2 Anaesthesia

Animals were anesthetised with isoflurane (IVAX Pharmaceuticals, UK) delivered via oxygen. Induction anaesthesia was administered within the induction glass chamber with 5% isoflurane delivered via 2 l/min of oxygen (Figure 2.1(b)). This allowed the rat to achieve a state of deep anaesthesia within 3-5mins. The rat was then moved from the induction chamber to the operating board, where the mouth and nose of the rat were placed in a conical rodent facemask designed to deliver the gas mixture (Figure 2.1(c)). Maintenance anaesthesia was delivered via this facemask using 2% of isoflurane via 1-2 l/min of oxygen, with alteration of the rate as required according to the heart rate, breathing pattern and general

appearance of the rat. At the end of the procedure, this anaesthesia delivery was turned off and the rat was moved to its cage to recover**.**

2.1.3.3 Operative technique (Chapter 3 experiments)

Following anaesthesia, the rat was weighed and the rat skin cleaned with chlorhexidine spray and shaved with the electric razor. The rat was positioned on the operating board and limbs retracted using rubber bands and pins secured on the operating board. A midline laparotomy incision was made using a 10' scalpel and the rectus sheath and peritoneum opened with mcindoe scissors. The abdominal wall was then retracted using paper clips designed as retractors as seen in Figure 2.1. The bowel was moved to the right side and the left renal pedicle was identified and clamped for 45mins using a vascular clip (IRI group). The kidney was visually assessed for both ischemia upon clamping and reperfusion upon release of the clamp. The kidneys are usually pink in colour and upon clamping they become purple/dark brown, confirming ischaemia. Following the clamp the wound was covered with saline soaked gauze. The kidney was frequently observed for its colour. The rat was monitored throughout, with particular emphasis on its heart rate, breathing pattern, and colour, allowing for adjustment of the anaesthesia as needed. Following 45mins of ischaemia, the vascular clip was removed to allow reperfusion (confirmed by the return of the kidney's colour to pink). The laparotomy wound was then closed with a continuous 4-0 vicryl suture to the rectus sheath/muscle layer and the skin was closed with a continuous 4-0 vicryl suture with the knots carefully buried so as to avoid any biting/chewing by the rat post-operatively (Figure 2.1(i)). The rat was then transferred to its cage, which was placed over a heating

pad, to allow recovery. Once it was mobile and comfortable, the cage was moved back to the conventional holding room.

Rats in the Sham group underwent the same operation without renal pedicle clamping. Animals in the IPC/IRI group underwent 15mins of left renal pedicle clamping followed by 20mins of reperfusion before the IRI.

Post-operatively, the rat was checked at the end of the day, and on 2 separate occasions the next day. The wound was reviewed to look for and document any signs of infection or dehiscence. Adequate breathing pattern, general movement at ease (without discomfort or limping) within the cage, urine and normal solid faeces production, and general appearance of the skin and its texture, were all signs of satisfactory post-operative recovery and behaviour. All of these checks were documented carefully in the notes.

At 48h, the rat underwent 'terminal' anaesthesia. This involved the same induction and maintenance, followed by exsanguination. Exsanguination was performed as follows. The laparotomy was re-opened and extended superiorly towards the sternum. The sternum was retracted with a clip to allow good access to the diaphragm. The diaphragm was opened and the heart was resected with the scissors to allow exsanguination. Following this, the left kidney was retrieved.

2.1.3.4 Operative technique (Chapter 4 experiments)

Following anaesthesia, a midline laparotomy incision was made, and the renal

pedicles were identified. Both renal pedicles were identified and clamped for 45mins using vascular clips (IRI group) (Figure 2.1(d)). The kidneys were visually assessed for both ischemia (change of colour to purple) upon clamping (Figure 2.1(e)-(f)) and reperfusion (return of colour to pink) upon release of the clamp (Figure 2.1(g)-(h)). Rats in the Sham group underwent the same operation without renal pedicle clamping. During the procedure, the abdominal cavity was covered with saline-soaked gauze. The kidneys were retrieved 48h after terminal anaesthesia. Terminal anaesthesia involved same induction and maintenance, followed by exsanguination as described before, prior to retrieval of the kidneys. Direct Kill animals involved terminal anaesthesia, midline laparotomy, exsanguination and retrieval of kidneys.

Experiments were also performed on 6 different groups of IPC/IRI animals: 3 were continuous and 3 pulsatile. The 3 continuous IPC regimes that were tested were as follows: (a) 10mins of ischaemia followed by 20mins of reperfusion (IPC-C 10-20) (n=4); (b) 15mins of ischaemia followed by 20mins of reperfusion (IPC-C 15-20) (n=4); and (c) 20mins of ischaemia followed by 20mins of reperfusion (IPC-C 20-20) (n=4) (Figure 2.2). The 3 pulsatile IPC regimes that were tested were as follows: (a) 3 cycles of 2mins ischaemia and 5mins reperfusion (IPC-P 2-5) (n=4); (b) 3 cycles of 5mins ischaemia and 5mins reperfusion (IPC-P 5-5) (n=4); and (c) 3 cycles of 10mins ischaemia and 5mins reperfusion (IPC-P 10-5) (n=4) (Figure 2.3). One of these regimes with the most favourable results (in terms of protection against injury) was chosen (IPC-P 2-5), and a further 4 animals underwent surgery to complete n of 8. Each of these regimes was performed prior to IRI. In addition to this, an extra group of animals

underwent IPC alone (3 cycles of 2mins ischaemia and 5mins reperfusion) without any subsequent IRI (n=8). Post-operative checks were the same as for Chapter 3 experiments as described in section 2.1.3.3.

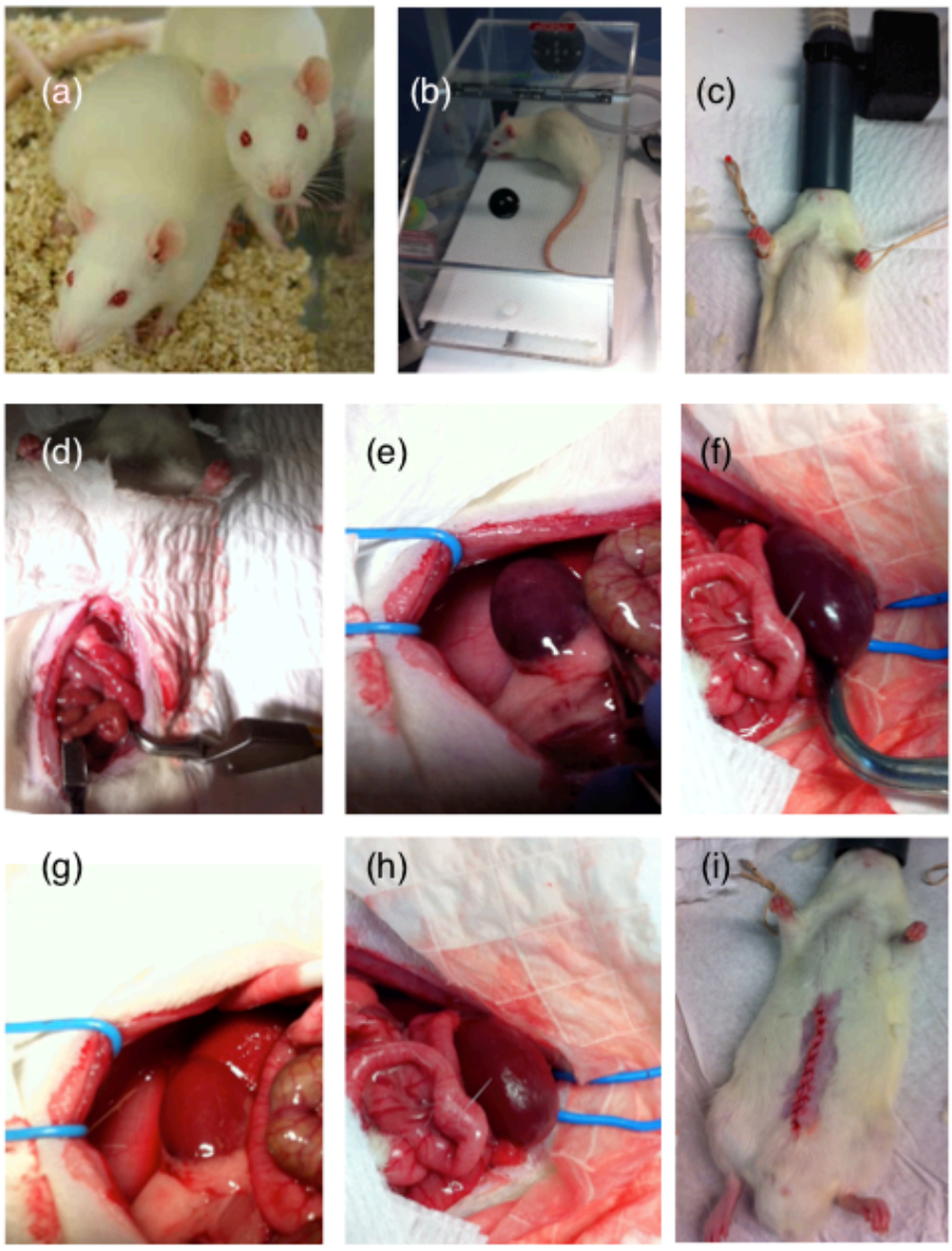

Figure 2.1: Photographic representation of the animal model of bilateral IRI

(a) Adult male Lewis rats (8-12 weeks old, approximately 200g in weight) were anaesthetised with isoflurane in a glass induction chamber (b) and transferred to the operating table for maintenance anaesthesia via a conical facemask (c). A midline laparotomy was performed and access gained to the peritoneal cavity, both kidneys were identified and the renal pedicles clamped with vascular clips (d). Upon clamping the kidneys became purple in colour ((e) and (f) representing clamped right and left

kidney respectively) confirming ischaemia. After 45mins of ischaemia the clamps were removed to allow reperfusion. The kidneys' colour returned to pink upon release of the clamp ((g) and (h) representing reperfused right and left kidney respectively). Following this, the rectus sheath/muscle layer and the skin were closed in 2 layers with 4-0 vicryl with the knot buried so as to prevent any biting/chewing by the rat post-operatively (i).

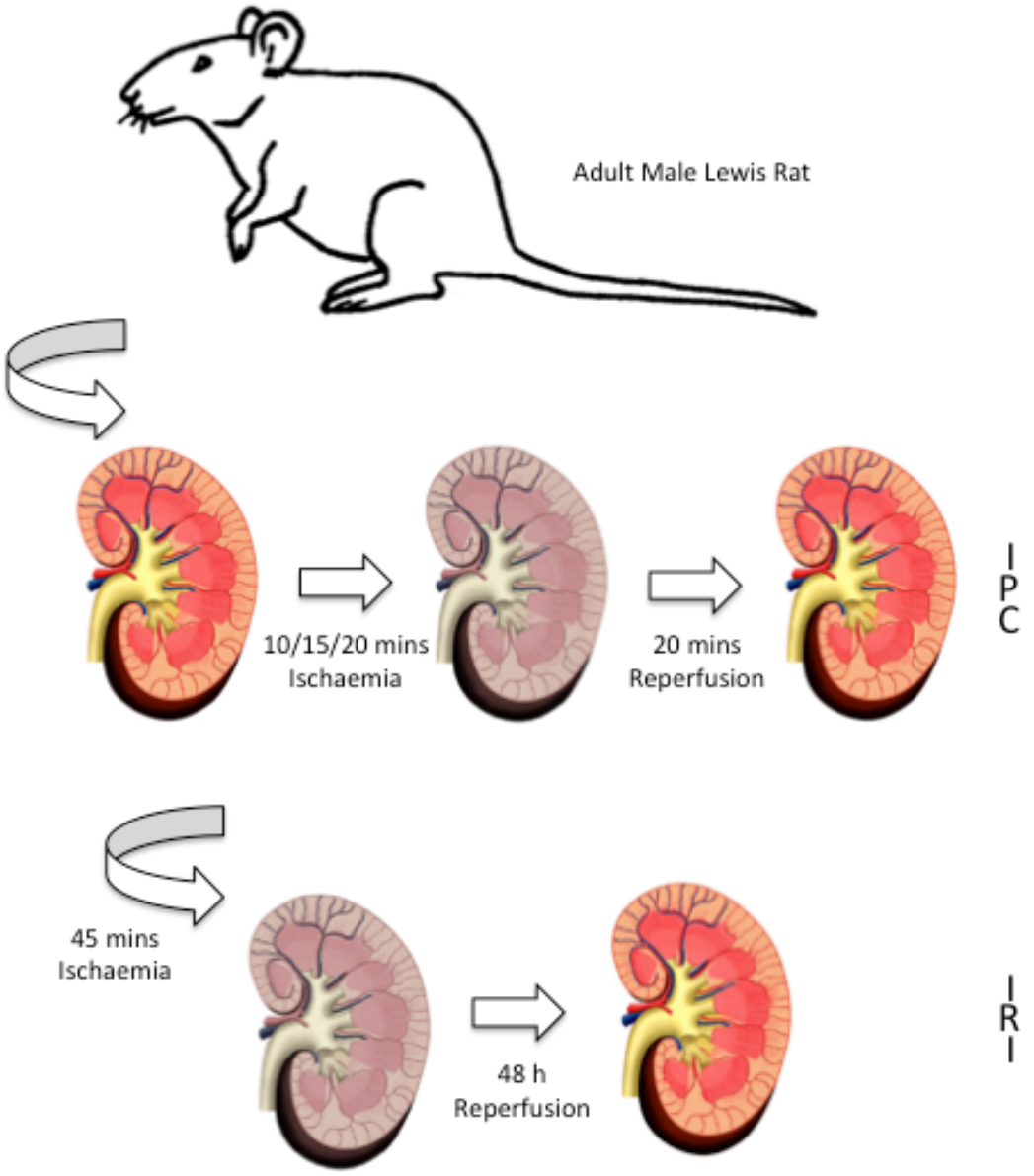

Figure 2.2: Continuous IPC regimes

Continuous IPC regimes as performed for Chapter 4 experiments

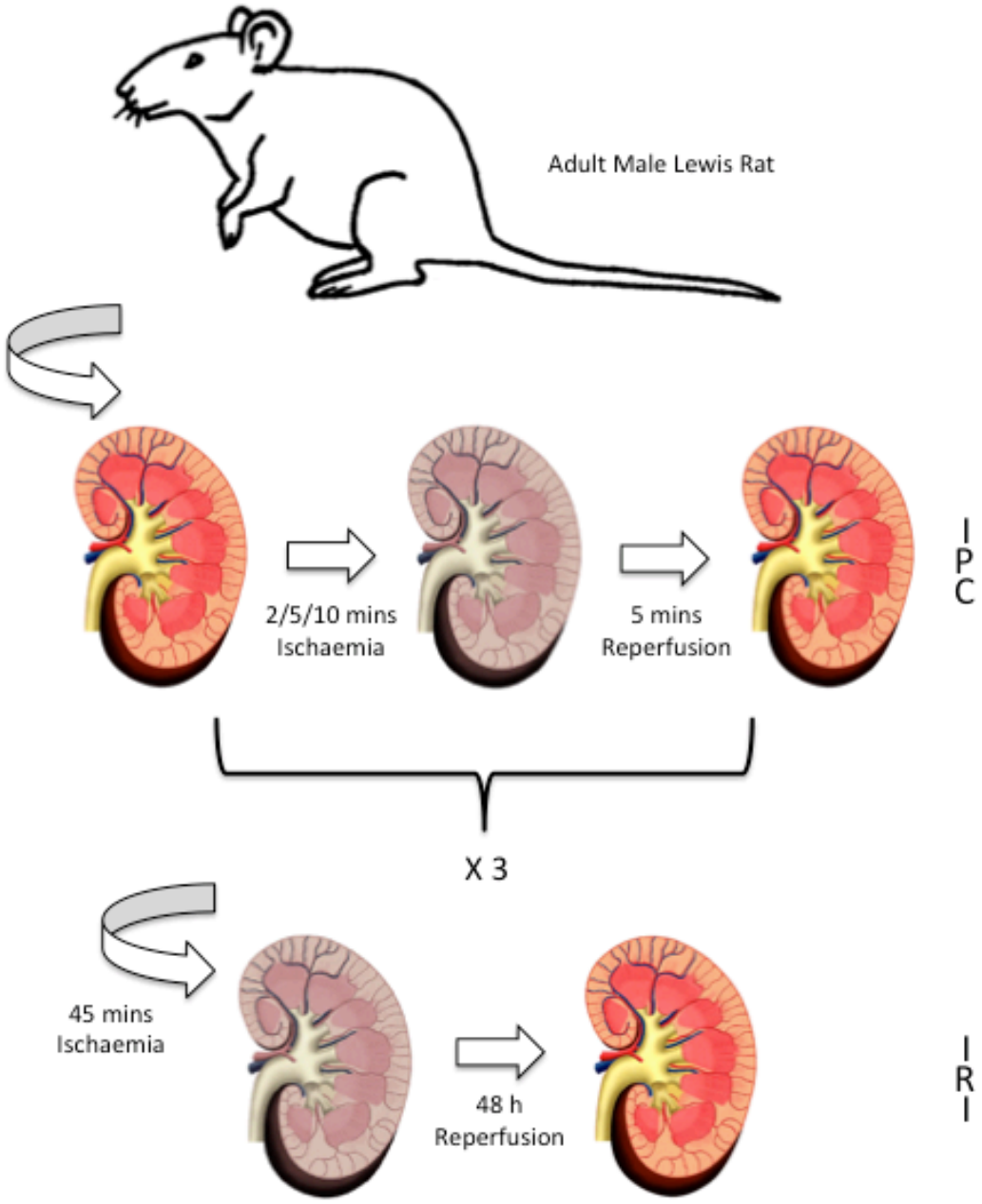

Figure 2.3: Pulsatile IPC regimes

Pulsatile IPC regimes as performed for Chapter 4 experiments

2.1.4 Blood collection

Up to 500ul of blood was collected at 2 time points from the animals in Chapter 4 experiments: pre-operatively at 0h (from the tail tip), and at time of retrieval (48h) (from the heart or central abdominal blood vessels). Pre-operatively a

small ‘incision’ was made on the rat-tail and blood ‘milked’ out from it. At 48h a syringe and needle was used to withdraw blood from the heart or central abdominal vessels. Blood was collected in small eppendorfs and delivered to the biochemistry lab immediately for serum creatinine analysis. Blood volume was calculated according to the guidelines published by The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3RS) (203). On average, rats have 64ml of blood per kg of bodyweight, and it is recommended that maximum blood volume that can be taken is <10% of total blood volume on any single occasion and <15% total blood volume in 28 days. Therefore, a rat that weighs 200g would have a total blood volume of 12.8 ml (64 x 0.2 = 12.8), allowing for a maximum of 1.28ml to be taken at any given time point. Therefore 500 ul (=0.5ml) was far below the maximum volume that could be taken.

2.1.5 Kidney retrieval and storage

Kidneys were retrieved at 48h. Upon retrieval, each kidney was cut using a scalpel into 3 pieces: 1 half and 2 quarters. The ‘half’ kidney was stored in formalin and sent to histopathology laboratory for paraffin wax embedding and sectioning, before being stained with haematoxylin and eosin. One ‘quarter’ kidney was placed immediately into an eppendorf of RNA later solution and stored at -80°C for RNA extraction at a later date. One ‘quarter’ kidney was ‘snap’ frozen in liquid nitrogen and stored within an eppendorf at -80°C for protein analysis at a later date.

2.1.6 Disposal of animal

Following terminal anaesthesia, exsanguination, and retrieval of kidneys, the animal carcasses were placed in a clinical waste bag and discarded into the animal carcass freezer.

2.1.7 Identification coding protocol and logbook

A unique coding protocol was employed to distinguish between the animals. A logbook was kept of all the animal experiments carried out, with full pre-, intra-, and post-operative details recorded. Coding protocol utilised the date of the experiment followed by a number signifying the order of animal experiments on that day, for example, the first animal on 5th January 2015 would be coded as 0501201501, and the 3rd would be coded as 0501201503. A unique mark was also placed on the tail of each rat using a permanent marker pen for identification purposes during the 48h observation period. In addition to this, a card was attached to each cage showing the rat identification details, operative procedure details, project licence number, my personal licence number, and my mobile phone number (in case of any emergency).

Each rat kidney or blood sample was also labelled, with chapter 3 rats labelled as 101 onwards, and chapter 4 rats labelled as 201 onwards. For the kidney samples, a prefix of R or L (R for Right kidney, L for Left kidney) was used. These codes were subsequently used for H&E, proteomics and RNA studies.

2.2 Histology

2.2.1 Haematoxylin & Eosin staining

Rat kidney tissue was embedded in paraffin, sectioned, and stained with haematoxylin and eosin. This was done by the department of pathology at University Hospital of Wales (Cardiff) according to their standard operating procedure.

2.2.2 Assessment of damage – The EGTI Histology damage score

All H&E slides were scored under blinded assessment by one histopathologist (Dr Gilda Pino-Chavez) according to the system detailed in Table 2.1. This comprehensive scoring system has been adapted specifically for animal research on kidney tissue in the context of injury and provides a quantitative measurement of the histological damage of the kidney. The widely adopted 'gold standard' scoring system for reporting histological damage in kidney injury is the Jablonski scoring system (204). This simple system is designed to give a rapid and objective assessment of kidney injury. In reality, however, it provides a limited score documenting predominantly the degree of necrosis within tubular cells only, ranging from 0 signifying 'no damage', to 4 'necrosis affecting all 3 segments of the proximal convoluted tubule'. It does not provide an assessment of the damage seen within the other cellular components, such as endothelial and glomerular cells. The Jablonski scoring system was originally described following a prolonged recovery period after ischaemic injury, and includes features of recovery (e.g. tubular cell mitoses) that may not be evident in the typical recovery periods used experimentally, such as in these experiments.

Therefore a more detailed scoring system was designed that included damage seen within the renal cortex to endothelial (E), glomerular (G), tubular (T), and interstitial (I) cellular compartments. This scoring system was devised based on literature evidence (35, 205-209) and has been validated in chapter 4 experiments, as described in section 4.2.1 and Figure 4.1.

Tissue type	Damage	Score
Tubular	No damage	0
	Loss of Brush Border (BB) in less than 25% of tubular cells. Integrity of basal membrane.	1
	Loss of BB in more than 25% of tubular cells, Thickened basal membrane	2
	(Plus) Inflammation, Cast formation, Necrosis up to 60% of tubular cells	3
	(Plus) Necrosis in more than 60% of tubular cells	4
Endothelial	No damage	0
	Endothelial swelling	1
	Endothelial disruption	2
	Endothelial loss	3
Glomerular	No damage	0
	Thickening of Bowman capsule	1
	Retraction of glomerular tuft	2
	Glomerular fibrosis	3
Tubulo/ Interstitial	No damage	0
	Inflammation, haemorrhage in less than 25% of tissue	1
	(Plus) necrosis in less than 25% of tissue	2
	Necrosis up to 60%	3
	Necrosis more than 60%	4

Table 2.1: The EGTI Histology scoring system

The EGTI histology soring system has been designed to provide a comprehensive assessment of the degree of architectural damage seen within the kidney cortex, with a cumulative score

ranging from 0 to 14. Median scores were used for statistical analyses.

2.2.3 Laser capture micro dissection of kidney tissue samples

For chapter 4 experiments, 12 paraffin embedded kidney blocks (Sham, IRI, IPC/IRI, and IPC alone groups each with n=3) were used to isolate glomeruli, proximal convoluted tubuli, distal convoluted tubuli, and vessel tissue (including endothelial cells), using the Arcturus Pixcell IIe infrared laser enabled laser capture micro dissection (LCM) system (Applied Biosystems). For each kidney block, two 6-µm sections were obtained. These were prepared by Mr Dilwyn Havard (senior pathology technician), at the department of pathology (University Hospital of Wales, Cardiff). The tissues were cut, placed in the middle third of an uncharged, uncoated glass slide (VFM White coat slides CellPath Ltd) and stained according to the method described by Espina et al (210). Using infrared laser, the target tissue was bonded to a polymer membrane located on a cap (Arcturus ® Capsure ® Macro LCM caps – Applied Biosystems) placed onto the slide which when lifted removed the highlighted/selected tissue. To differentiate between the different tissue types (in particular to differentiate between proximal and distal convoluted tubuli), these experiments were done with the help and expert guidance of Dr Gilda Pino-Chavez, an experienced histopathologist.

2.3 Patient Urine samples

2.3.1 Wales Kidney Research Tissue Bank consent

All patients recruited into the Chapter 5 experiments were consented into the Wales Kidney Research Tissue Bank (WKRTB) prior to their transplantation using the WKRTB consent form. Upon admission for their kidney transplantation, patients were given information sheets explaining the role of WKRTB and the purpose of the study prior to a written informed consent. The WKRTB consent form used can be found in Appendix 1.

2.3.2 Collection of urine samples

The urine samples were then collected from new kidney transplant patients who had provided written informed consent into the WKRTB from day 1 up to and including day 7. The samples were collected in a 20ml sterile universal container. Following a kidney transplant, most patients had a urinary catheter in situ for at least 5 days post-operatively, and therefore samples were collected from this using a sterile technique. After the catheter was removed, patients were asked to provide mid-stream samples into the universal containers. In patients who had passed very little urine, a smaller volume was collected from the urinary catheter. In total 33 patients were recruited into the Chapter 5 study. Samples were also collected from 10 healthy volunteers, as baseline controls.

2.3.3 Processing and storage

Upon collection of the urine samples in Universal containers, they were centrifuged at 2000*g* for 10mins at 4°C in order to remove any living cells. The

supernatant was then divided into 3 aliquots of 350µl each and stored at -80°C until RNA extraction. The remainder of the urine was stored in a fresh sterile universal container and stored at -80°C.

2.3.4 Clinical Data Collection

Demographic data were collected on the donors (age, gender, type of donor (living, DBD or DCD), cause of death for deceased donors, cold ischaemic times) and recipients (age, gender, cause of renal failure, dialysis status), Human Leucocyte Antigen (HLA) mismatch, duration of hospital stay, and estimated glomerular filtration rate (eGFR) at 3, 6, 9, 12 and 24 months. This data was collected from clinical patient databases ('Vital Data' and Clinical Portal), hospital admission notes, operation notes, observation and urine charts, and Human Tissue Authority (HTA) retrieval forms.

2.4 RNA Analysis

All general reagents were purchased from Sigma-Aldrich Corp. (Poole, UK), Life Technologies, Inc. (Paisley, UK), or New England Biolabs (Ipswich, MA, USA), unless otherwise stated. Oligonucleotides were purchased from Life Technologies. RNA was extracted from whole kidney tissue, LCM tissue and urine samples, and then analysed with RT-qPCR for microRNAs or mRNAs.

2.4.1 RNA Extraction

2.4.1.1 Tissue RNA extraction

Whole kidney tissue was homogenised following the addition of TRI-zol reagent (Life Technologies), with 1ml of TRI-zol reagent per 50-100mg of tissue. Following homogenisation, the solution was left for 5mins to allow total cell lysis. 1ml of the resulting solution was used for the next step, with the rest of the solution stored at -80°C for use in the future. 0.2ml of chloroform was added to 1ml of homogenised solution. After mixing by inversion, samples were incubated for 3mins at room temperature, then centrifuged at 12000rpm for 15mins at 4°C. The colourless upper aqueous phase (containing the RNA) was transferred to a clean micro centrifuge tube and the lower phase (containing DNA and protein) discarded. RNA precipitation was achieved by addition of 0.5ml isopropanol to each sample, and incubated for 10mins at room temperature. The samples were then briefly vortexed and centrifuged at 12000rpm for 10mins at 4°C to form the RNA pellet. The supernatant from the tube was then pipetted out leaving only the RNA pellet at the bottom of the tube. The RNA pellet was then washed thrice with 1ml of ethanol (75%) and centrifuged at 7500rpm for 5mins at 4°C

following each wash. Ethanol was then removed and pellet allowed to air dry for 15-30mins before re-suspension in 50μl of nuclease-free water.

2.4.1.2 Determination of Tissue RNA purity, concentration and quality

RNA concentration was determined using the Nano drop Spectrophotometer (Thermo Fischer Scientific), at the Henry Wellcome Building, Cardiff. Equal volume (1μl) of sample was placed on Nano drop reader and absorbance measured at 230nm, 260nm and 280nm. A 260/280 ratio of above 1.8 was indicative of a sufficiently pure sample in terms of its purity for RNA. A second ratio 260/230 was calculated to determine the purity of nucleic acid from potential contaminants like phenol. A 260/230 ration of above 1.8 was indicative of a pure sample. Concentration was calculated as ng/μl. This was done according to the manufacturer's recommendations and with the guidance of previous PhD students and post-docs.

RNA quality was also assessed using the Agilent Technologies 2100 Bio analyser with RNA 6000 Nano chips (Palo Alto, CA, USA). This provided an RNA Integrity Number (RIN), a measure of the quality of RNA, with RIN values above 8 deemed as high quality RNA suitable for microRNA analyses. Appendix 2 and Appendix 3 show the Agilent Bio analyser results for a subset of samples from Chapter 3 and Chapter 4 experiments respectively.

2.4.1.3 RNA extraction from urine samples

A previous PhD student (C Beltrami) had tested several extraction kits for RNA extraction from urine samples and concluded that the Qiagen miRNeasy mini kit

(Qiagen, Cat. No. 217004) was the best method. RNA extraction was performed according to the manufacturer's recommendations with a few minor modifications as recommended by Andreason et al and C Beltrami (211, 212), namely the use of carrier RNA (MS2 RNA, Roche Cat. No. 10165948001) per 750µl of QIAzol reagent. Urine samples from transplant patients and control subjects were processed according to the manufacturer's recommendations. The following modifications were made, according to C Beltrami's PhD work. The urine sample (350µl) was mixed with 750µl of QIAzol plus 1µg of carrier RNA (MS2 RNA, Roache), and the mixture incubated at room temperature for 5mins, after which 0.5ρM of spike in *Caenorhabditis elegans* (cel-miR-39) (Ambion, Cat. No. 4464066, Part No. MC10956) was added to each sample. Subsequently, 200µl of chloroform was added per sample, and samples were incubated at room temperature for 2mins and then spun for 15mins at 4°C. At that point, the manufacturer's protocol was followed, with the entire aqueous phase from each sample loaded onto a single affinity column. RNA extracts were then stored at -80°C until analysis.

2.4.1.4 RNA extraction from LCM tissue samples

The polymer membrane on the LCM caps was removed and placed into an eppendorf. RNA was then extracted using the RecoverALL™ Total Nucleic Acid Kit (Ambion, Cat. No. AM1975) according to the manufacturer's recommendations and protocol, except for 2 modifications: (1) The deparaffinisation step was not carried out, as this had already been performed before the LCM procedure; and (2) 1µg of RNA carrier (MS2 RNA, Roche) was

added during the nucleic acid isolation stage. This modification was advised by 2 recent PhD students (C Carrington and C Beltrami).

2.4.2 Messenger RNA detection

2.4.2.1 Reverse Transcription (RT)

Following total RNA extraction and quantification from the kidney tissue samples, cDNA was generated from 1µg of RNA in each sample, using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Cat. No. 4368814), according to the manufacturer's recommendations. Essentially, 1µg of total RNA in 10µl of water, was added to 10µl of RT master mix. RT master mix consisted of the following:

RT Master mix component	**Volume in µl**
10 x RT Buffer	2
25 x dNTP Mix (100 mM)	0.8
10 x RT Random Primers	2
Multiscribe™ Reverse Transcriptase	1
RNase inhibitor	1
Nuclease-free water	3.2

Table 2.2 Components of RT master mix for mRNA

Each sample was then placed on the thermo cycle with the following thermal profile: 10mins at 25°C, 2h at 37°C, and 5 sec at 85°C, followed by cooling at 4°C. Following this, the cDNA was diluted by adding 60µl of water, and used for qPCR or stored at -20°C for later use.

2.4.2.2 Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) was performed on a 7900-HT Fast Real-Time PCR System (Life Technologies). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), IL-17, interleukin 18 (IL-18), and TNF-α reaction products were quantified using POWER SYBR® Green PCR Master Mix (Life Technologies, Cat. No. 4367659) with 300nM gene-specific primers. The amplification of a single PCR product was confirmed by melting curve analysis. Expression of NGAL, KIM-1, IL-17, IL-18, and TNF-α was normalised to GAPDH. The relative changes in gene expression were analysed by the 2 to the power of minus delta delta cycle threshold ($2^{-\Delta\Delta CT}$) method (213). The primers were designed and provided to me as a kind gift by Dr R Jenkins, using Primer-BLAST, against mRNA sequences taken from the NCBI database, to amplify all known splice-variants. Primers were designed to span intron-exon junctions and PCR product length was ideally around 100-150 base pairs. The nucleotide sequences of the used primer pairs were as follows:

Gene	Forward Primer	Reverse Primer
GAPDH	5'-CCTCTGACTTCAACAGCGACAC-3'	5'-TGTCATACCAGGAAATGAGCTTGA-3'
NGAL	5'-GGGCTGTCCGATGAACTGA-A-3'	5'-CATTGGTCGGTGGGAACAGA-3'
KIM-1	5'CGGCTAACCAGAGTGACTTGT-3'	5'-TACAGAGCCTGGAAGAAGCAG-3'
IL-17	5'-CCATCCATGTGCCTGATGCT-3'	5'-GTTATTGGCCTCGGCGTTTG-3'
IL-18	5'-GACCGAACAGCCAACGAATC-3'	5'-ATAGGGTCACAGCCAGTCCT-3'
TNF-α	5'-ATGGGCTCCCTCTCATCAGT-3'	5'-GCTTGGTGGTTTGCTACGAC-3'

Table 2.3 Forward and Reverse primer sequences for mRNA genes

2.4.3 MicroRNA detection

MicroRNAs were analysed in whole rat kidney tissue samples, LCM tissue samples, and urine samples.

2.4.3.1 Reverse Transcription (RT)

Following RNA extraction, RT was performed to generate cDNA, using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Cat. No. 4368814), according to the manufacturer's recommendations. The RT master mix per reaction consisted of the following:

RT Master mix component	**Volume in µl**
10 x RT Buffer	1.5
25 x dNTP Mix (100 mM)	0.15
MicroRNA-specific 5 x RT-primer	3
Multiscribe™ Reverse Transcriptase	1
RNase inhibitor	0.1
Nuclease-free water	4.25

Table 2.4 Components of RT master mix for microRNAs

To the 10µl of master mix, 5µl of RNA was added (for tissue RNA - the 5µl contained 10ng of total RNA; for urine RNA and LCM tissue samples - 1µl of RNA was added to 4µl water). The RT master mix and RNA mixture was incubated on ice for at least 5mins. The RT non-template negative controls substituted RNA with water to make an equal volume for the reaction. Each sample (containing a volume of 15µl) was then placed on the thermo cycle with the following thermal

profile: 30mins at 16°C, 30mins at 42°C, 5mins at 85°C, followed by cooling at 4°C. Following this, the cDNA was diluted by adding 30µl of water, and used for qPCR or stored at -20°C for later use.

2.4.3.2 Quantitative polymerase chain reaction (qPCR)

For each microRNA analysed, the PCR master mix per sample consisted of:

- 1µl of microRNA-specific set of PCR-primers and Taqman probe (designed and supplied by Applied Biosystems)
- 5µl of water
- 10µl of Taqman Universal Master Mix II with No AmpErase UNG (composed of an optimized solution of themostable DNA polymerase, deoxynucleotides, and the passive reference dye ROX (Applied Biosystems, Cat. No. 4440047)

This made a total of 16µl of microRNA-specific master mix per sample, which was added to 4µl of pre-diluted microRNA-specific cDNA per appropriate well, on an Optical 96-well Fast Plate (Applied bio systems) for qPCR. Water was added for the non-template controls instead of cDNA. The plate was sealed with a MicroAmp Optical Adhesive Film (Applied Biosystems) and qPCR was performed on a ViiA7 Real-Time PCR System (Life Technologies), using the manufacturer's recommended parameters: 10mins at 95°C, followed by 40 cycles of 15secs at 95°C and 1min at 60°C.

Expression of microRNAs was normalised to miR-16 or miR-cel-39. The relative changes in gene expression were analysed by the $2^{-\Delta\Delta CT}$ method (213). The

Taqman assays used in this study are shown in Table 2.5 below. The catalogue number for each of these assays was 4427975 (Life Technologies).

MicroRNA assay	**MicroRNA Assay ID**
hsa-miR-9	000583
hsa-miR-10a	000387
hsa-miR-16	000391
hsa-miR-21	000397
hsa-miR-29a	000412
hsa-miR-191	002299
hsa-miR-221	000524
hsa-miR-222	002276
hsa-miR-375	000564
hsa-miR-429	001024
hsa-miR-506	001050
hsa-miR-574-3p	002349
hsa-miR-cel-39	000200

Table 2.5 Taqman microRNA assays

2.5 MicroRNA profiling

2.5.1 Urine Taqman Low Density Array (TLDA)

Taqman low density array (TLDA) was performed to profile microRNAs in urine samples from transplant patients (Chapter 5). The Megaplex RT Primers Human Pool A v.2.1 which consists of 381 RT primers (377 unique microRNAs and 4 controls) was used to perform the reverse transcription for microRNAs for 8 RNA samples (LD-No DGF (n=4), CD-DGF (n=4)) followed by a pre-amplification step using Megaplex PreAmp primers, according to the manufacturer's recommendations (Life technologies).

For each RT reaction, a fixed volume of 3µl of RNA solution (from the 50 µl of RNA solution) was used. The RT reaction was performed according to the manufacturer's recommendations (0.8µl of Pooled Primers combined with 0.2µl of 100mmol/L dNTPs with dTTP, 0.8µl of 10x Reverse-Transcription Buffer, 0.9µl of MgCl2 (25mmol/L), and 1.5µl of Multiscribe Reverse Transcriptase (50U/µl) and 0.1µl of RNAsin (20U/µl). The RT reaction was done using a 7900HT thermo cycle (Applied Biosystems) with the following cycle parameters: 16°C for 2mins, 42°C for 1min and 50°C for 1sec for 40 cycles followed by incubation at 85°C for 5mins. The RT reaction products were then amplified using the Megaplex PreAmp Primers (Primers A v2.1). A 2.5µl aliquot of the RT product was combined with 12.5µl of Pre-amplification Master mix (2x) and 2.5µl of Megaplex PreAmp Primers (10x). The pre-amplification reaction was performed under the following parameters: 95°C for 10mins, 55°C for 2mins and 72°C for 2mins, followed by 12 cycles of 95°C for 15secs and 60°C for 4mins.

Finally, samples were heated at 99.9°C for 10mins to ensure enzyme inactivation. Pre-amplification reaction products were diluted to a final volume of 100µl.

A TLDA Human MicroRNA Panel Card A v.2.0 was used to quantify and determine the expression profiles of 377 unique microRNAs for these samples using a 7900-HT Fast Real-Time PCR System, according to the manufacturer's recommendations (Life Technologies).

In each array, three endogenous controls and a negative control were included. Card A was selected as it focuses on the more highly characterised microRNAs. PCR reactions were performed using 450µl of the Taqman Universal PCR Master Mix No AmpErase UNG (2x) and 9µl of the diluted pre-amplification product. Aliquots of 100µl of the PCR master mix were dispensed to each port of the Taqman microRNA Array card. The fluidic card was then centrifuged and mechanically sealed prior to qPCR.

2.5.2 Microarray

RNA samples extracted from the kidney tissue of 4 groups from chapter 4 experiments (Sham, IRI, IPC/IRI and IPC Alone), was sent to an external provider, Exiqon, for microRNA profiling hybridisation microarray (n=5 in each group). The samples were labelled using the miRCURY LNA™ microRNA Hi-Power Labelling Kit, Hy3™/Hy5™ and hybridised on the miRCURY LNA™ microRNA Array (7th Gen) following a dual-colour experimental design. An outline of the workflow from Exiqon is displayed in Figure 2.4.

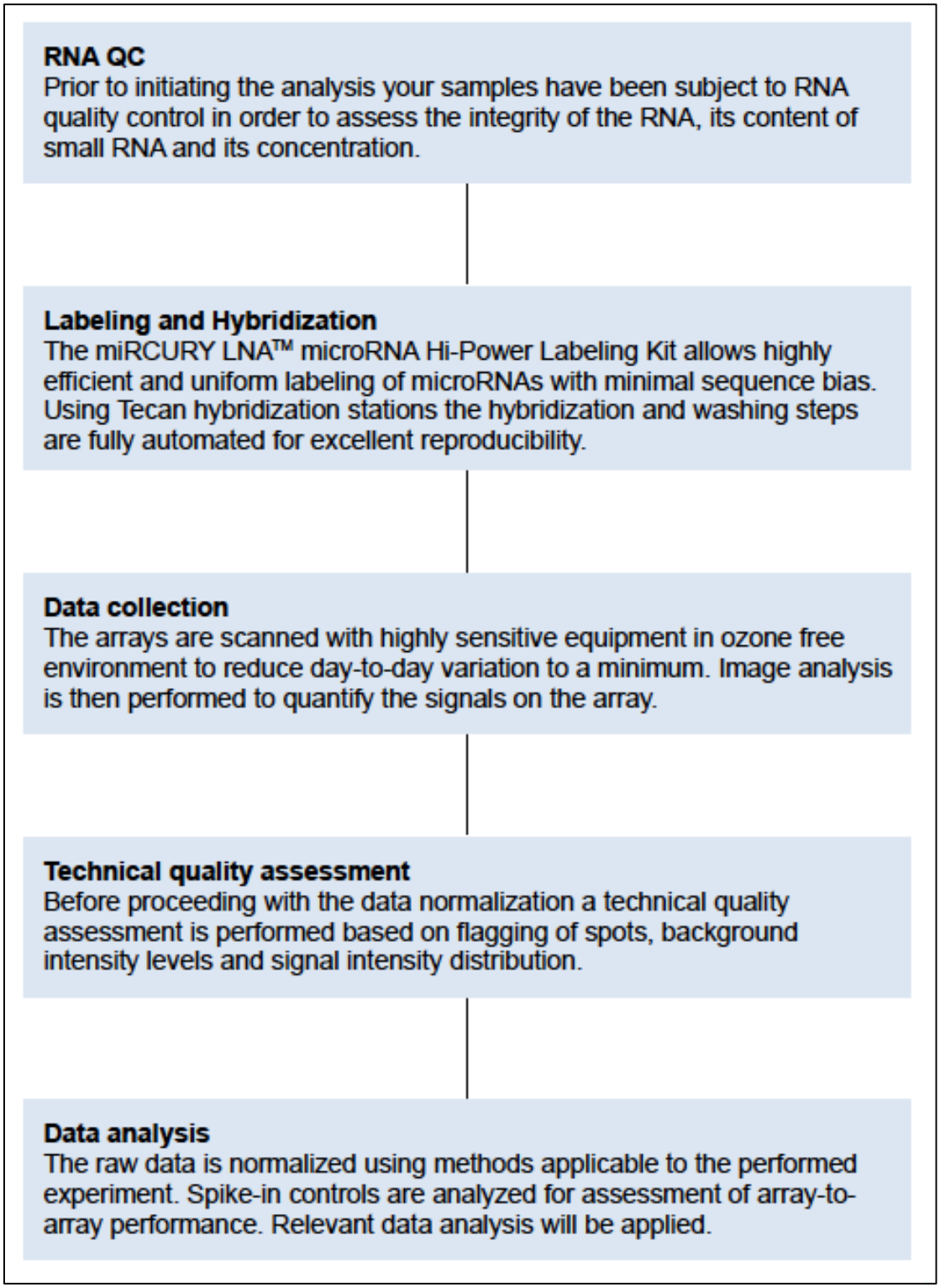

Figure 2.4: Exiqon Hybridisation microarray Workflow outline

The threshold of detection was calculated for each individual microarray slide as 1.2 times the 25th percentile of the overall signal intensity of the slide.

MicroRNAs with intensities above threshold in less than 20% (or 2) of the samples were removed from the final dataset used for expression analysis. The number of microRNAs detectable above background threshold was identified for each sample (out of a total of 714 possible microRNAs) and comparison between groups was made.

2.5.3 Next Generation Sequencing

Next Generation Sequencing (NGS) was performed by Exiqon on RNA extracted from kidney tissue from 4 groups (Sham, IRI, IPC/IRI and IPC alone) (pooled n=1 for each group). The raw sequencing files were received from Exiqon, and subsequently mapped with normalised read counts calculated. The normalised read counts were analysed by chi-square and pair-wise comparison. Chi-square p-values were significant for the majority of the data set. Hierarchical clustering was performed using the GENE-E software and marker selection used to interrogate the pair-wise comparisons. In order to interpret the data set, the normalised read counts were filtered to exclude values below 1000, and the data set was filtered to exclude fold change values of <1.5 increase and decrease. The new data set was then tabulated and visualised on XY plots. These data are described in section 4.2.5.

2.6 Serum Creatinine analysis

Blood samples taken from the rat before operation and at time of retrieval (at 48h) for the chapter 4 experiments were sent to the department of biochemistry (University Hospital of Wales, Cardiff). Serum creatinine was measured from these samples using the Jaffe reaction according to their standard operating procedure.

2.7 Statistical Analysis

Statistical analyses were performed using Graph Pad Prism Version 6 software (La Jolla, CA, USA) for majority of the dataset. Data were expressed as either mean (± SEM) or median (and range) depending on whether the data was parametric or non-parametric. The data was assessed for statistical significance by 'unpaired t test' or 'one-way ANOVA (with post-hoc Bonferroni correction)' for parametric data, and 'Mann-Whitney U test' or 'Wilcoxon test' for non-parametric data. Differences with $p < 0.05$ were considered statistically significant. Level of significance was portrayed as below:

P-value	**Description**	**Summary**
≥0.05	Not significant	ns
0.01 – 0.05	Significant	*
0.001 – 0.01	Very Significant	**
<0.001	Extremely Significant	***

Table 2.6 P-values and description

Linear regressions and correlation analyses were also performed using Graph Pad Prism Version 6 software, and significance values reported as above.

For the microRNA profiling experiments, the following were used:

- TLDA Array (Chapter 5): In addition, to Graph Pad, Norm Finder algorithm was applied to the expression data in order to identify a suitable reference gene. Several genes were tested and all were found to be quite variable and therefore not suitable as a reference gene. In the

end, miR-cel-39 was used as the reference gene for the chapter 5 and chapter 6 experiments. Volcano plots were created in Graph Pad.

- Microarray (Chapter 4): The heat map and PCA analyses and graphs were obtained from Exiqon. Volcano plots were created in Graph Pad using the raw data obtained from Exiqon.
- NGS (Chapter 4): The raw sequencing files received from Exiqon were mapped and normalised read counts calculated. The normalised read counts were analysed by chi-square and pair-wise comparison using Graph Pad. Hierarchical clustering was performed with GENE-E software.

Chapter 3 – The Effects of a Localised IPC Regime in a Unilateral IRI Rat Model

3.1 Introduction

Reducing IRI is arguably the largest and most important challenge in kidney transplantation. There are several approaches to reduce or ameliorate IRI, and IPC has been shown to be one such treatment strategy. IPC is an interesting therapeutic strategy that involves a brief period of ischaemia and reperfusion that is thought to allow the organ of interest to develop a degree of 'tolerance' to a subsequent prolonged period of ischaemia. Animal studies have showed variable results, with most showing a clear benefit in reducing renal IRI (95), whilst some have shown that it confers no benefit (98). Several IPC methods have been described, including having the stimulus localised at the target organ or remotely from it, having one continuous episode of ischaemia or several cycles of brief ischaemia and reperfusion (pulsatile IPC), and varying the interval between the IPC stimulus and the index ischaemia (90, 95, 96). Most IPC investigations in kidneys have used an adult male rat as the animal model and 45-min IRI period has been shown to be robust and reliable in studies that have evaluated the effects of treatment on IRI (214, 215).

For an IPC regime to be a practical and clinically transferrable therapeutic practice in kidney transplantation, a relatively short period of ischaemia and reperfusion localised to the organ is the most desirable option. Indeed, the meta-analysis described in the Section 1.4 showed that most studies have used localised IPC (95). Of particular note, a continuous regime of 15 minutes of IPC before injury has been shown to result in histological and functional protection (96).

Several studies now suggest the increasing importance of microRNAs in CKD and AKI from various causes, including IRI (164, 216, 217). In particular microRNA-21 (miR-21) has been implicated in preventing tubular epithelial cell death (32). In one study, 15mins of localised IPC significantly increased miR-21 expression, resulting in attenuation of IRI 4 days later, whereas knockdown of miR-21 significantly increased tubular cell apoptosis (100).

The aim of this chapter was to determine whether a 15-min localised IPC regime attenuated injury in a rat model of unilateral IRI, and to investigate associated changes in cytokines and miR-21 expression levels.

To this end, the established rat model of IRI within this department was utilised (cross-clamping of the left renal pedicle) and the effect of IPC was evaluated on:

1. histological architectural damage to the renal cortex
2. the expression of AKI markers: KIM-1 and NGAL
3. the expression of cytokines (IL-17, IL-18, and TNF-α)
4. the expression of miR-21

3.2 Results

Fifteen adult male Lewis rats underwent a midline laparotomy and were divided into 3 groups (n=5 each): (1) Sham operation; (2) Left unilateral IRI (45 minutes of cross-clamping of the renal pedicle); and (3) 15mins of ischaemia followed by 20mins of reperfusion (IPC) prior to 45-min IRI (IPC/IRI group). Kidney tissue was retrieved 48h later, sectioned, and stained with haematoxylin and eosin for histological assessment. RNA was extracted for RT-qPCR analysis of AKI markers, cytokines, and miR-21.

3.2.1 Histological Architecture

Forty-five minutes of unilateral IRI in the rat caused marked histological damage at 48h when compared with sham controls. Figure 3.1 shows the changes seen in the renal cortex in sham (A), IRI (B) and IPC/IRI (C) groups. Sham animals showed normal histological architecture of the renal cortex with no damage seen in endothelial, glomerular, tubular or tubulo-interstitial cellular components. Both the IRI and IPC/IRI groups showed extensive damage to the cellular compartments of the renal cortex. The changes seen included, endothelial cell disruption and cell loss; thickening of Bowman's capsule and in some cases evidence of glomerular tuft retraction; tubular cell damage (loss of brush border, cellular inflammation, cast formation and necrosis); and tubulo-interstitial damage (inflammation, haemorrhage, and necrosis) (Figure 3.1 A – C). Histologically there was no significant difference in the damage seen between the IRI and IPC/IRI groups.

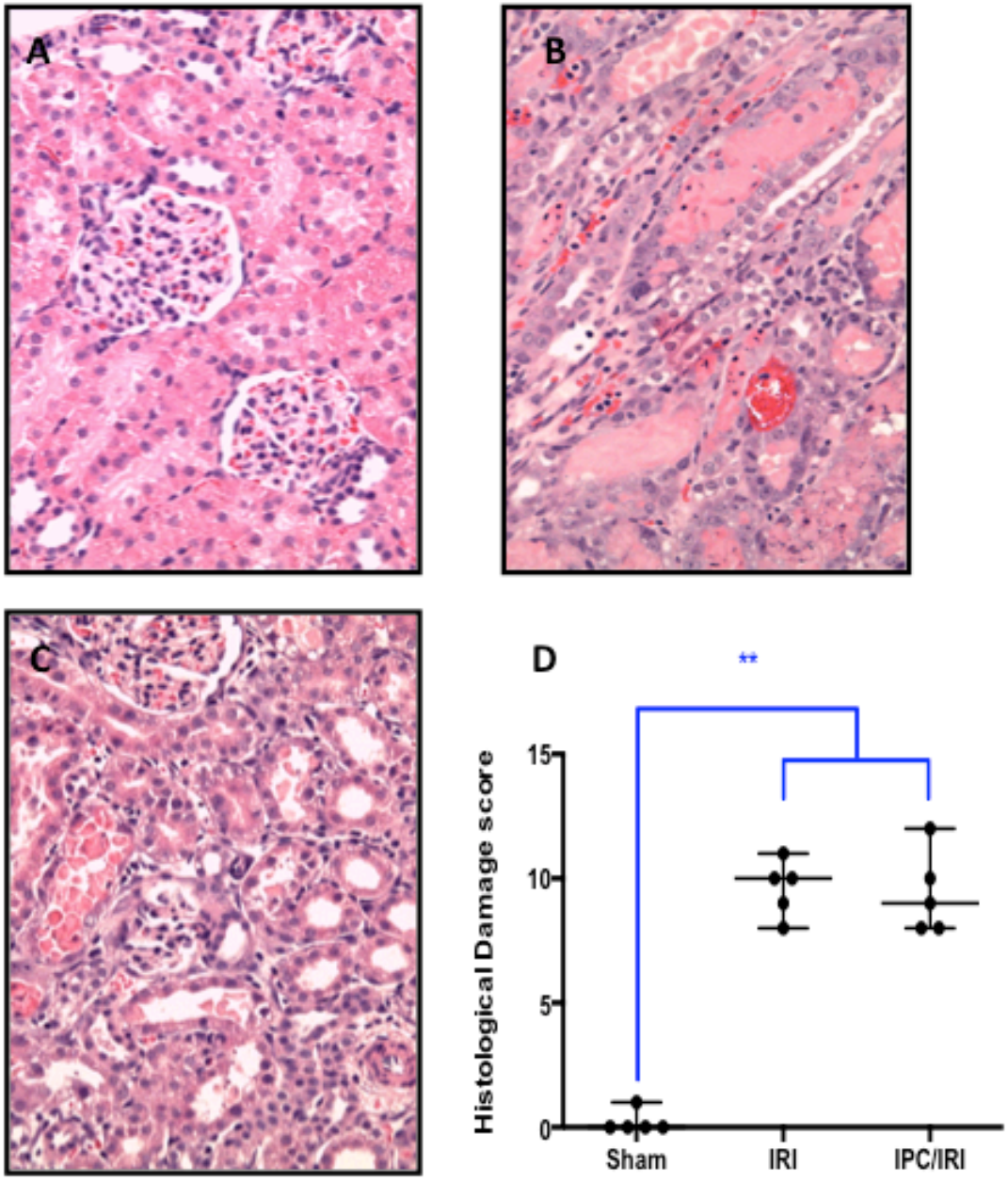

Figure 3.1: Histological characterisation of the renal cortex in unilateral sham, IRI and IPC/IRI rats

Photomicrographs (x200) of H&E staining of renal cortex sections from **(A)** sham, **(B)** 45min unilateral IRI, and **(C)** IPC (15min ischaemia and 20min reperfusion) prior to IRI, in rats at 48h after reperfusion. **(A)** shows normal histological architecture of the renal cortex with no damage seen in endothelial, tubular, glomerular or interstitial cell compartments. **(B)** and **(C)** show damage to the cellular compartments of the renal cortex including tubular cell necrosis. **(D)** H&E sections were assessed and scored using a comprehensive histological damage scoring system comprising Endothelial, Glomerular, Tubular, and Interstitial cell damage. Histological Damage scores are plotted as median and range.

Numbers of animals in each group: Sham (n=5), IRI (n=5), IPC/IRI (n=5).

Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

The median (and range) histology score was 0 (0-1), 10 (8-11) and 9 (8-12) in Sham, IRI and IPC/IRI groups respectively. The difference between sham and IRI was significant ($p = 0.0079$), however the IRI and IPC/IRI groups were not significantly different ($p = 0.8095$) (Figure 3.1 D).

No mortality was associated with any of the animals.

3.2.2 Acute kidney injury markers

NGAL and KIM-1 were selected for analysis because several studies have reported that they are accurate and robust biomarkers of AKI (218-220). As shown in Figure 3.2, NGAL mRNA synthesis increased by 30-fold and KIM-1 mRNA synthesis increased by 300-fold in the IRI group when compared with Sham ($p < 0.0001$). No statistical significant difference was found between the IRI and the IPC/IRI groups in the mRNA expression of NGAL ($p = 0.0610$) and KIM-1 ($p = 0.7903$).

The histological and acute kidney injury marker results shown in Figures 3.1 and 3.2 suggest that this IRI model is reliable, producing significant non-fatal kidney injury, and the IPC regime does not ameliorate this injury.

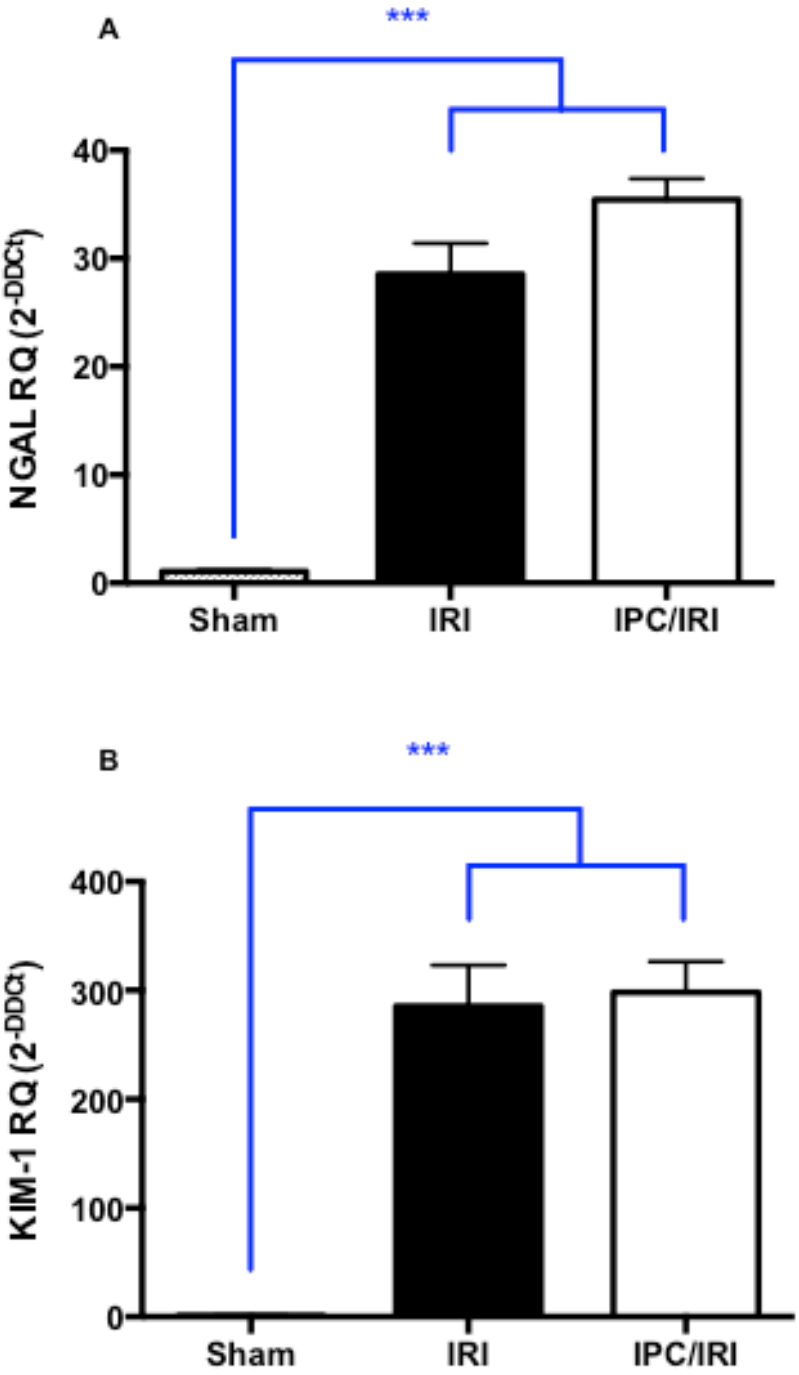

Figure 3.2: Expression of Acute Kidney Injury markers in unilateral sham, IRI and IPC/IRI rats

RT-qPCR analysis of **(A)** NGAL and **(B)** KIM-1 in sham, 45min unilateral IRI, and IPC (15min ischaemia and 20min reperfusion) prior to IRI, in rats at 48h after reperfusion. Expression is normalised to GAPDH and plotted as mean ± SEM.

Numbers of animals in each group: Sham (n=5), IRI (n=5), IPC/IRI (n=5).

Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.3 Cytokine profile

IL-17, IL-18, and TNF-α were selected for analysis because previous studies have reported that they play an important role in the pathophysiology of IRI, including kidney IRI (221-223). As shown in Figure 3.3, mRNA expression levels of IL-17 ($p = 0.4614$), IL-18 ($p = 0.3807$), and TNF-α ($p = 0.0757$) were all increased in IRI compared with Sham but not significantly.

There was an overall increase in the mRNA expression of IL-17, IL-18, and TNF-α in the IPC/IRI group compared with both the sham and IRI groups. For IL-17 this difference was not statistically significant between sham and IPC/IRI ($p = 0.0960$), and between IRI and IPC/IRI ($p = 0.2401$) (Figure 3.3 A).

There was a nearly 4-fold increase in mRNA synthesis of IL-18 in IPC/IRI group compared with Sham and this was statistically significant ($p = 0.0303$). Although, there was an increase in mRNA synthesis of IL-18 in IPC/IRI compared with IRI, this difference was not statistically significant ($p = 0.1683$) (Figure 3.3 B).

The expression of TNF-α was increased 4-fold in the IPC/IRI group compared with Sham group ($p = 0.0013$), and 2-fold in IPC/IRI compared with the IRI group ($p = 0.0328$) (Figure 3.3 C).

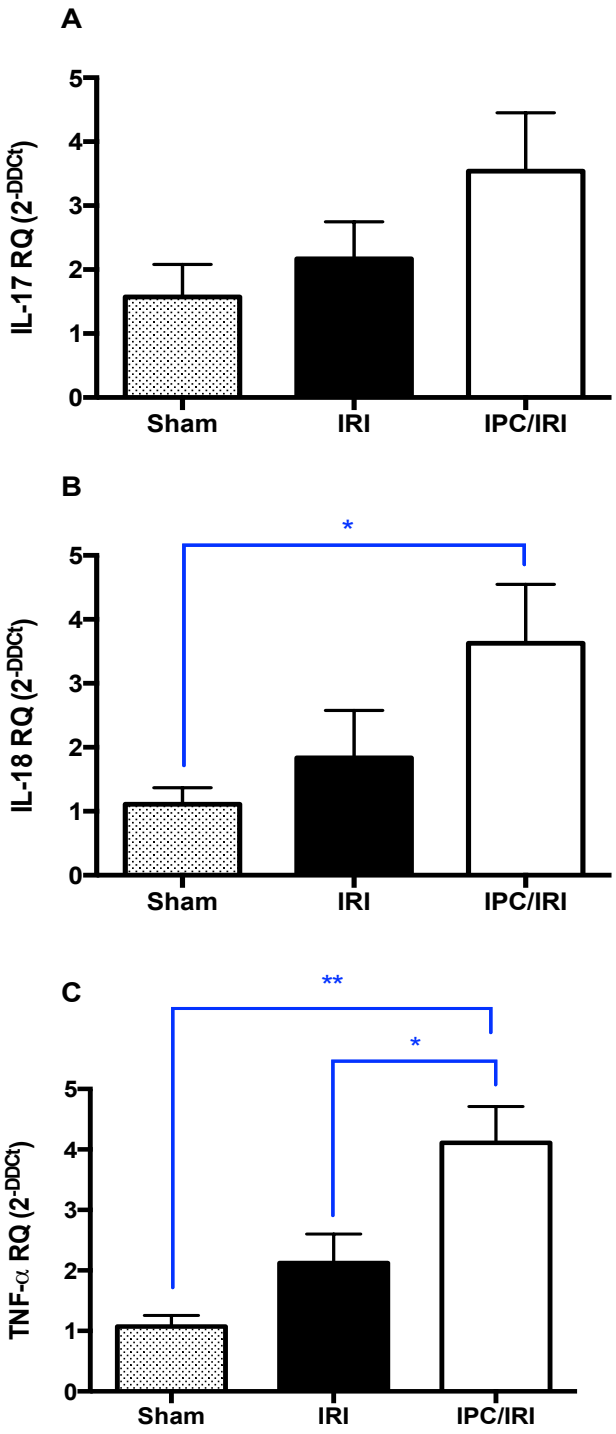

Figure 3.3: Expression of Cytokines (IL-17, IL-18, and TNF-α) in unilateral sham, IRI and IPC/IRI rats

RT-qPCR analysis of **(A)** IL-17, **(B)** IL-18, and **(C)** TNF-α in sham, 45min IRI, and IPC (15min ischaemia and 20min reperfusion) prior to IRI, in rats at 48h after reperfusion. Expression is normalised to GAPDH and plotted as mean ± SEM.

Numbers of animals in each group: Sham (n=5), IRI (n=5), IPC/IRI (n=5).

Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.4 MicroRNA-21 changes

Several reports have suggested that microRNA expression changes are important in IRI, with particular emphasis on the role of miR-21. There was a 2-fold statistically significant increase in the expression of miR-21 in the IRI group compared with the sham group (p = 0.0347) (Figure 3.4), and a 2.5 fold increased in the IPC/IRI group compared with the Sham group (p = 0.0058). There was a slight increase in the expression of miR-21 from IRI to IPC/IRI. However, this increased miR-21 expression shown in the IPC/IRI group was not significantly different compared with the IRI group (*P* = 0.4519) (Figure 3.4).

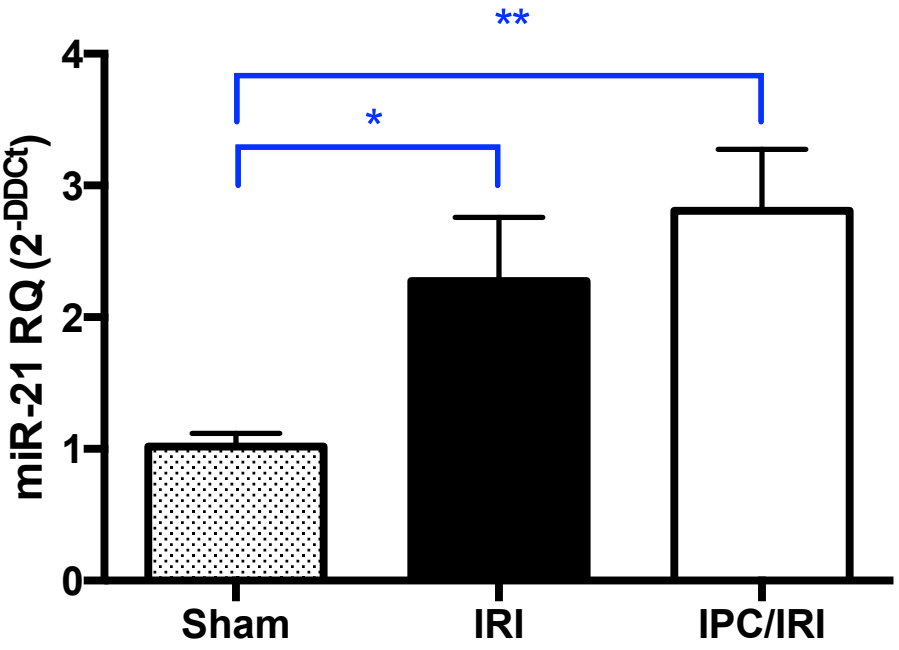

Figure 3.4: Expression of miR-21 in unilateral sham, IRI and IPC/IRI rats

RT-qPCR analysis of miR-21 in sham, 45min unilateral IRI, and IPC (15 min ischaemia and 20min reperfusion) prior to IRI, in rats at 48h after reperfusion (n=5 in each group). Expression is normalised to miR-16 and plotted as mean ± SEM.

Numbers of animals in each group: Sham (n=5), IRI (n=5), IPC/IRI (n=5).

Statistical significance: * p<0.05, ** p<0.01, *** p<0.001.

3.3 Discussion

This chapter has demonstrated that:

(1) 45mins of ischaemia followed by 48h of reperfusion to the left kidney in the rat causes:

(a) Significant damage to the renal cortex; (b) Increased expression of established markers of AKI; and (c) Increased expression of inflammatory cytokines shown to be implicated in IRI.

(2) The immediate localised continuous IPC regime (15mins ischaemia and 20mins reperfusion prior to the IRI) used did not protect the rat kidney against IRI. This is supported by no significant differences seen histologically to the renal cortex architecture, nor of the levels of molecular markers of kidney injury between IRI and IPC/IRI groups.

(3) There is significant up-regulation of TNF-α mRNA synthesis in the IRI and IPC/IRI groups, which may be suggestive of this inflammatory cytokine's a role in the early mechanisms of IPC.

(4) There is significantly increased expression in IRI of miR-21, a microRNA that has recently been shown to play a vital role in the underlying molecular mechanisms of IRI, however there was no difference between IRI and IPC/IRI groups.

The rat model used is a well-established *in vivo* model of kidney injury/IRI (218, 224) and cross-clamping of the renal pedicle for 45mins produces significant but not fatal ischemic injury, reported in the literature (214, 215). In these experiments, the 48h reperfusion period led to clear histological and molecular

changes, which are in agreement with previous studies (96, 98, 225). Therefore, it is not surprising that IRI as utilised in this rat model produced significant non-fatal injury to the kidney, and consequentially it is a robust model of acute kidney injury.

The finding that this IPC regime does not affect histological or molecular markers of renal injury in this model should be placed in the context of the current controversy as to whether IPC protects from subsequent renal IRI. Although several investigators have found IPC to be effective in protecting against renal IRI in various animal species (90-94), other studies have found no evidence that IPC is protective (98, 99, 226). Nevertheless, a recent meta-analysis by Wever and colleagues (95) analysed 58 animal studies, concluding that IPC is associated with significantly improved histological injury scores and renal function (serum creatinine, blood urea nitrogen) following IRI. Therefore, other types of IPC regimes are worthy of further investigation.

The significant increase of TNF-α that we measured in our model (Figure 3.3 C) suggests that IPC had an additive inflammatory and potentially harmful effect. Although not demonstrated in these experiments, one may speculate that this effect may be part of the IPC hypothetical series of events that would eventually confer protection, as seen in other studies that used a longer interval between IPC and IRI. This hypothesis, if followed, requires further investigation.

MiR-21 was selected because several studies have reported its significant role in kidney IRI (32, 187, 227). Tubular cell apoptosis contributes significantly to

renal IRI, and although the role of miR-21 remains to be fully elucidated, some evidence is suggestive of a protective as well as a pathological role for miR-21, in this context (228). Indeed, anti-apoptotic functions have also been attributed to miR-21 (32, 229). In this chapter's experiments, miR-21 expression was significantly increased in IRI, supporting its potential as a biomarker of IRI-mediated kidney injury. However, results observed between IRI and IPC/IRI groups were not significantly different. This is in contrast to Xu's study, in which a 15-min IPC regime decreased IRI induced 4 days later, in association with an up regulation of miR-21 and hypoxia-inducible factor 1α (HIF-1α) expression (100). Similarly to this chapter's experiments, they used an IPC regime of 15mins of continuous ischemia, but the extended period of 4 days between IPC and IRI may be key in terms of understanding the difference in outcomes. A beneficial effect after this extended recovery may be due to improvements in tissue robustness to subsequent injury, which may take hours rather than minutes to set in place. MicroRNAs act predominantly by post-transcriptional repression of their targets, leading to diminished target mRNA (and as a result) protein synthesis. This diminished synthesis may lead to a delayed alteration in tissue phenotype or response, requiring first a change in microRNA expression or activity and then the time required for expression of key protein targets to "decay" to the necessary threshold level for the subsequent effect, as determined by the degree of post-transcriptional repression elicited and the stability of the protein target. What is abundantly clear is that there is a huge scope for further detailed microRNA analysis studies in the context of renal IRI and IPC/IRI.

One major limitation of these experiments is that the rat model of unilateral IRI does not allow for changes in overall kidney function to be accurately measured. This is because the contralateral (right) kidney was either not subjected to nephrectomy nor to ischemia, therefore it was not possible to provide a meaningful measurement of functional markers such as serum creatinine or blood urea nitrogen. However, despite this shortcoming, the histological damage correlated well with 2 molecular markers of AKI (NGAL and KIM-1). Nevertheless, it is obvious that further investigations that assess different IPC regimes (or other therapies) are needed in a model, which allows for measurement of functional assessment of the renal response to IRI. For example, a rat model of bilateral IRI or a model in which the contralateral kidney was removed would be a way to move forward.

3.4 Concluding Remarks

The next set of experiments must:

(1) Develop a rat model of IRI that allows for accurate functional measurement of renal function.

(2) Methodically investigate several different regimes of IPC to identify one that confers benefit to renal IRI.

(3) Perform a thorough review and analysis of the microRNA profile in renal IRI and IPC.

Chapter 4 – The role of microRNAs in IPC in a Bilateral IRI Rat Model

4.1 Introduction

In the previous chapter, a unilateral model of IRI in the rat was used and it was shown that one IPC regime (15mins of ischaemia and 20mins of reperfusion) did not confer any benefit, as demonstrated by the histological evaluation of renal cortex, mRNA synthesis of AKI markers (NGAL and KIM-1), and mRNA synthesis of inflammatory cytokines. In addition to this, there was no effect on the expression of miR-21 in response to IPC stimulus.

Although the above findings are in keeping with literature, the vast majority of studies on IPC have shown that it is beneficial in reducing injury in IRI. It is therefore prudent that different IPC regimes are tested to identify one that would confer benefit in the rat model and also be easily transferrable to clinical practice in transplantation.

Secondly, one of the most important things in kidney IRI, in the context of transplantation and other clinical settings, is determining kidney function as well as structural damage. To determine function, a rat model that would either render ischaemia to both kidneys, or to one kidney (after performing a contralateral nephrectomy) was clearly needed.

With this background in mind, the first aims of this chapter were:

(4) To develop a rat model of IRI that allows for accurate measurement of renal function. For this a model of bilateral IRI was chosen.

(5) To methodically investigate several different regimes of IPC to identify one that confers benefit to renal IRI.

Following on from this, once a suitable rat model of IRI had been successfully developed and a beneficial regime of IPC identified, the next aims of this chapter would be:

(6) To perform microRNA profiling experiments to identify potential target microRNAs within IPC and IRI.

(7) To determine the cellular origin of these potential target microRNAs within the kidney

4.2 Results

Fifty-eight adult male Lewis rats underwent a midline laparotomy and were divided into 4 main groups: (1) Bilateral Sham operation (n=8); (2) Bilateral IRI (45mins of cross clamping of both renal pedicles); (3) Direct Kill (n=5); and (4) Several different IPC regimes, described in sections 4.2.2 – 4.2.4 (n=36). Kidney tissue was retrieved 48h later, sectioned, stained with haematoxylin and eosin for histological assessment. Blood samples were taken pre-operatively and at 48h (at time of retrieval) for serum creatinine measurement using the Jaffe reaction. RNA was extracted from the kidney tissue for RT-qPCR analysis of AKI markers, and microRNA experiments.

4.2.1 Animal Model of IRI

4.2.1.1 Bilateral IRI Model

Forty-five minutes of bilateral IRI in the rat caused marked histological damage at 48 hours when compared with sham controls (Figure 4.1). The median (and range) EGTI histology score (Table 2.1) was 8 (5 – 9) in the IRI group compared to 0 (0 – 2) in the sham group ($p<0.0001$) (Figure 4.2(A)). Mean serum creatinine at 48 hours was 76.63 (±13.36) μmol/l in the IRI group compared with 31.90 (±0.25) μmol/l in the sham group ($p=0.0067$) (Figure 4.2(B)). The mRNA expression of NGAL and KIM-1 was increased 14-fold and 150-fold in the IRI group respectively ($p<0.0001$) (Figure 4.2(C)-(D)). The individual components of the EGTI score were also significantly higher in the IRI group compared with sham (Figure 4.3).

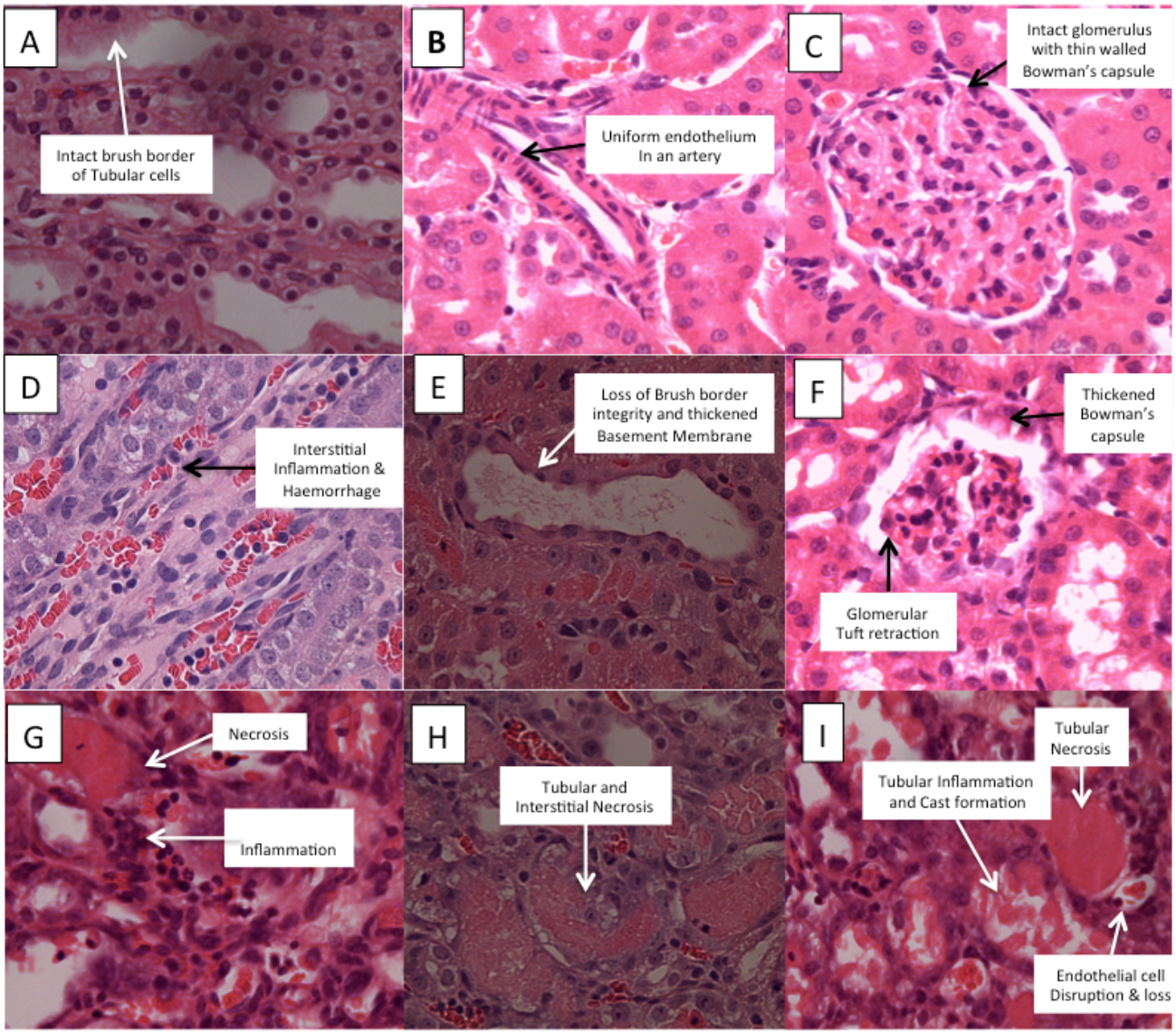

Figure 4.1: Histological images of rat renal cortex sections

Renal cortex H&E (x200) paraffin sections, from sham and 45-min bilateral IRI in rats, at 48h after reperfusion (Sham n=8, IRI n=9), were assessed for Endothelial, Tubular, Glomerular and Interstitial cell damage, using the EGTI histology scoring system. [A] – [C] show normal appearance of the renal cortex in a Sham rat. [A] shows that the brush border of the tubular cells is intact with no thickening of the basal membrane. No inflammation or necrosis is seen (Tubular score 0). There is no visible interstitium signifying no damage/abnormality within the interstitial compartment (Interstitial score 0). [B] shows a uniform endothelium with no swelling or disruption of the endothelial cells (Endothelial score 0). [C] shows an intact glomerulus with thin walled Bowman's capsule and no tuft retraction (Glomerular score 0). [D] – [I] show varying degrees of damage of the renal cortex in 45-minute bilateral IRI rats. [D] shows inflammation and haemorrhage within the interstitium, which is present in less than 25% of the tissue without any evidence of necrosis (Interstitial score 1). [E] shows thickened basal membrane of the tubular

cells with loss of the brush border in more than 25% of the tubular cells, without the presence of cast formation or necrosis (Tubular score 2). [F] shows a glomerulus with evidence of thickened Bowman's capsule (Glomerular score 1), and glomerular tuft retraction (Glomerular score 2). [G] shows inflammation and haemorrhage within the interstitial compartment, with necrosis in up to 60% of the cells (Interstitial score 3). [H] shows complete necrosis in tubular and interstitial cellular compartments (Tubular score 4, Interstitial score 4). [I] shows evidence of inflammation and cast formation within the tubules with evidence of necrosis in up to 60% of tubular cells (Tubular score 3), and endothelial cell disruption and loss (Endothelial score 3).

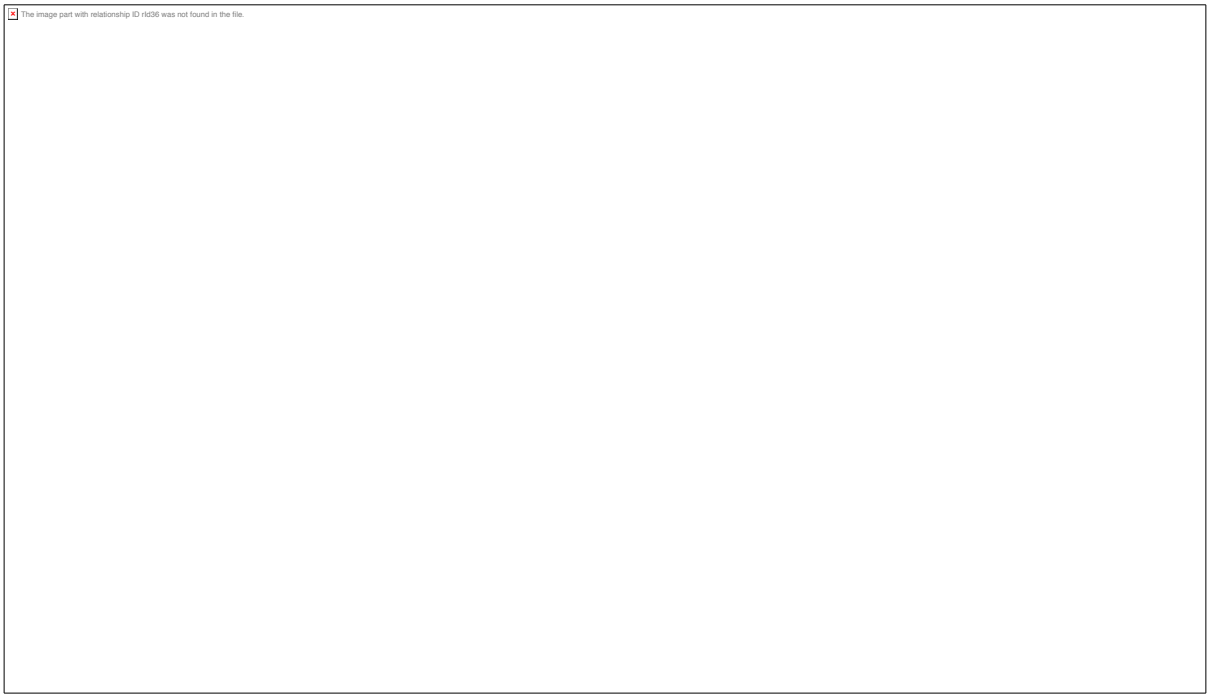

Figure 4.2: Effect of bilateral IRI on EGTI Histology score, serum creatinine and expression of NGAL and KIM-1

(A) Renal cortex sections from Direct kill, Sham and 45min bilateral IRI, in rats at 48h after reperfusion were stained with H&E and assessed using the EGTI scoring system comprising of Endothelial, Glomerular, Tubular, and Interstitial cell damage. EGTI Histology scores are plotted as median and range. **(B)** Serum creatinine was measured pre-op and at 48h in sham and IRI rats and is plotted as mean ± SEM. **(C and D)** RT-qPCR analysis of NGAL and KIM-1 was performed. Expression is normalised to GAPDH and plotted as mean ± SEM.

Numbers of animals in each group: Direct Kill (n=5), Sham (n=8), IRI (n=9).

Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

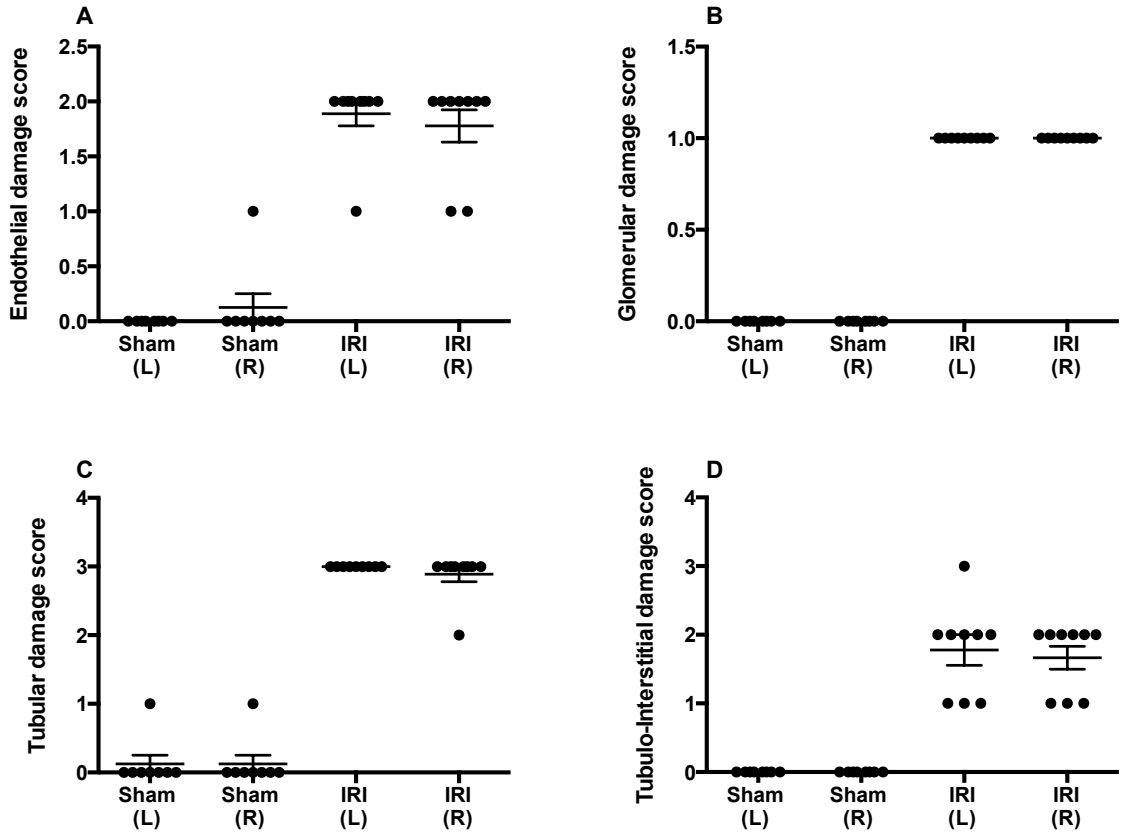

Figure 4.3: Effect of bilateral IRI on Endothelial, Glomerular, Tubular, and Tubulo-Interstitial damages scores

Renal cortex sections from sham and 45-min bilateral IRI in rats at 48h after reperfusion were stained with H&E and assessed using a comprehensive scoring system comprising of Endothelial, Glomerular, Tubular, and Interstitial cell damage (Sham n=8, IRI n=9). Individual cellular component scores are plotted as median and range for Endothelial (A), Glomerular (B), Tubular (C), and Tubulo-Interstitial (D) components.

4.2.1.2 Validation of EGTI histology scoring system

These sham and IRI experiments also allowed for the evaluation of the EGTI scoring system as a suitable assessment tool of histological damage of the renal cortex. The EGTI histology scoring system, developed specifically for this research and described in section 2.2.2 was designed to comprehensively document the damage seen within the renal cortex of an *in vivo* model of AKI/IRI. The widely adopted 'gold standard' scoring system for reporting histological

damage in kidney injury is the Jablonski scoring system (204). This simple system is designed to give a rapid and objective assessment of kidney injury. In reality, however, it provides a limited score documenting predominantly the degree of necrosis within tubular cells only, ranging from 0 signifying 'no damage', to 4 'necrosis affecting all 3 segments of the proximal convoluted tubule'. It does not provide an assessment of the damage seen within the other cellular components, such as endothelial and glomerular cells. The Jablonski scoring system was originally described following a prolonged recovery period after ischaemic injury, and includes features of recovery (e.g. tubular cell mitoses) that may not be evident in the typical recovery periods used experimentally, such as in these experiments. Therefore a more detailed scoring system was designed that included damage seen within the renal cortex to endothelial (E), glomerular (G), tubular (T), and interstitial (I) cellular compartments, as detailed in Table 2.1.

EGTI and Jablonski scores were correlated with NGAL, KIM-1 and serum creatinine. Both EGTI and Jablonski scores correlated significantly with serum creatinine at 48 hours, and expression of NGAL and KIM-1 (Figure 4.4), signifying that both scores are highly predictive of kidney function and structural damage. In addition to this, the association between each component on the EGTI histology scoring system and serum creatinine at 48h, and expression of NGAL and KIM-1 was examined using univariate logistic regression analyses. All 4 cellular components were independently associated with serum creatinine at 48h ($p<0.05$), and NGAL and KIM-1 ($p<0.0001$).

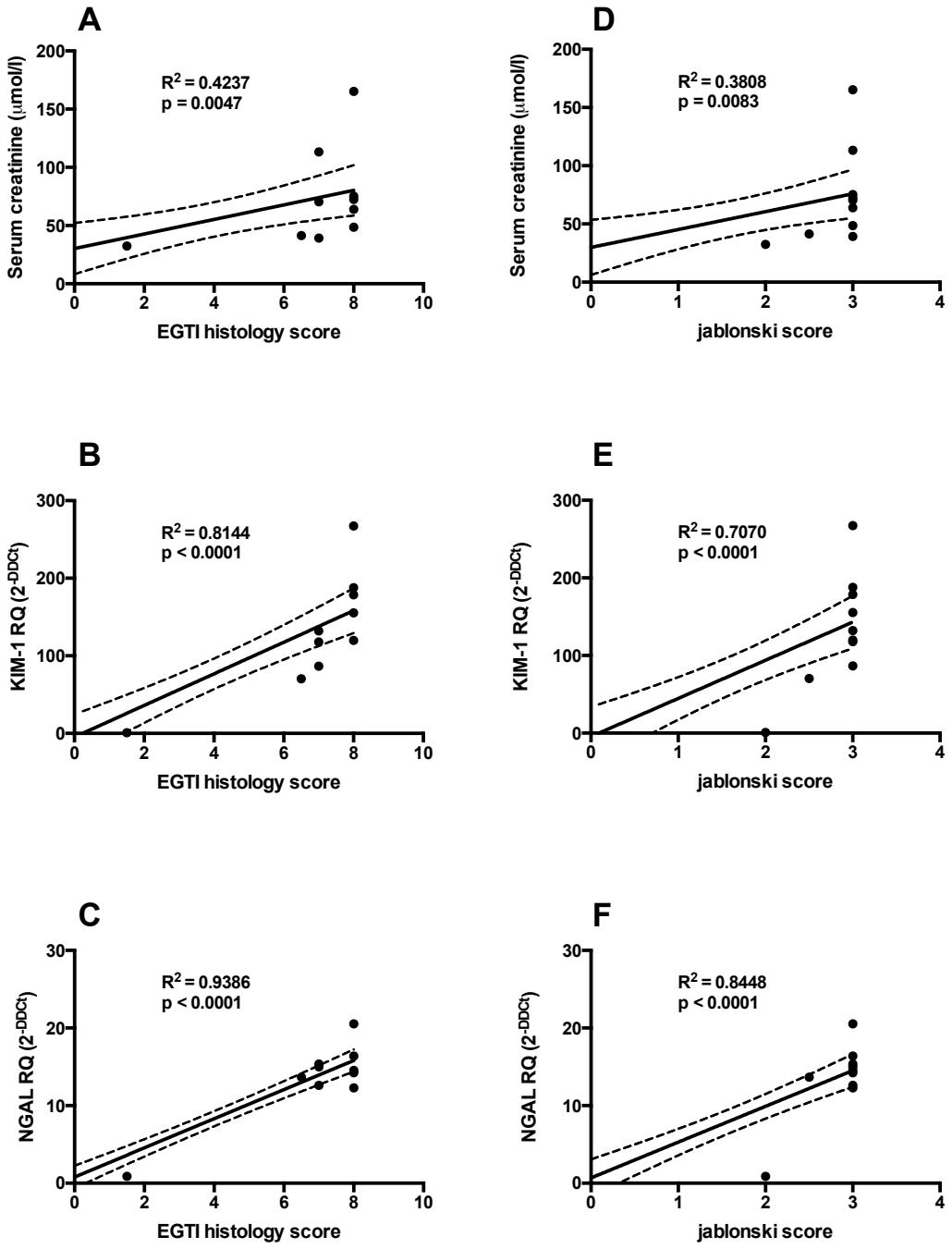

Figure 4.4: Correlation of EGTI and Jablonski scores with serum creatinine, KIM-1, and NGAL

Renal cortex sections from sham and 45-min bilateral IRI in rats at 48h after reperfusion (Sham n=8, IRI n=9) were stained with H&E and scored using the EGTI and Jablonski scoring systems. Serum creatinine was measured at 48h and RT-qPCR analysis of KIM-1 and NGAL was performed, with expression normalised to GAPDH. EGTI and Jablonski scores were both plotted and correlated with serum creatinine (A and D), KIM-1 (B and E), and NGAL (C and F).

4.2.2 The Effect of IPC in the bilateral IRI model

Having established that the bilateral IRI rat model was robust and reliable, the effect of 6 different localised IPC regimes was tested. Localised regimes were selected, as they would be easily transferrable to clinical practice. Three continuous and three pulsatile regimes were chosen. The continuous regimes consisted of 10, 15 and 20mins of ischaemia (n=4 each) followed by 20mins of reperfusion prior to IRI (Figure 2.2). The pulsatile regimes consisted of 2, 5 or 10mins of ischaemia followed by 5mins of reperfusion, in 3 cycles respectively, prior to IRI (n=4 each) (Figure 2.3).

4.2.2.1 The Effect of continuous IPC regimes

Three continuous IPC regimes were tested (n=4 each): 10mins of ischaemia followed by 20mins of reperfusion (IPC-C 10-20); 15mins of ischaemia followed by 20mins of reperfusion (IPC-C 15-20); and 20mins of ischaemia followed by 20mins of reperfusion (IPC-C 20-20). Neither of these regimes reduced injury (Figure 4.5). There was marked histological damage to the renal cortex with 45mins of bilateral IRI and with all of the 3 continuous IPC regimes. The median (and range) EGTI histology scores were 8 (6 – 9), 7 (7 – 9), and 7 (7 – 8) in the 'IPC-C 10-20', 'IPC-C 15-20', and 'IPC-C 20-20' groups compared with 8 (5 – 9) in the IRI group (p = ns) (Figure 4.5(A)). Mean serum creatinine at 48h was 154.5 (±73.79) μmol/l, 110.2 (±15.21) μmol/l, and 115.9 (±28.83) μmol/l in the 'IPC-C 10-20', 'IPC-C 15-20', and 'IPC-C 20-20' groups compared with 76.63 (±13.36) μmol/l in the IRI group (p = ns) (Figure 4.5(B)). The mRNA expression of NGAL was increased at least 2-fold in all of the 3 continuous IPC groups compared with the IRI group ($p<0.0001$) (Figure 4.5(C)). The mRNA expression of KIM-1 was

increased 2-fold in the 'IPC-C 15-20' group compared with the IRI group (p = 0.0018), but not significantly increased in the IPC-C 10-20' and 'IPC-C 20-20' groups (Figure 4.5(D)).

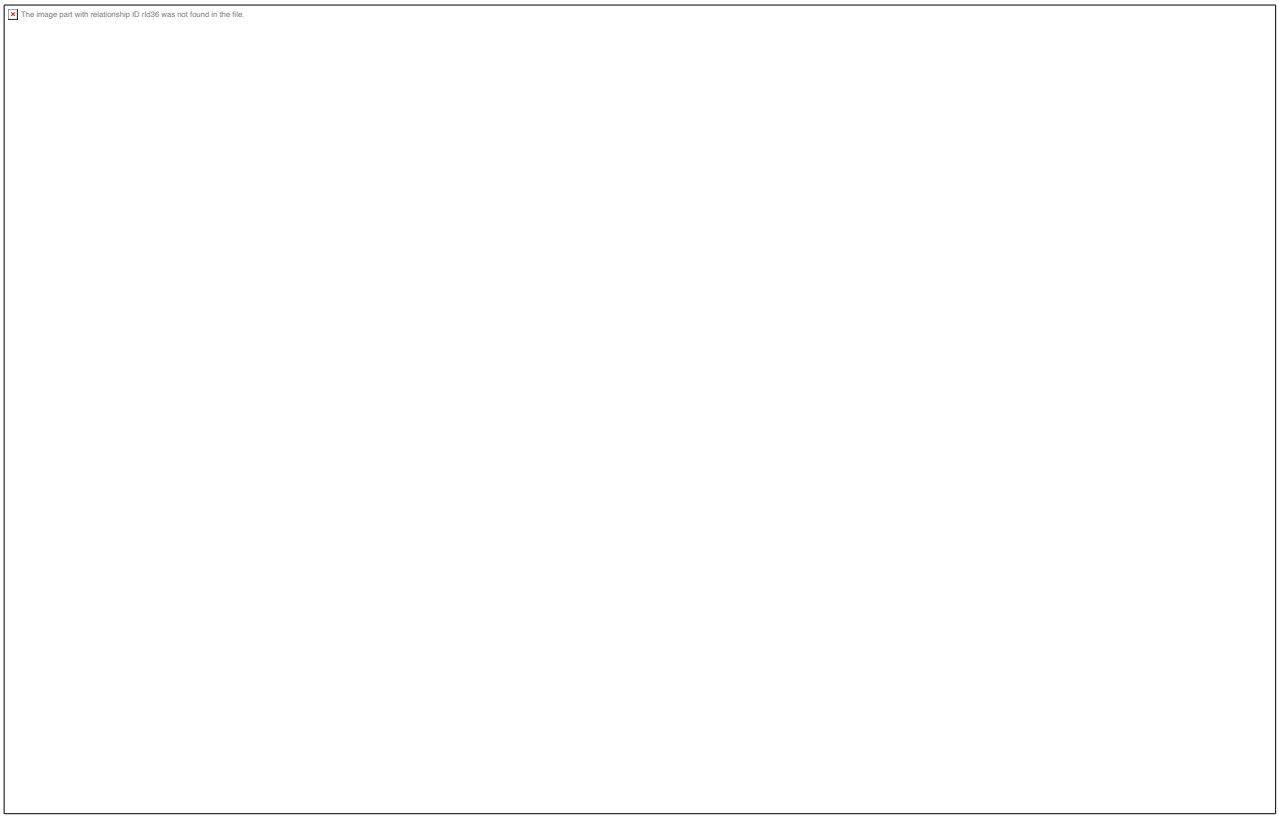

Figure 4.5: Effect of continuous IPC on EGTI score, serum creatinine and expression of NGAL and KIM-1

(A) Renal cortex sections from Direct Kill, Sham, 45min bilateral IRI, and 3 localized continuous IPC regimes (10, 15 and 20min ischaemia and 20min reperfusion (IPC-C 10-20, IPC-C 15-20, and IPC-C 20-20 respectively) prior to IRI, in rats at 48h after reperfusion, were stained with H&E and assessed using a comprehensive scoring system comprising of Endothelial, Glomerular, Tubular, and Interstitial cell damage. EGTI Histology scores are plotted as median and range. **(B)** Serum creatinine was measured pre-op and at 48h and is plotted as mean ± SEM. **(C and D)** RT-qPCR analysis of NGAL and KIM-1 was performed. Expression is normalised to GAPDH and plotted as mean ± SEM.

Numbers of animals in each group: Direct Kill (n=5), Sham (n=8), IRI (n=9), IPC-C 10-20 (n=4), IPC-C 15-20 (n=4), and IPC-C 20-20 (n=4).

Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.2.2 The Effect of pulsatile IPC regimes

Three pulsatile IPC regimes were tested (n=4 each): 3 cycles of 2mins ischaemia and 5mins reperfusion (IPC-P 2-5); 3 cycles of 5mins ischaemia and 5mins reperfusion (IPC-P 5-5); and 3 cycles of 10mins ischaemia and 5mins reperfusion (IPC-P 10-5). There was less histological damage to the renal cortex with the 3 pulsatile IPC regimes compared with IRI group. The median (and range) EGTI histology scores were 7 (3 – 7), 6.5 (0 – 7), and 7 (6 – 7) in the 'IPC-P 2-5', 'IPC-P 5-5', and 'IPC-P 10-5' groups compared with 8 (5 – 9) in the IRI group (Figure 4.6(A)). Mean serum creatinine at 48h was 40.43 (±3.35) μmol/l, 55.65 (±6.88) μmol/l, and 59.75 (±5.65) μmol/l in the 'IPC-P 2-5', 'IPC-P 5-5', and 'IPC-P 10-5' groups compared with 76.63 (±13.36) μmol/l in the IRI group ($p = 0.014$, $p = 0.339$ and $p = 0.435$ respectively) (Figure 4.6(B)). The mRNA expression of NGAL was not increased or decreased significantly compared with the IRI group (p = ns) (Figure 4.6(C)). The mRNA expression of KIM-1 was decreased 2-fold in the 'IPC-P 2-5' and 'IPC-P 10-5' groups compared with the IRI group ($p = 0.0009$ and $p = 0.026$ respectively), however it was not decreased or increased in the 'IPC-P 5-5' group compared with the IRI group ($p = 0.359$) (Figure 4.6(D)).

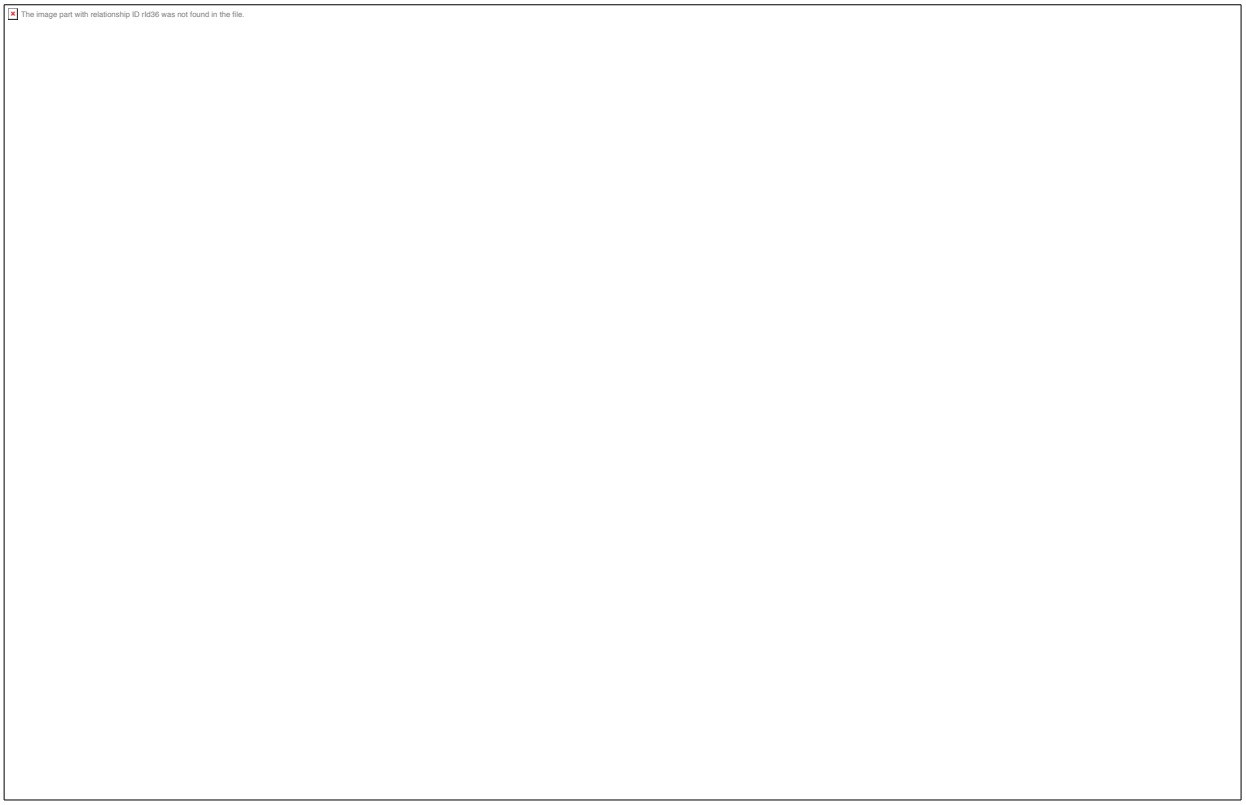

Figure 4.6: Effect of localised pulsatile IPC on EGTI score, serum creatinine and expression of NGAL and KIM-1

(A) Renal cortex sections from 45min bilateral IRI, and 3 localised pulsatile IPC regimes (3 cycles of 2, 5 and 10min ischaemia and 5min reperfusion (IPC-P 2-5, IPC-P 5-5, and IPC-P 10-5 respectively) prior to IRI, in rats at 48h after reperfusion were stained with H&E and assessed using a comprehensive scoring system comprising of Endothelial, Glomerular, Tubular, and Interstitial cell damage. EGTI Histology scores are plotted as median and range. **(B)** Serum creatinine was measured pre-op and at 48h and is plotted as mean ± SEM. **(C and D)** RT-qPCR analysis of NGAL and KIM-1 was performed. Expression is normalised to GAPDH and plotted as mean ± SEM.

Numbers of animals in each group: Direct Kill (n=5), Sham (n=8), IRI (n=9), IPC-P 2-5 (n=4), IPC-P 5-5 (n=4), and IPC-P 10-5 (n=4).

Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.3 The Chosen IPC regime

It became clear from the above results that the pulsatile regimes were more beneficial in reducing injury to the kidneys. Having analysed the results, the 'IPC-P 2-5' regime was chosen to proceed with and complete the number of animals

in the group to make n of 8, named the 'IPC/IRI' group. This IPC regime was also done alone without any subsequent IRI, 'IPC Alone' group (n = 8). Overall this IPC regime reduced structural and functional damage to the kidney (Figure 4.7).

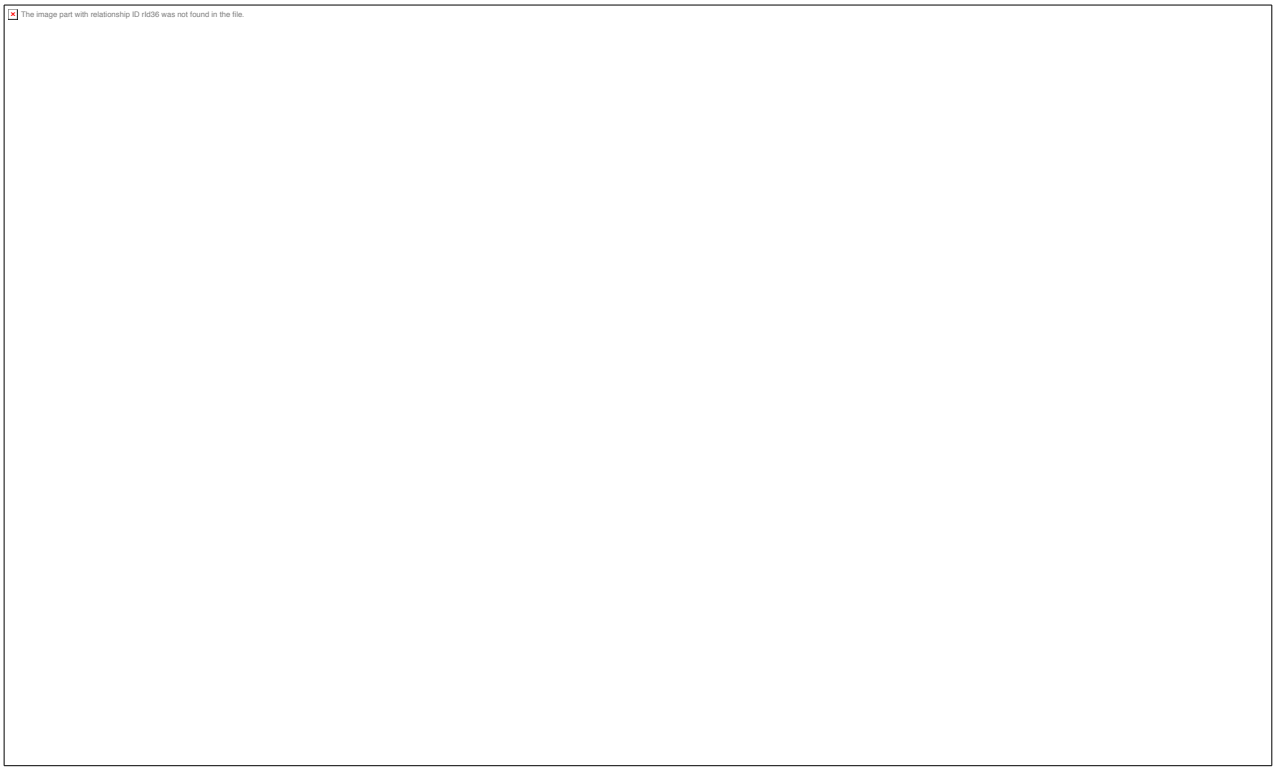

Figure 4.7: Effect of IPC on EGTI score, serum creatinine and expression of NGAL and KIM-1

(A) Renal cortex sections from Direct Kill, Sham, 45min bilateral IRI, localised pulsatile IPC (3 cycles of 2min ischaemia and 5min reperfusion) prior to IRI, and localized pulsatile IPC (3 cycles of 2min ischaemia and 5min reperfusion) alone without any subsequent IRI, in rats at 48h after reperfusion were stained with H&E and assessed using a comprehensive scoring system comprising of Endothelial, Glomerular, Tubular, and Interstitial cell damage. EGTI Histology scores are plotted as median and range. **(B)** Serum creatinine was measured pre-op and at 48h and is plotted as mean ± SEM. **(C and D)** RT-qPCR analysis of NGAL and KIM-1 was performed. Expression is normalised to GAPDH and plotted as mean ± SEM.

Numbers of animals in each group: Direct Kill (n=5), Sham (n=8), IRI (n=9), IPC alone (n=8), and IPC/IRI (n=8).

Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Three cycles of 2mins ischaemia and 5mins reperfusion prior to 45-min IRI caused significantly less histological damage with a median (and range) EGTI histology score of 2.5 (0 – 7) compared with 8 (5 – 9) in the IRI group ($p < 0.005$) (Figure 4.7(A)). Mean serum creatinine at 48h was 40.31 (±1.59) μmol/l compared with 76.63 (±13.36) μmol/l in the IRI group ($p = 0.023$) (Figure 4.7(B)). The mRNA expression of NGAL and KIM-1 was decreased 4-fold and 1.5-fold respectively in the IPC/IRI group compared with the IRI group ($p = 0.0195$ and $p < 0.0001$ respectively) (Figure 4.7(C)-(D)).

4.2.4 MicroRNA Profiling

RNA extracted from the kidney tissue from 4 groups (Sham, IRI, IPC/IRI and IPC Alone) was analysed by an external provider, Exiqon, for microRNA profiling experiments. Hybridisation microarray (n = 5 in each group) and NGS (pooled n = 1 for each group) were both performed.

4.2.4.1 Microarray

Samples were analysed by Exiqon using the miRCURY LNA™ microRNA Hi-Power Labelling Kit and hybridised on the miRCURY LNA microRNA Array (7th Gen) following a dual-colour experimental design. It was designed to identify and profile microRNAs that are differentially expressed in the different samples. Following this, unsupervised analysis was conducted using a method called principal component analysis (PCA). PCA is a method designed to reduce the dimensions of large data sets and explore the naturally arising sample classes based on the expression profile. The top 50 microRNAs with the largest variation

across all the samples were included allowing for a plot which shows an overview of how the samples cluster based on this variance. Any 'biological' difference between the samples allows for a primary component of the variation, which leads to separation of samples in different regions of the PCA plot according to their underlying biology.

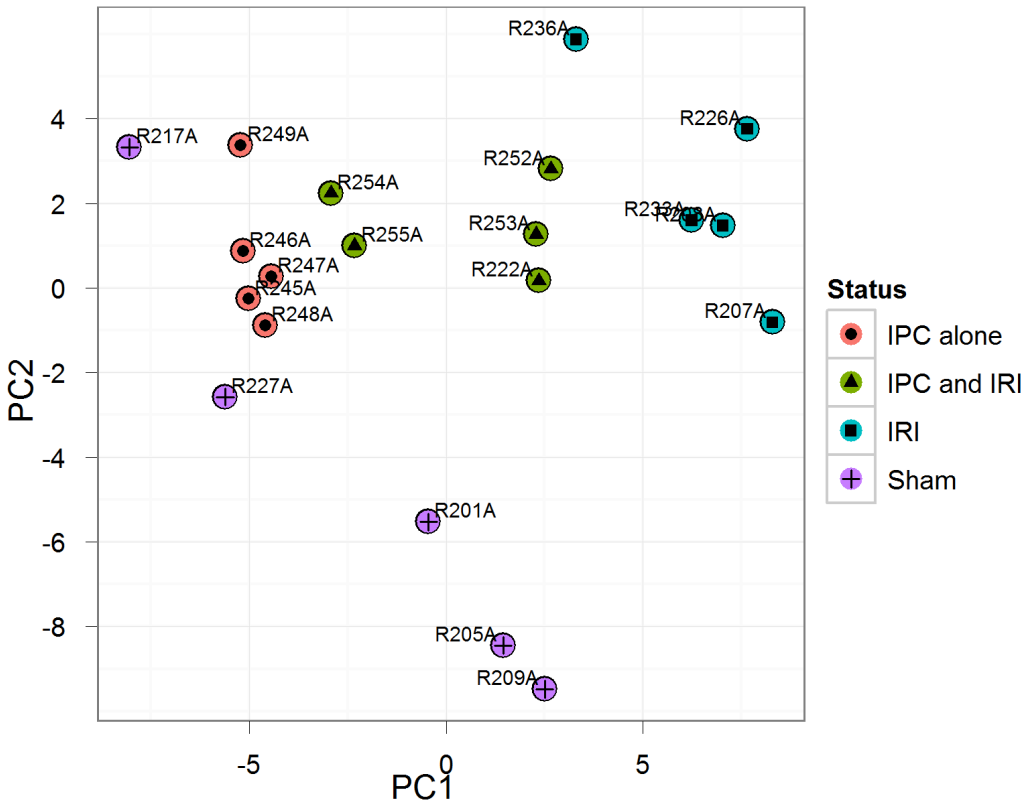

Figure 4.8: Traditional Principal Component Analysis (PCA) from microarray profiling analysis of bilateral sham, IRI, IPC alone and IPC/IRI rats

RNA extracted from kidney tissue of bilateral sham, 45min IRI, IPC alone, and IPC/IRI in rats at 48h after reperfusion was profiled for microRNA analysis using Exiqon microarray. This shows a traditional PCA for the top 50 microRNAs with the highest standard deviation. It provides an overview of how the samples cluster based on their variance and leads to separation of samples in different regions of a PCA plot corresponding to their underlying microRNA biology.
Numbers of animals in each group: Sham (n=5), IRI (n=5), IPC alone (n=5), and IPC/IRI (n=5).

The PCA plot (Figure 4.8) shows that although there is more variation in the sham group samples, overall all the samples cluster according to their biological group. This indicates that the microRNA differences between the groups are the

largest contributors to variation. A heat map diagram was also made which shows the results of a two-way hierarchical clustering of microRNAs and samples (Figure 4.9). The clustering is done using the complete-linkage method together with the euclidean distance measure. As with the PCA plot, the heat map diagram shows that the samples cluster according their biological group.

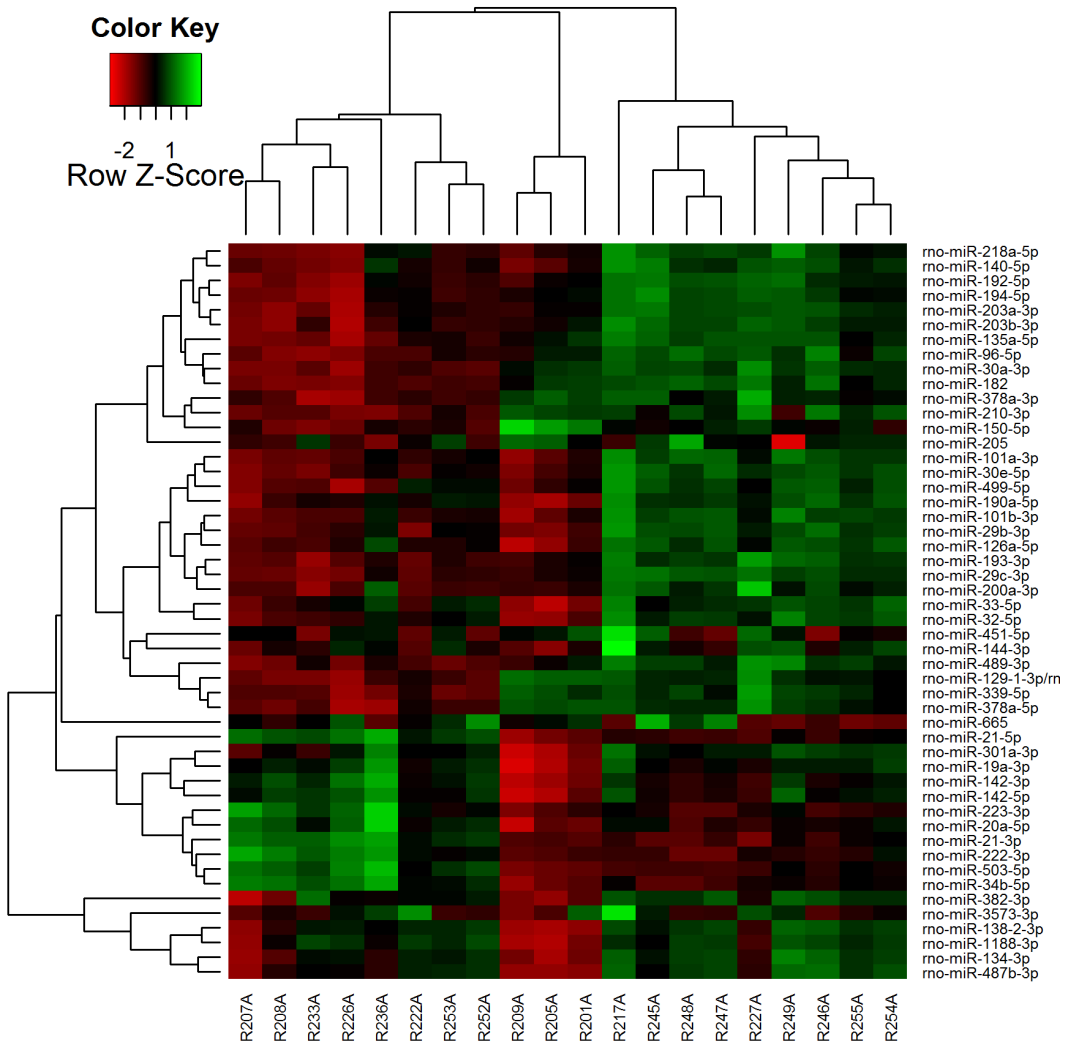

Figure 4.9: Heat Map of unsupervised hierarchical clustering from microarray profiling analysis of bilateral sham, IRI, IPC alone and IPC/IRI rats

The heat map diagram shows the results of a two-way hierarchical clustering of microRNAs and samples. Clustering was performed on the top 50 microRNAs with the highest standard deviation. Each row represents a microRNA and each column represents a sample. The

microRNA clustering tree is shown on the left and the sample clustering tree on the top. The colour scale illustrates the relative expression level of a microRNA across all the samples. Red colour represents an expression level below the mean and green colour represents expression level above the mean.

From the microarray dataset, comparison analyses were conducted between the different groups. Due to the high number of microRNAs being tested in parallel, there is a tendency towards high false positive results, therefore to overcome this, multiple statistical tests were conducted, including the 'Benjamini and Hochberg multiple testing adjustment method'. From this, volcano plots were constructed. When comparing IRI and Sham groups, 125 unique microRNAs were identified as being significantly differentially expressed (27 up-regulated and 98 down-regulated in IRI) (Figure 4.10(A)). An arbitrary cut off for log fold change of 0.3 and -0.3 was used to narrow down the list of differentially expressed microRNAs to 97 (21 up-regulated and 76 down-regulated in IRI). When comparing IRI and IPC/IRI groups, 30 unique microRNAs were identified as being significantly differentially expressed (16 up-regulated and 14 down-regulated in IPC/IRI) (Figure 4.10(B). When applying the fold-change cut-off of >0.3 and <-0.3, this list was reduced to 21 microRNAs (11 up-regulated and 10 down-regulated in IPC/IRI). When comparing IPC alone and sham groups, no microRNAs were identified as being significantly differentially expressed (Figure 4.10(C)).

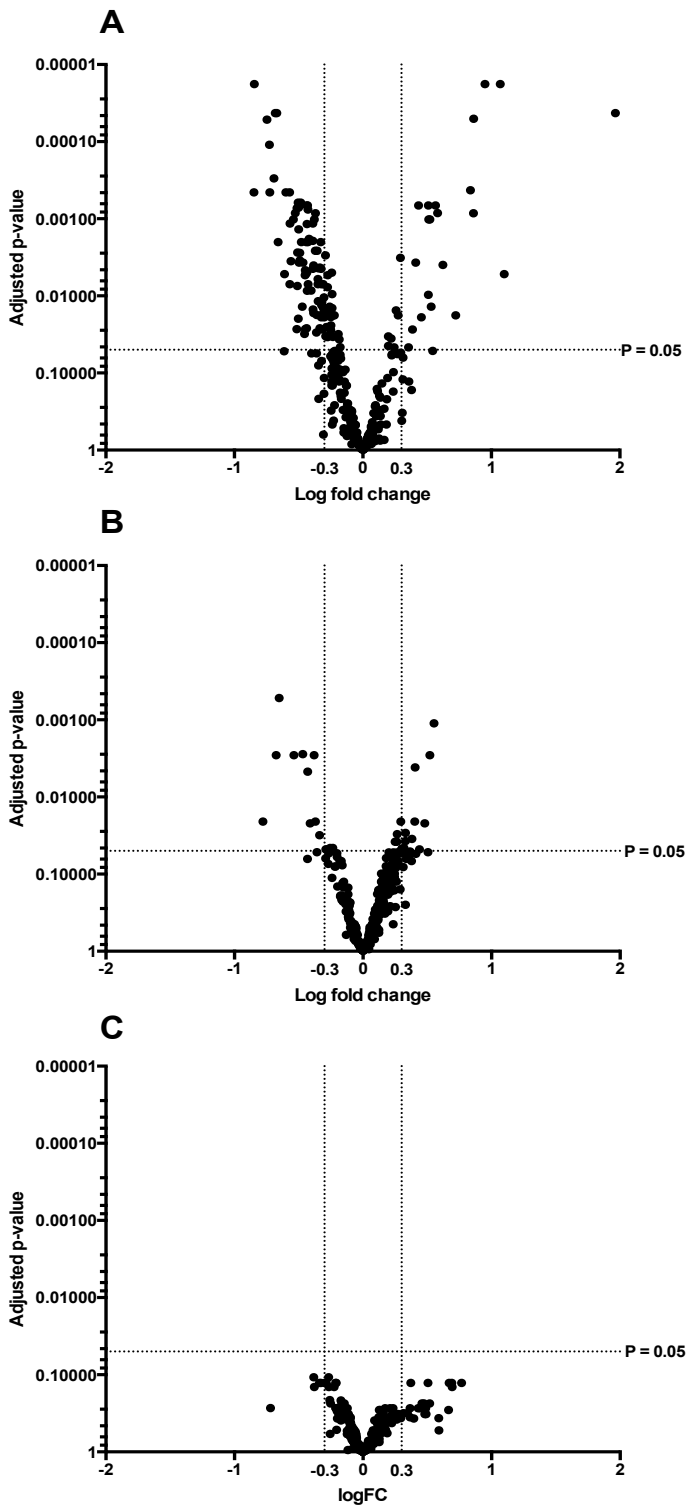

Figure 4.10: Volcano Plots from Microarray analysis

RNA extracted from kidney tissue of bilateral sham, 45min IRI, IPC alone, and IPC/IRI in rats at 48h after reperfusion was sent to Exiqon for Microarray quantification to profile the expression of 309 unique microRNAs (n=5 each). Volcano plots of statistical significance (adjusted p-value) against Log fold change were plotted for **(A)** 'IRI v Sham', **(B)** 'IPC/IRI v IRI', and **(C)** 'IPC Alone' v Sham' demonstrating the most differentially expressed microRNAs.

4.2.4.2 Next Generation Sequencing (NGS)

NGS was performed by Exiqon on RNA extracted from kidney tissue from 4 groups (Sham, IRI, IPC/IRI and IPC alone) (pooled n=1 for each group). The raw sequencing files were received from Exiqon, and subsequently mapped with normalised read counts calculated. The normalised read counts were analysed by chi-square and pair-wise comparison. Chi-square p-values were significant for the majority of the data set. Hierarchical clustering was performed using the GENE-E software and marker selection used to interrogate the pair-wise comparisons. In order to interpret the data set, the normalised read counts were filtered to exclude values below 1000, and the data set was filtered to exclude fold change values of <1.5 increase and decrease. The new data set was then tabulated and visualised on XY plots. When comparing IRI and Sham groups, 37 unique microRNAs were identified as being differentially expressed (18 up-regulated, 19 down-regulated in IRI) (Figure 4.11(A)). When comparing IRI and IPC/IRI groups, 12 unique microRNAs were identified as being differentially expressed (3 up-regulated, 9 down-regulated in IPC/IRI) (Figure 4.11(B)).

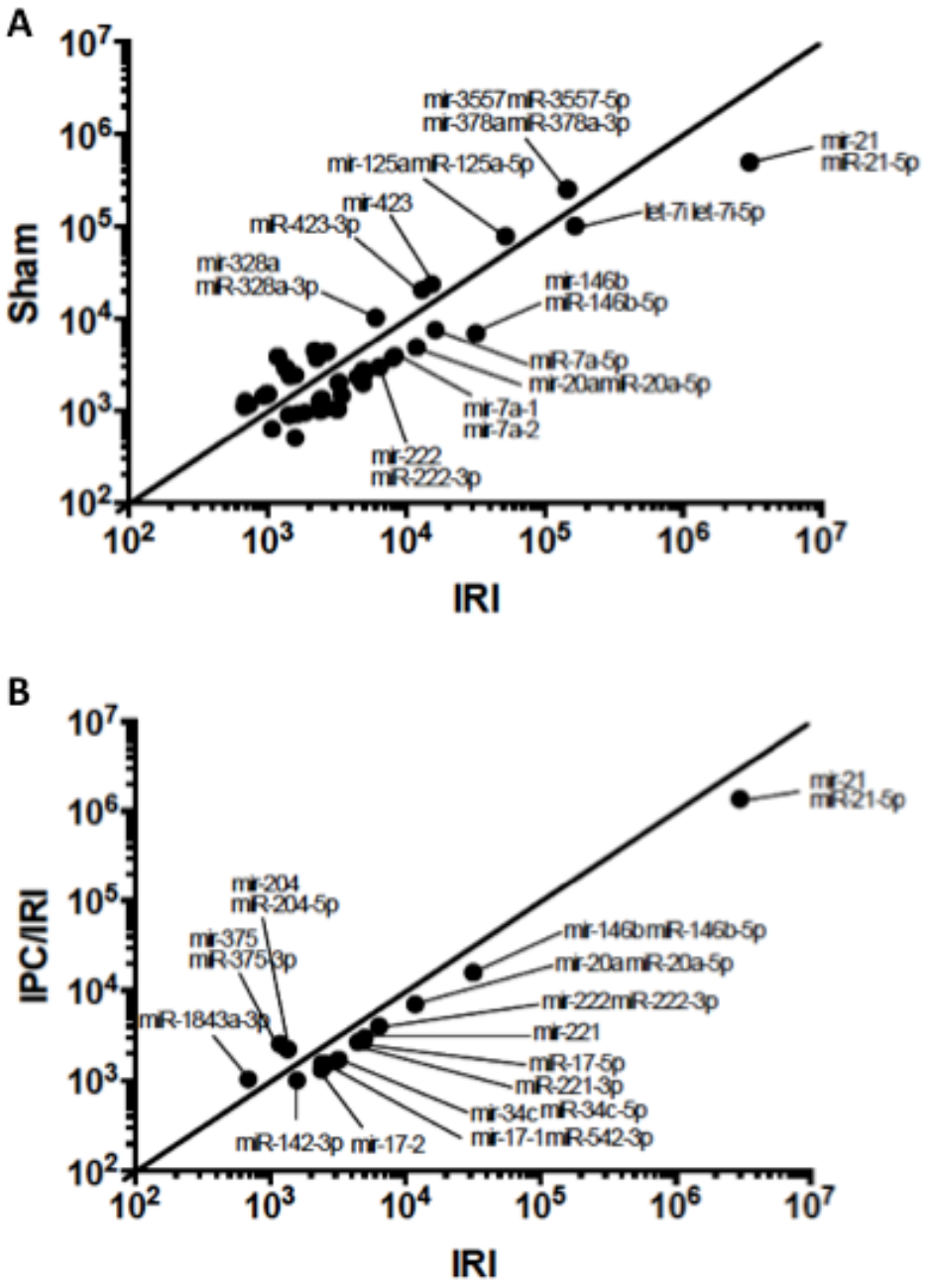

Figure 4.11: Pairwise comparisons from Next Generation Sequencing analysis

RNA extracted from kidney tissue of bilateral sham, 45min IRI, IPC alone, and IPC/IRI in rats at 48h after reperfusion was sent to Exiqon for Next generation Sequencing (NGS) (pooled samples n=1 each). The raw sequencing files received were mapped and normalised read counts calculated, which were then filtered to exclude values below 1000. Hierarchical clustering was performed with GENE-E software and marker selection used to interrogate the pair-wise comparisons. The new data was then filtered to exclude fold change values <1.5 increase or decrease, tabulated and visualised on the above XY plots (**A** and **B** show pair-wise comparisons of 'Sham v IRI', and 'IRI v IPC/IRI' respectively.

4.2.4.3 Comparison of Microarray and NGS data

The microRNA NGS data was compared to the microarray data to identify and narrow selection of candidate target microRNAs for further analysis. Shared regulated microRNAs were identified as 17 and 4 in 'Sham v IRI' and 'IRI v IPC/IRI' comparisons respectively (Figure 4.12).

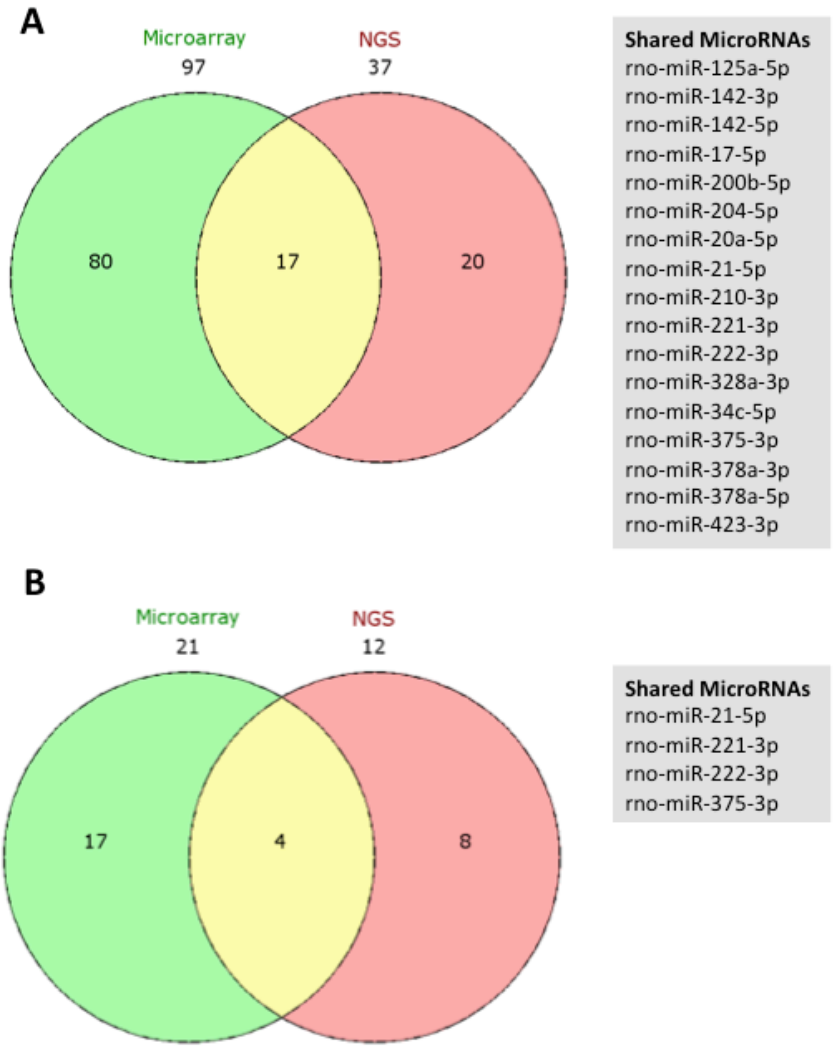

Figure 4.12: Comparison of Microarray and Next Generation Sequencing analyses

RNA extracted from kidney tissue of bilateral sham, 45min IRI, IPC alone, and IPC/IRI in rats at 48h after reperfusion underwent Exiqon Microarray (n=5 each) and Next generation Sequencing (NGS) analyses (pooled samples n=1 each). Venn diagrams **(A)** and **(B)** show the overlap between Microarray and NGS of differentially expressed microRNAs in 'Sham v IRI' and 'IRI v IPC/IRI' respectively.

4.2.5 Target microRNAs

From the NGS and microarray data analyses, the 4 microRNAs that overlapped in the 'IRI v IPC/IRI' analysis (Figure 4.12 (B)) were further analysed. These 4 microRNAs were miR-21-5p, miR-221-3p, and miR-222-3p (up regulated in IRI and down regulated in IPC/IRI), and miR-375-3p (down-regulated in IRI and up-regulated in IPC/IRI).

4.2.5.1 Confirmatory RT-qPCR Analyses

Confirmatory RT-qPCR analysis was then conducted in all of the samples in those 4 groups, showing a highly significant up regulation in IRI for miR-21, miR-221, and miR-222 (Figure 4.13(A)-(C)). Although in both the NGS and microarray analyses miR-375 was down regulated in IRI and up regulated in IPC/IRI, no statistical difference was found between the groups when the confirmatory RT-qPCR was performed in all of the samples for miR-375 (Figure 4.13(D)).

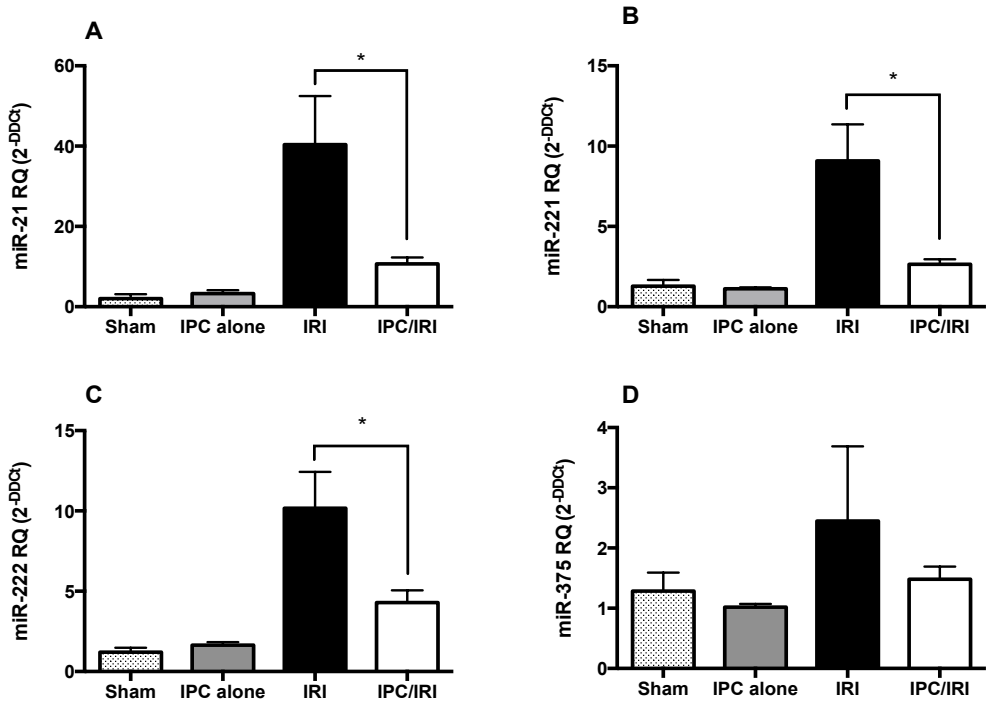

Figure 4.13: Effect of IPC on expression of miR-21, -221, -222, and -375

Confirmatory RT-qPCR analyses of miR-21 (**A**), -221 (**B**), -222 (**C**), and -375 (**D**) in sham, 45min bilateral IRI, localised pulsatile IPC (3 cycles of 2min ischaemia and 5min reperfusion) prior to IRI, and localised pulsatile IPC (3 cycles of 2min ischaemia and 5min reperfusion) alone without any subsequent IRI, in rats at 48h after reperfusion. Expression is normalised to miR-16 and plotted as mean ± SEM. Numbers of animals in each group: Sham (n=8), IRI (n=9), IPC alone (n=8), and IPC/IRI (n=8). Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.5.2 Laser capture micro dissection

Following this, the origin within the nephron of these target microRNAs was tested. For this, a technique called laser capture micro dissection (LCM) was utilised to dissect the following 4 sub-compartments of the nephron: glomeruli, proximal convoluted tubuli, distal convoluted tubuli, and vessels. RNA was extracted and RT-qPCR analyses performed for the target microRNAs (miR-21, miR-221, miR-222, and miR-375) (Figure 4.14).

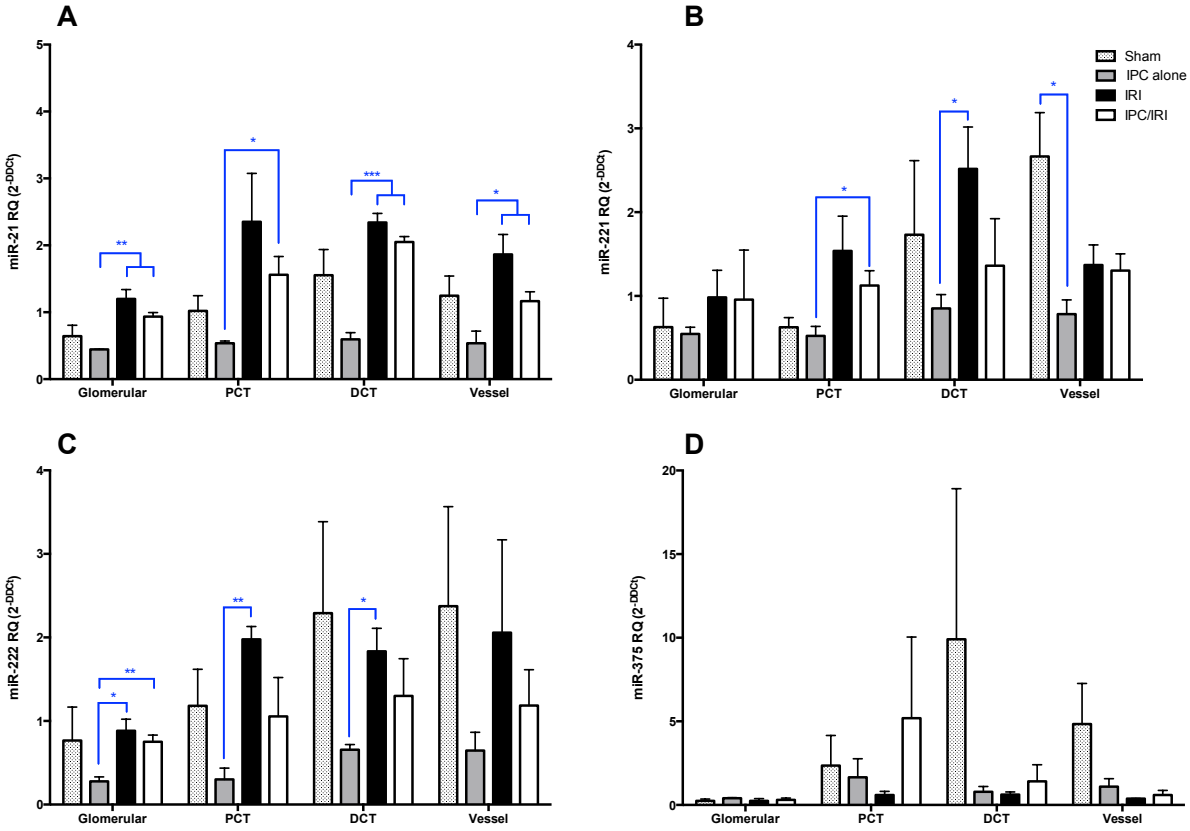

Figure 4.14: Localisation of miR-21, -221, -222, and -375 in different nephron sub-compartments

Renal cortex sections (6 microns) from Sham, 45min bilateral IRI, localised pulsatile IPC (3 cycles of 2min ischaemia and 5min reperfusion) prior to IRI, and localised pulsatile IPC (3 cycles of 2min ischaemia and 5min reperfusion) alone without any subsequent IRI, in rats at 48h after reperfusion were stained with H&E for laser capture micro dissection (LCM). LCM was performed to extract 4 nephron sub-compartments: Glomerular (G), Proximal convoluted tubuli (PCT), Distal convoluted tubuli (DCT), and Vessel (V) for RNA extraction and RT-qPCR analysis of miR-21 (**A**), -221 (**B**), -222 (**C**), and -375 **(D**). Expression is normalised to miR-16 and plotted as mean ± SEM.

Numbers of animals in each group: Sham (n=3), IRI (n=3), IPC alone (n=3), and IPC/IRI (n=3).

Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

These analyses showed that these microRNAs were largely found in each of the 4 sub-compartments of the nephron and that an increased amount of these microRNAs was expressed following IRI. Broadly speaking, an increased amount

of these microRNAs was found in proximal and distal convoluted tubular tissue compared with glomerular and vessel tissue. In addition to this, these analyses showed that there was very little amount of miR-375 expressed within all of these tissues.

4.3 Discussion

The main findings of this chapter are that:

(1) The bilateral IRI model used proved to be robust and reliable in that it produced significant but non-fatal kidney injury, as shown by the histological damage seen to the renal cortex, the mRNA synthesis of AKI markers, and functional assessment with measurement of serum creatinine.

(2) An IPC regime (Three cycles of 2mins ischaemia and 5mins reperfusion) prior to 45-min IRI was found to be beneficial in reducing the structural damage to the kidney and improving kidney function.

(3) Pulsatile IPC regimes were better than continuous IPC regimes

(4) Kidney IRI in the rat was shown to have a unique microRNA signature as demonstrated by 2 separate microRNA profiling techniques.

(5) This microRNA signature of kidney IRI was diminished by IPC, suggesting that it is an injury signature.

(6) The cellular location of these microRNAs within the nephron was largely tubular cells.

The 45-min bilateral IRI rat model used in these experiments has been shown to be a well-established *in vivo* model of kidney injury/IRI (230). It was shown in this chapter and the previous chapter and studies within the literature, that cross-clamping of the renal pedicle for 45mins produces significant but not fatal ischemic injury. In these chapter 4 experiments, the 48h reperfusion period led to clear histological, functional and molecular changes, which are in agreement

with previous studies. Based on all of this, the renal IRI *in vivo* model, as utilised in this chapter, is a robust and reliable model of significant (and potentially reversible) acute kidney injury.

Several IPC regimes were tested in this chapter's experiments. Localised regimes were selected, as they would be easily transferrable to clinical practice. All 3 continuous regimes conferred no benefit and in fact it could be argued that they perhaps worsened the injury to the kidney, based on the higher serum creatinine, higher histological damage scores, and significantly increased expression of AKI markers. This is agreement with some literature, as studies have reported that IPC may not be protective (98, 99, 226). Indeed, these results are also supported by the findings of chapter 3, where a similar continuous IPC regime (15mins ischaemia and 20mins reperfusion prior to IRI) showed no benefit. One study (96) that investigated several continuous localised regimes in a bilateral IRI model (using 40mins as the index ischaemia period) identified that the best IPC regime that offered both structural and functional protection was 15mins ischaemia and 10mins reperfusion. They also found that prolongation of the reperfusion period to 20 or 40mins abolished that protective effect of IPC. This may explain the reason why the 3 continuous regimes, as used in this chapter's experiments, offered no protection, as the reperfusion time was 20mins. Nevertheless, one study by Xu et al identified that a similar IPC regime (15mins ischaemia and 20mins reperfusion) reduced IRI 4 days later (100). Although the IPC regime was similar to the one employed in these experiments, the extended period of 4 days between IPC and IRI may be an important factor in understanding the difference in outcomes.

Several studies have found IPC to be effective in protecting against renal IRI in various animal species (90-94), and this chapter identified some regimes that were protective. Overall, the pulsatile regimes as delivered in this chapter, were beneficial in protecting the kidney from injury. In particular one of these pulsatile IPC regimes, which was chosen, conferred the most protection both structurally and functionally. This is supported by the meta-analysis of Wever and colleagues (95) which concluded that majority of the studies have reported that IPC is associated with significantly improved histological kidney injury scores and renal function (serum creatinine, blood urea nitrogen) following IRI. It may be that the shorter reperfusion time, as utilised for the pulsatile regimes in this chapter's experiments, is an important factor in determining protection.

Within these experiments, its unclear why pulsatile regimes may be better. However, one can speculate that the pulsatile nature of the IPC regime appears to set up the protective signal without itself predisposing to injury. A key question to address in future studies would be how this signal is initiated and propagated to confer its protection. One important observation that can be made from these experiments is that the length of the ischaemia and reperfusion components of the IPC regime appear to be important, as well as the interval between IPC and IRI. Within the literature, a great deal of variation in IPC protocols can be seen, and each of the approaches whether its localised or remote, early or late, and continuous or pulsatile, has been shown in different studies to confer protection to the kidney.

Furthermore these experiments have shown that kidney IRI in the rat has a unique microRNA signature as confirmed by 2 separate profiling techniques (microarray and NGS). Within the literature, 18 *in vivo* studies have been conducted that have evaluated the role of microRNAs within kidney IRI. Of these, only 3 have been conducted in the rat, and 2 (within mice) have investigated IPC in this context. Most of these studies (including this chapter's experiments) have identified miR-21 to be one of the most abundant and important microRNAs within kidney IRI, with significant role being shown in apoptosis (32, 179), autophagy (185), IPC (100, 175) and as a biomarker of kidney injury (172). Although the role of miR-21 still remains to be further fully elucidated, evidence is suggestive of a protective as well as a pathological role (228). In this chapter's experiments, miR-21 expression was significantly increased in IRI, and reduced by IPC, supporting its role as a reliable and useful biomarker of IRI-mediated kidney injury. In other studies however, miR-21 expression is increased by IPC. Xu et al showed that 15mins IPC protected the kidney from injury 4 days later, termed delayed IPC (100). This delayed IPC up regulated miR-21 and was thought to be protective due to the anti-apoptotic properties of miR-21 (100). Another study also showed up regulation of miR-21 was associated with the protective effects of Xenon preconditioning to the kidney (175). A recent study showed that delayed IPC associated with up regulation of miR-21 protected the kidney from injury via HIF-1α by inhibiting its target 'prolyl hydroxylase domain protein 2' (PHD2) (231). This chapter has shown that IPC as employed in these experiments, reduced the expression of miR-21. In all of the above studies, the IPC used has been delayed IPC, and the difference in those studies and this chapter's experiments further proves the fact that differences in IPC regimes

have a significant impact at the molecular level. This could also explain why there is such a difference in results from various studies evaluating IPC. In addition to this, as the role of miR-21 is being elucidated further in various studies, it is becoming clearer that it has a significant role in regulating both protective and pathological molecular pathways (228).

The microRNAs miR-221 and miR-222, were also found to be significantly increased in IRI and decreased by IPC. They are both clustered genes that have identical seed sequences and often work in synergy (232). Neither of these microRNAs has been previously reported within the literature in the context of kidney IRI. Therefore it is a unique and novel finding of this chapter's experiments. It has been reported within the literature that these microRNAs, when elevated, are poor prognostic indicators within renal carcinoma (233), and that they have an important role in regulation of vascular calcification (232). Future studies will need to investigate the role of these microRNAs (on their potential targets) within the context of kidney injury, and may involve manipulation of these microRNAs experimentally to better understand their function. Nevertheless, it can be safely concluded that the experiments undertaken within this chapter have identified a unique microRNA profile of rat kidney IRI, some of which is novel and unique to the literature.

The microRNA profiling techniques used in this chapter's experiments were optimal at the present time. Both 'hybridisation microarray' and 'NGS' techniques were used to profile microRNAs within these experiments, followed by confirmatory RT-qPCR analyses of selected microRNAs. Using both profiling

techniques allowed for the detection of microRNAs that would be robust and reliable for further studies. Both techniques have their advantages and disadvantages. The hybridisation microarray is an established method which when compared with NGS is fairly low-cost and has high throughput in that a larger number of samples are able to be processed per day (234). However, it has lower specificity than NGS and it is not possible to identify novel microRNAs. NGS is able to achieve high accuracy and can distinguish between microRNAs that are very similar in sequence, as well as novel microRNAs and is therefore more unbiased and arguably more robust (235). From this chapter's experiments and previous studies (235, 236), it seems that although there is an overlap between the microRNAs that are profiled between the 2 techniques, this is not a complete overlap and there are clear differences. This difference/lack of complete overlap may be due to the fact that each of the approaches may preferentially select a specific cohort of microRNAs. For the purposes of this chapter's experiments, the microRNAs that overlapped between the 2 profiling methods in 'IRI vs. IPC/IRI' groups were chosen as the candidate microRNAs. Nevertheless, the other microRNAs identified with either of the techniques should not be ignored and may play an extremely important role in kidney IPC and IRI.

It was also shown in this chapter's experiments that the microRNAs were found in glomerular, tubular (proximal and distal) and vascular tissue components of the renal cortex, although they were in higher concentration within tubular cells. This would be in keeping with the fact that miR-21 is likely to be an injury marker that is released following injury and that because tubular cells are more

prone to damage following IRI, more miR-21 is released by them. In addition to this, a therapeutic strategy of IPC has been shown to diminish this microRNA signal, suggesting that it is indeed an injury signal. This raises the intriguing possibility that these microRNAs are injury biomarkers, which could potentially be measured in the urine. Therefore one of the next questions that seem pertinent to investigate is the role of microRNAs as biomarkers of kidney injury in the clinical context of kidney transplantation.

Chapter 5 - The urinary microRNA expression profile of Delayed Graft Function in Human Kidney Transplantation

5.1 Introduction

In the previous chapter, an *in vivo* model of kidney IRI was established, and it was shown that IPC, a therapeutic strategy, reduced kidney injury both structurally and functionally. In addition to this, it was shown that IRI has a unique microRNA profile and IPC changes that profile, suggesting that microRNAs are functionally important in the kidney's response to ischaemic injury, and maybe useful as biomarkers of kidney injury. The next objective of this thesis was to evaluate the utility of these microRNAs as biomarkers of kidney injury in the context of transplantation. A biomarker that informed about presence and extent of renal IRI would have significant value within the field of kidney transplantation, as the increased use of ECD, DCD and marginal donors has increased the impact of IRI and consequent risk of DGF and PNF (11-13). DGF is an important clinical consequence of IRI and patients with DGF have increased complications and poorer long-term outcomes (27-29). Identifying a (non-invasive) biomarker of the extent of IRI and hence DGF, will allow prediction of outcome and tailored management for transplant patients. Development of this biomarker will also allow for testing of potential treatments to attenuate IRI and reduce the risk of DGF.

Having identified a profile of microRNAs differentially regulated in IRI and IPC in Chapters 3 and 4, and knowing that microRNAs are tissue-specific, and stable in many mediums including serum and urine, they seem supreme candidates as biomarkers. Urine is an excellent bio fluid in the context of kidney pathology, as its molecular composition has been shown to strongly reflect intra-renal events

(237). It is also very attractive because it is readily available, easy to collect and hence allows for multiple large volume collections. This research group has developed a robust laboratory-based methodology for the isolation and quantification of urinary microRNAs (237, 238). At the time of these experiments, no one had evaluated microRNAs as biomarkers of DGF in this context. Studies had evaluated urinary microRNAs as biomarkers of acute rejection, suggesting that microRNA changes post-transplantation can be easily detected in urine and provide clinically useful information. Therefore, to undertake this study, I decided to measure microRNAs within urine samples of patients immediately post-transplantation in a cohort of patients that went on to develop DGF and one that did not.

The aims of this chapter were to:

(1) Determine the microRNA expression profiles in urine samples from kidney transplant patients 24 hours post-transplantation;
(2) To assess the utility of these data to discriminate between patients with and without DGF.

5.2 Results

Thirty-three consecutive kidney transplant recipients were recruited at the Cardiff Transplant Unit. Recruitment took place until there were at least 10 patients per group: Living donor kidney transplant without DGF (LD-No DGF) (n=10), Cadaveric donor kidney transplant without DGF (CD-No DGF) (n=10), and Cadaveric donor kidney transplant with DGF (CD-DGF) (n=13). All patients gave informed consent for samples to be collected into the All Wales Kidney Research Tissue Bank. Urine samples were collected on days 1 to 5 post-transplant and RNA was extracted for profiling and RT-qPCR analysis of microRNAs.

5.2.1 Patient population

Donor and Recipient demographics are described in Table 5.1. The overall median age of the donors was 48 (range 12 – 76). 'L-No DGF' donors were younger than both cadaveric groups, as expected, although the oldest 'LD-No DGF' donor was 66, reflecting the current practice at Cardiff Transplant Unit. Of the deceased donors, 11 (48%) were DBD and 12 (52%) DCD organs. Overall median cold ischaemic time (CIT) was 636 minutes. However, as expected, for the 'LD-No DGF' group, the CIT was more than 4 times less than of both the cadaveric groups. The median length of stay in the hospital following transplantation was 6, 7 and 19 days for 'LD-No DGF', 'CD-No DGF', and 'CD-DGF' groups respectively. Two patients were lost to follow up (moved to out of area) and the median duration of follow up was 27 months (range 24 – 29).

		Overall	Living donor without DGF	Cadaveric donor without DGF	Cadaveric donor with DGF
Donor median age in years (range)		48 (12 – 76)	43.5 (27 – 66)	54 (20 – 75)	65 (12 – 76)
Donor gender	Male	17	3	4	10
	Female	16	7	6	3
Donor cause of death	ICH	10	-	3	7
	HBI	2	-	2	0
	ICT	2	-	1	1
	Other	9	-	4	5
Median Cold Ischaemic Time in minutes (range)		636 (46 – 1512)	192 (46 – 253)	870.5 (425 – 1512)	884 (456 – 1270)
Deceased Donor Type	DCD	12	-	3	9
	DBD	11	-	7	4
Recipient median age in years (range)		53 (18 – 76)	50.5 (18 – 61)	43 (19 – 74)	56 (39 – 76)
Recipient gender	Male	23	6	7	10
	Female	10	4	3	3
Dialysis Status	Pre-dialysis	5	5	0	0
	HD	18	3	5	10
	PD	10	2	5	3
Median Length of hospital stay in days (range)		8 (4 – 44)	6 (4 – 12)	7 (5 – 15)	19 (8 – 44)
Recipient cause of renal failure	DM	3	0	1	2
	IgA N	4	2	0	2
	GN	4			4
	APKD	6	4	1	1
	FSGS	5	2	2	1
	Other	12	3	6	3
Median no. of HLA mismatches (range)		4 (0 – 6)	4 (0 – 5)	4 (0 – 6)	4 (0 – 6)

Table 5.1: Donor and Recipient Demographics

Consecutive kidney transplant patients were recruited and separated into 3 groups: living donor kidney transplant without DGF (LD-No DGF); cadaveric donor kidney transplant without DGF (CD-No DGF); and cadaveric donor kidney transplant with DGF (CD-DGF). Donor and recipient demographics are described in the table above.

5.2.2 Clinical Outcomes

There was no patient mortality during the follow-up period, however there were 4 graft failures. One patient had a dual kidney transplant with thrombosis of one of his kidney grafts at 2 days postop and failure of the other graft at 2 months postop; 2 failures were due to recurrence of primary disease at 11 and 22 months; and 1 failure was secondary to acute rejection at 12 months. Eight (24%) patients experienced at least one episode of acute rejection following their transplant within the first year. Rejection rates were 30%, 10%, and 31% in the 'LD-No DGF', 'CD-No DGF', and 'CD-DGF' groups respectively ($p = 0.452$). Median (and range) eGFR at 6 and 12 months post-transplantation was 58.5 (18 – 94) ml/min and 56 (15 – 100) ml/min. There was no significant difference in eGFR during the follow up period between the 3 groups ($p > 0.9$) (Figure 5.1).

5.2.3 MicroRNA profile in DGF

Following RNA extraction, from urine samples collected at 24h post-transplantation, a Taqman Low Density Array (TLDA) was performed to quantify and determine the expression of 381 microRNAs (377 unique and 4 control microRNAs) in a "discovery set" comprising urine of 4 transplant recipients from 'LD-No DGF' group compared to 4 from 'CD-DGF' group. This profiling quantification showed that microRNAs were differentially expressed and that majority of these 377 unique microRNAs profiled, were up-regulated in the 'CD-DGF group' compared with the 'LD-No DGF' group (Figure 5.2).

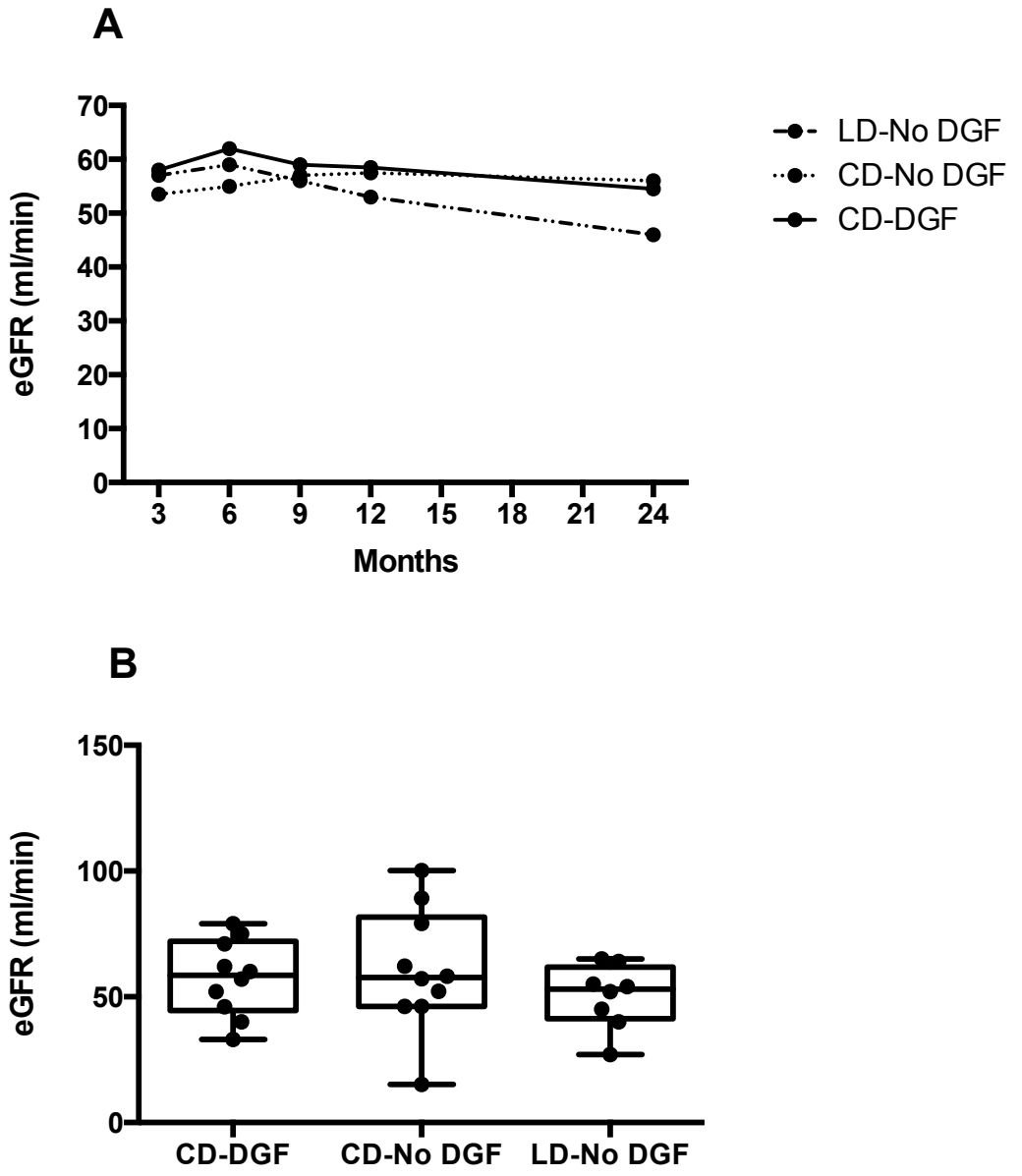

Figure 5.1: Estimated glomerular filtration rate post-transplantation

Consecutive kidney transplant patients were recruited into 3 groups: Living donor kidney transplant without DGF (LD-No DGF); Cadaveric donor kidney transplant without DGF (CD-No DGF); and Cadaveric donor kidney transplant with DGF (CD-DGF). The estimated glomerular filtration rate (eGFR) (in ml/min) was measured using the Modification of Diet in Renal Disease (MDRD) formula at several time points post-transplantation, and median values plotted in **[A]**. Median eGFR (ml/min) at 12 months was also plotted **[B]**. The boxes indicate first and third quartiles and the median, the whiskers indicate the maximum and minimum of the data, and individual values are shown as dots. Number of patients in each group: LD-No DGF (n=10); CD-No DGF (n=10); and CD-DGF (n=13).

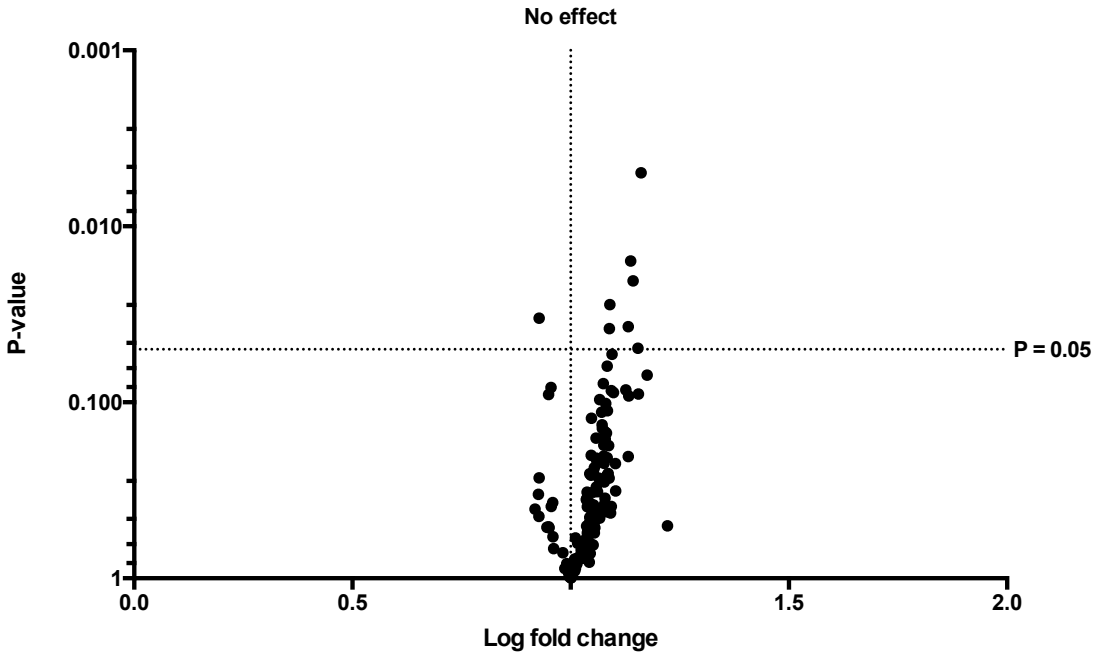

Figure 5.2: Volcano plot comparing microRNA expression in first pass urine from cadaveric donors subsequently developing versus not developing delayed graft function (DGF)

Taqman Low Density Array (TLDA) quantification was performed to profile the expression of 377 microRNAs in urine collected 24 hours post-transplantation in a "discovery set" comprising first pass urine of 4 recipients of living donor kidneys who did not subsequently develop DGF (LD-No DGF) compared to 4 recipients of Cadaveric (Deceased after Cardiac Death (DCD)) kidneys with DGF (CD-DGF). Independent profiling was performed on each urine sample before normalisation to miR-cel-39. Relative expression was calculated by the delta delta Ct method (213). MicroRNAs that were undetectable in more than 1 sample were excluded, leaving 137 microRNAs displayed in this plot. A volcano plot of statistical significance (p-value) against fold-change was plotted between 'LD-No DGF' and 'CD-DGF'. The most differentially expressed microRNAs were selected as candidates for further analysis (miR-9, -10a, -21, -29a, -221, -429, -506, and -574-3p).

Moreover, 7 microRNAs were significantly up-regulated and 1 microRNA significantly down-regulated, in the 'CD-DGF' group when compared with the 'LD-No DGF' group. These 8 microRNAs that were most differentially expressed based on fold change and significance level were selected as candidates for further analysis/validation in a larger sample set.

5.2.4 Validation of target microRNAs

From the TLDA profiling quantification, 8 microRNAs (miR-9, -10a, -21, -29a, -221, -429, -506, and -574-3p), which were most differentially expressed in 'CD-DGF' group compared with 'LD-No DGF' group 24h post-transplantation, were chosen for confirmatory RT-qPCRs in all patient urine samples. MiR-9 was down-regulated and the remaining 7 microRNAs up-regulated in the 'C-DGF' group compared with 'L-No DGF' group in the TLDA quantification. However, when all of the patient samples were analysed for confirmatory RT-qPCR analysis, miR-9 was also significantly up regulated in the 'CD-DGF' group compared with both 'LD-No DGF' and 'CD-No DGF' groups. Expression of miR-9, -10a, -29a, -221, and -429 was significantly up-regulated with ≥5-fold change in the 'CD-DGF' group compared with both 'LD-No DGF' and 'CD-No DGF' groups, and expression of miR-21 and -574-3p was significantly up regulated with ≥20-fold change in the 'CD-DGF' group compared with both 'LD-No DGF' and 'CD-No DGF' groups, at 24h post-transplantation (Figure 5.3).

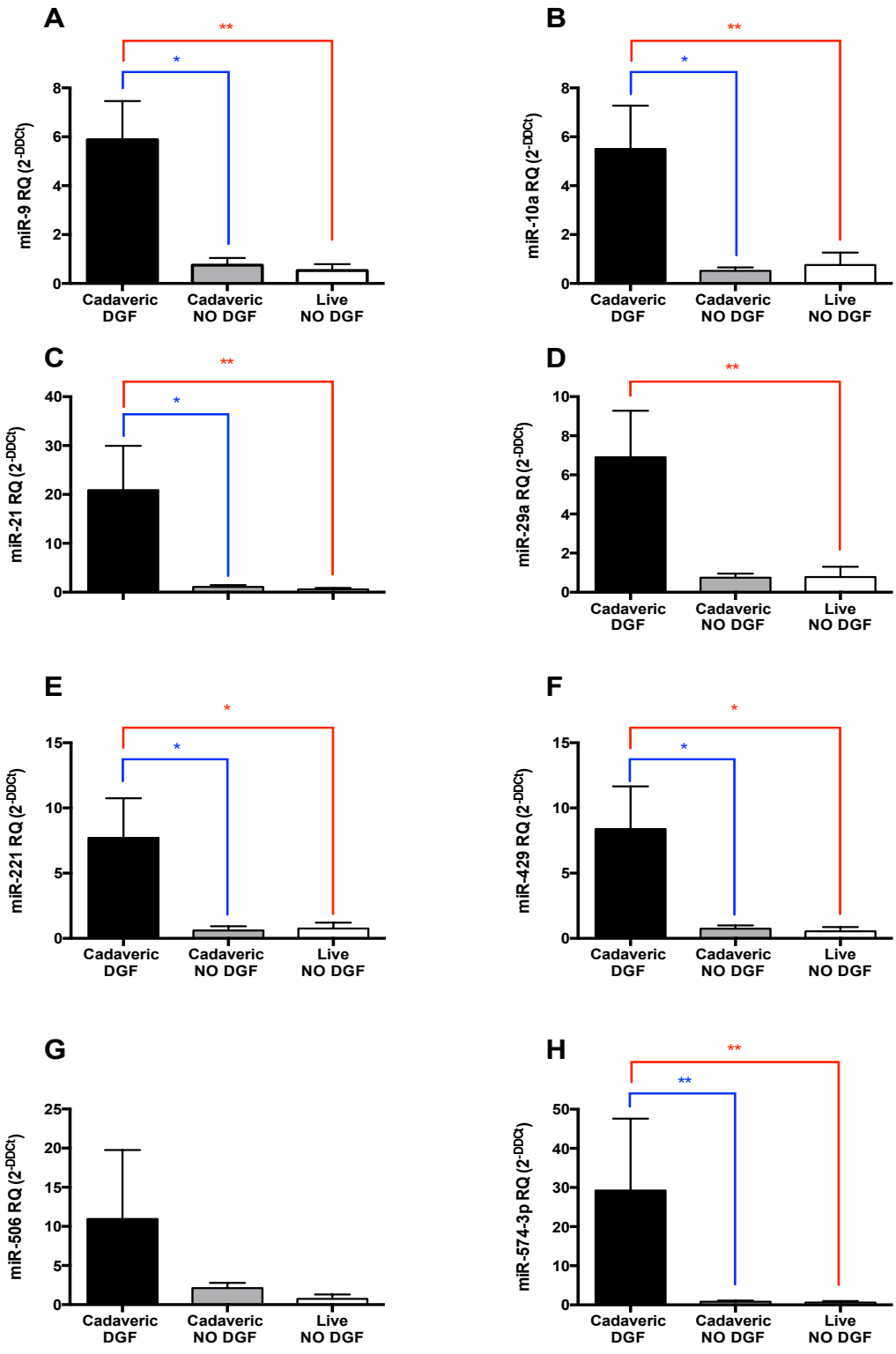

Figure 5.3: RT-qPCR analysis of microRNAs differentially expressed in DGF

MicroRNAs identified as differentially expressed in TLDA profiling (Figure 32) were quantified by RT-qPCR in first pass urine from the full set of 33 patients, whose donor and recipient characteristics are described in Table 4. MicroRNAs were quantified by Taqman individual microRNA assay, and data are normalised to miR-Cel-39 before calculation of relative expression by the delta delta Ct method (213). 6 of 7 up regulated microRNAs were validated. Mir-9, found

to be down regulated by TLDA analysis, was actually significantly up regulated in the CD-DGF group upon this confirmatory RT-qPCR analysis.

Data are plotted as mean ± SEM. Number of patients in each group: LD-No DGF (n=10); CD-No DGF (n=10); and CD-DGF (n=13). Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.2.5 Correlation with graft function

The expression of the target microRNAs identified from the TLDA profiling quantification (miR-9, -10a, -21, -29a, -221, -429, -506, and -574-3p) at 24h post-transplantation, was correlated with eGFR at 6, 12 and 24 months post-transplantation. No significant correlation was identified (R^2 <0.1, p = ns).

5.2.6 MiR-21 expression profile in the 1st 5 days post-transplantation

The above data has shown clear differences between the DGF and 'No DGF' groups at 24 hours post-transplantation. Following this, the time course of these changes post-transplantation was assessed, as that would be relevant for their potential use as urinary biomarkers in this context. MiR-21 was measured because it has been shown to be an important microRNA biomarker of IRI from our previous studies and within the literature, particularly in animal studies (32, 100, 228). The expression profile of miR-21 was determined within urine of transplanted patients in the immediate 5 days post-transplantation in the 3 groups. The expression of miR-21 in urine was significantly up-regulated in the 'CD-DGF' group compared with both the 'No DGF' groups during the 1st five days post-transplantation ($p < 0.05$) (Figure 5.4). When the 'CD-No DGF' and 'LD-No DGF' groups were compared, no significant difference in miR-21 expression levels was found.

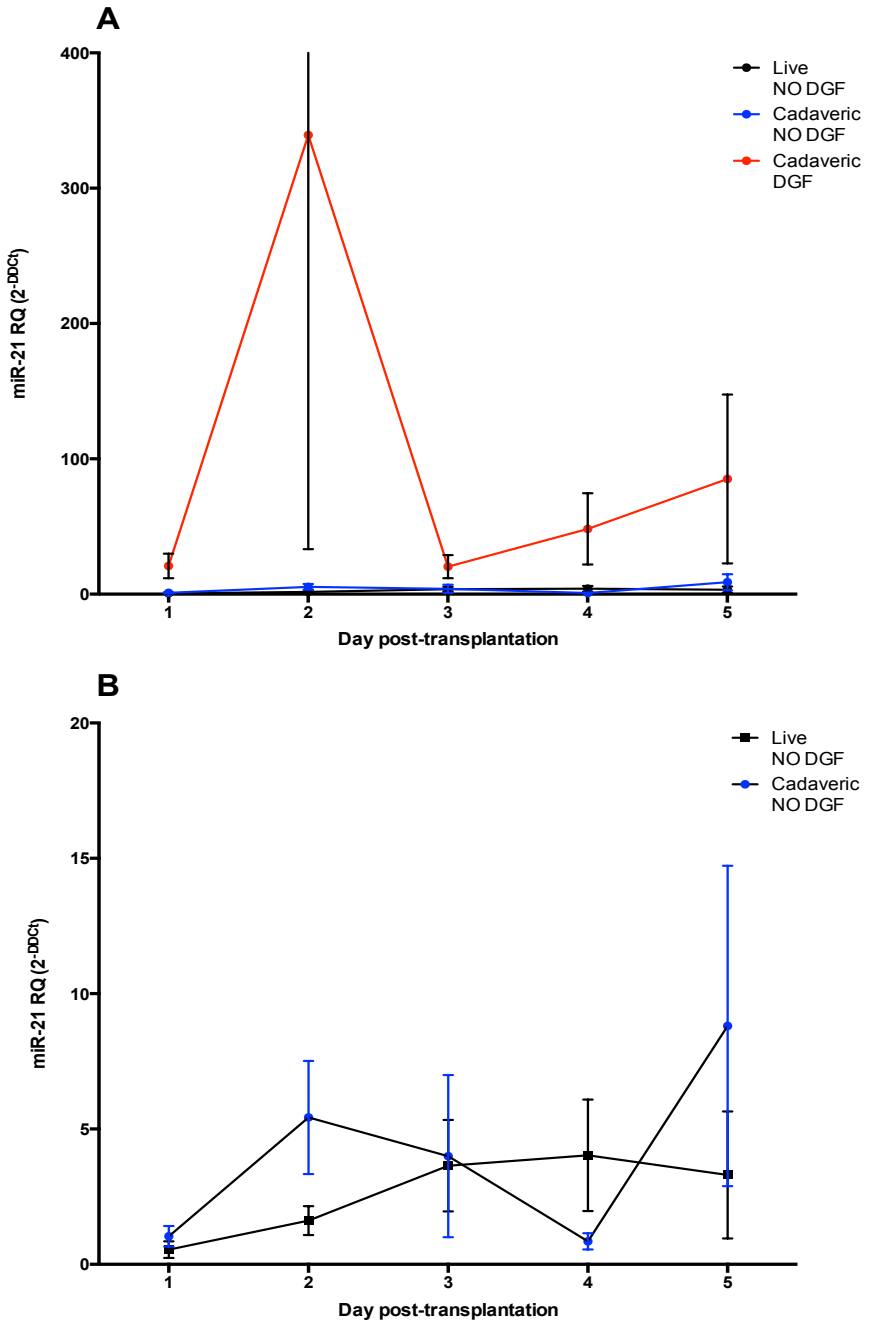

Figure 5.4: The urinary expression profile of miR-21 post-transplantation

MicroRNAs identified as differentially expressed in TLDA profiling (Figure 32) were quantified by RT-qPCR in first pass urine from the full set of 33 patients, whose donor and recipient characteristics are described in Table 4. MicroRNAs were quantified by Taqman individual microRNA assay, and data are normalised to miR-Cel-39 before calculation of relative expression by the delta delta Ct method (213). 7 up regulated microRNAs were validated, of which miR-21 was one of the most differentially expressed at 24h post-transplantation. RT-qPCR was performed for miR-21 to determine its expression profile in the first 5 days post-transplantation. Data are plotted as mean ± SEM. Number of patients in each group: LD-No DGF (n=10); CD-No DGF (n=10); and CD-DGF (n=13). Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.3 Discussion

MicroRNAs are clearly emerging as important biomarkers in the context of kidney injury and transplantation. This chapter shows that the expression of miR-9, -10a, -21, -29a, -221, -429, and -574-3p in urine on day 1 post-transplantation is predictive of DGF. Moreover, the expression profile of miR-21 remained strongly predictive of DGF in the first 5 days post-transplantation.

To the best of my knowledge, this is the first study that has evaluated the urinary microRNA profile in transplant patients in the immediate post-transplantation period, and indeed it is the only study that has evaluated the microRNA profile within DGF.

Development of these microRNAs into non-invasive biomarkers of injury has the potential for profound implications in the field of kidney transplantation. Predicting outcomes post-transplantation currently relies on clinical examination, serum creatinine measurement, and invasive biopsy of the kidney graft. Blood and urine testing occur daily in all post-transplant patients, and invasive biopsy of the kidney graft is frequently employed in order to differentiate DGF from other possible causes of graft dysfunction such as acute rejection. Biopsy of the graft is an invasive procedure that is uncomfortable and painful for the patient, and can cause significant bleeding potentially leading to graft loss, the risk of which is increased in the immediate post-transplant period (20).

MicroRNAs detected within the urine largely arise from the urogenital tract. This is supported by recent work from our laboratory, which has shown that: (i) MicroRNAs are readily detected in the urine of kidney failure patients (237); (ii) Urinary microRNAs are very informative about important clinical changes in diabetes (in preparation); and (iii) that microRNAs are stabilised in the urine by Ago2 and exosome association (212).

There is accumulating evidence that microRNAs play a key role in AKI of various aetiologies including IRI ((32, 164, 216) and Chapters 1-2). MiR-21 in particular has been shown to have a significant role in kidney IRI. Tubular cell apoptosis is a hallmark of kidney IRI, and although the full role of miR-21 is yet to be determined, it has been attributed to anti-apoptotic and metabolic functions (32, 227, 239). Indeed we have shown in Chapters 1 and 2, that kidney IRI in an *in vivo* model is associated with significantly increased expression levels of miR-21, supporting its role as a marker of IRI-mediated kidney injury (240). Consistent with this, in the present study miR-21 expression levels within urine of DGF patients was significantly increased. Moreover, this expression level remained significantly increased compared to the 'No DGF' groups for the first 5 days post-transplantation. In addition, miR-221 was found to be up regulated within both DGF (in this study) and IRI in the rat kidney model (Chapter 2).

MicroRNAs have been investigated as potential biomarkers in determining acute rejection in transplant patients (188-190). One study in particular identified 3 microRNAs, which were dys-regulated in acute rejection: miR-10a was up regulated, and miR-10b and -210 down regulated. The study went on to conclude

that urinary miR-210 in particular was able to discriminate between treated and un-treated acute rejection (190). Indeed in the present study, we also showed that miR-10a was significantly up regulated in the DGF group. This supports the fact the miR-10a may be a useful biomarker of AKI in transplant patients, however non-discriminative in whether the AKI is secondary to DGF or rejection. Another study also identified urinary miR-10a as sensitive and specific biomarker of AKI in a mouse model of kidney damage secondary to IRI or stretozotocin-induced diabetes (174), further supporting evidence that it is a useful biomarker of AKI secondary to a variety of pathologies.

In terms of clinical outcomes within this cohort of patients, it is clearly evident that overall this group of patients had a good graft function with no patient mortality during the follow-up period. There was no statistical difference in eGFR between the 3 groups, suggesting that even those patients with DGF recovered well with eventual good function. It would be interesting to note their graft function over a longer period of time. It is perhaps because of such good and indifferent graft function between the groups that the expression levels of the microRNAs did not correlate with eGFR.

Profiling techniques are continuously developing, and in the future it may be possible to apply alternative approaches including 'Next Generation Sequencing' (NGS) to profile microRNAs in low abundance clinical samples, such as I have done here. However, a previous attempt to do this for RNA from laser capture micro dissected glomeruli in this laboratory was not successful, despite working collaboratively with one of the world-leading authorities in this technique (C

Carrington, personal communication). Therefore I selected the TLDA quantification method for profiling the microRNAs was selected in this cohort of patients, which had recently been optimised in this laboratory for use with urine samples. TLDA quantification is a 384-well plate format that allows the simultaneous determination of the expression of 377 unique microRNAs (Human MicroRNA Panel Card A v.2.0).

RNA is extracted from urine is in low quantities, and our current established methodology for RNA extraction from urine requires the use of a carrier RNA, hence making it difficult for an accurate measurement of RNA quantity within the sample. Hence, miR-cel-39 is included as a spike in control, allowing evaluation of extraction efficiency between samples. Choice of normaliser is also a challenge in studies such as this. At present there is no single "gold standard" to overcome this limitation, and my approach in this study/set of experiments is deemed as acceptable practice (241).

One limitation of this study is the relatively small sample size within the 3 groups, increasing the possibility of a type 2 statistical error. Previous studies that have investigated microRNAs as biomarkers in kidney transplantation have also had relatively small number of patients, with one study reporting their findings with n of 3 in each group (188). The substantial fold-changes and high level of statistical significance that I have found in this dataset is suggestive that these findings represent real and important differences in the groups that have been studied. Furthermore, the link to outcome (risk of DGF) highlights the potential utility of these as novel biomarkers. It will be important to evaluate

these further in larger sample cohorts, and to this end the laboratory is in discussion with the tissue banks led by Prof Neil Sheerin in Newcastle University and Prof Rutger Ploeg in Oxford University, to enable this next phase of the work.

It is clearly evident that microRNAs are emerging as important biomarkers in multiple disease processes including kidney injury and transplantation. Further refinement of the microRNA data reported in this study is needed to identify a non-invasive biomarker panel that discriminates reliably between DGF and other pathologies, such as acute rejection. The identification and refinement of a biomarker that is specific to kidney injury in the context of transplantation would allow for its potential use in determining outcomes prior to transplantation. Such a thing would so advantageous to the transplant surgeon, as given the fact that the organs used are increasingly from ECD, DCD, marginal and older donors, any methodology that allows for accurate prediction of outcomes prior to their implantation, would aid in the decision making process of usage or discard and selection of most suitable recipient.

Chapter 6 - Can microRNA-21 expression in hypothermic machine perfusate predict early outcomes in Clinical Kidney Transplantation?

6.1 Introduction

In the previous chapter, microRNAs were profiled in urine samples 24h post kidney transplantation in patients subsequently developing DGF and in those not developing DGF. The previous chapter's findings were that 7 microRNAs were significantly up regulated in DGF and were/are therefore potentially useful biomarkers of kidney injury in this context. Moreover, it was shown that miR-21, one of these 7 microRNAs, was not only a highly sensitive biomarker of DGF but that it remained significantly up regulated in the first five days post-transplantation within the DGF group. It was also shown that miR-21 is highly expressed in the kidney and prominent in the signature of microRNAs increased by IRI (Chapters 3 and 4).

Currently in the field of kidney transplantation, with the increased usage of marginal and ECD organs, there is a real need for a reliable measure of organ quality prior to transplantation. The lack of such a biomarker limits the capability of the transplant team to predict graft performance following transplantation, especially in the case of marginal donor kidneys. Such a biomarker would potentially be 'revolutionary' as it would aid transplant surgeons in the decision making process of organ usage or discard, and selection of the most suitable recipient.

Hypothermic machine perfusion is a technique used to preserve organs and has been shown to reduce the incidence of DGF when compared with static cold storage (76). There is great interest at present in potential measures of organ

quality that may be assayed from the hypothermic machine perfusate (HMP) solution, and that may inform about the quality of the donated organ prior to making the decision to commit to transplantation. MicroRNAs have not previously been studied in this context. With this background in mind, the aims of this chapter were:

1. To determine whether microRNAs could be extracted from HMP by adapting the laboratory's established methods for RNA extraction from urine samples.
2. To evaluate miR-21 quantification in HMP as a sentinel for outcome following kidney transplant.

6.2 Results

6.2.1 Stability of microRNAs in HMP samples

To test the stability of microRNAs within HMP, samples were first collected from 2 donor kidneys (1 DCD, 1 DBD) placed on the hypothermic machine perfusion system at 1 h after perfusion. Samples were centrifuged at 2,000*g* for 10 min at 4°C at 0h and then maintained at room temperature, with aliquots of 350µl taken at 0, 6, 12 and 24h. Aliquots of 350µl were also taken at these time points from samples not subjected to centrifugation. Samples showed stability of microRNA signal at time points up to 6h following collection. Twelve to twenty-four h samples showed increasing variation in retained microRNA signal, consistent with partial degradation, with delay of >6 hours between sample collection and RNA extraction, but stability of endogenous microRNAs at earlier time points (Figure 6.1). The urine development work done in this laboratory has shown the importance of a centrifugation step for preservation of microRNA signal (C Beltrami and K Simpson, personal communication) and I therefore evaluated microRNA signal for time points to 24h following HMP sampling both with and without centrifugation. However, for the remainder of the experiments, I chose to use the established protocol, which included centrifugation as an important step.

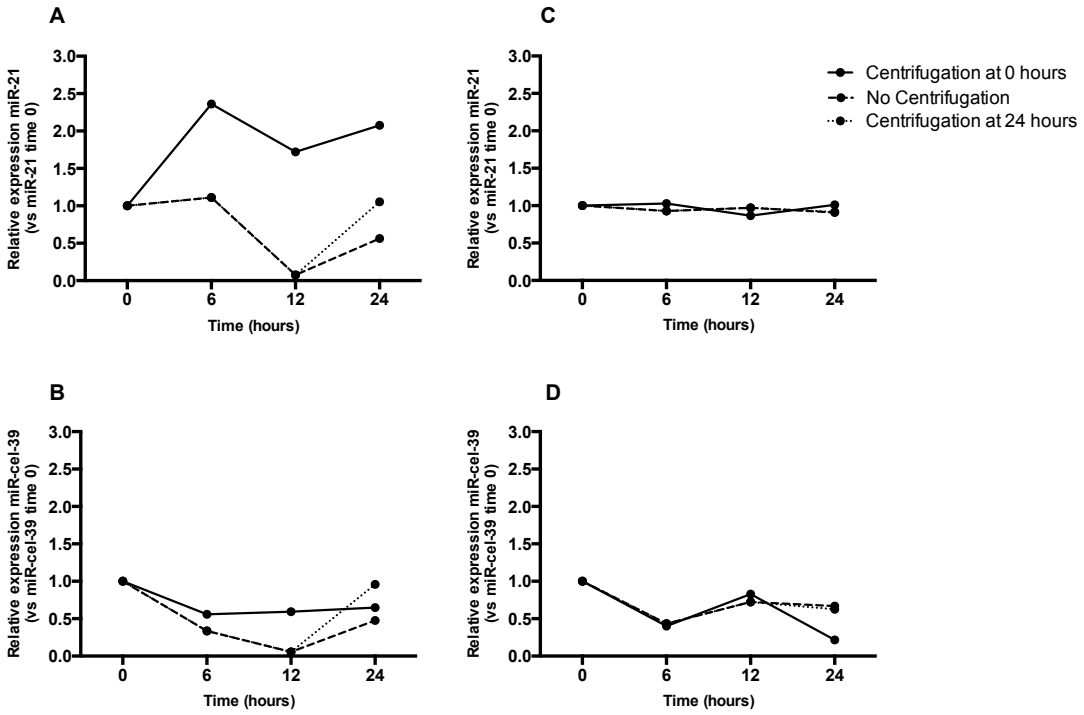

Figure 6.1: MicroRNA recovery from HMP samples

HMP samples were taken from 2 donor kidneys (1 DBD, 1 DCD) placed on the Life Port® at 1h after perfusion. Following RNA extraction using miRNeasy Mini Kits (Qiagen), RT-qPCR analysis was performed for miR-21 and cel-miR-39 at 0, 6, 12 and 24h at room temperature for samples which were centrifuged at 0h, 24h or not centrifuged. Relative expression of endogenous miR-21 and exogenous miR-cel-39 were plotted over 24h for 1 DBD [A, B] and 1 DCD [C, D] kidney respectively.

6.2.2 Patient Population

Eleven ECD kidneys were placed on the Life Port® hypothermic machine perfusion system following backbench preparation for a median 627mins (range 117 – 1027) using Kidney Preservation Solution-1 (KPS-1) (Organ Recovery Systems, Chicago, USA), prior to transplantation at Cardiff Transplant Unit (University Hospital of Wales, Cardiff). HMP samples were taken 1h after perfusion. HMP samples were centrifuged at 2,000 *g* for 10 mines at 4°C to remove debris. The supernatant was divided into 350µl aliquots that were

stored at -80°C prior to RNA extraction. RT-qPCR was performed for quantification of miR-21, and correlated to clinical outcomes.

Patient characteristic	
Donor median age in years (range)	66 (27–69)
Donor gender	
Male	6
Female	5
Donor median BMI (range)	27 (21–34)
Donor median creatinine (range)	86 (53–108)
Donor cause of death	
ICH	6
HBI	5
Donor comorbidities	
CVA	2
Hypertension	4
Diabetes	0
Median cold ischemic time in minutes (range)	1077 (502–1512)
Median agonal time in minutes (range) for DCD kidneys	27 (18–155)
Median 1st warm ischemic time in minutes (range) for DCD kidneys	13 (9–18)
Median hypothermic machine perfusion time in minutes (range)	627 (117–1027)
Donor type	
DCD	10
DBD	1
Recipient median age in years (range)	62 (34–74)
Recipient gender	
Male	8
Female	3
Recipient median BMI (range)	30 (21–39)
Dialysis status	
HD	8
PD	3
Recipient cause of renal failure	
DM	4
GN	3
APKD	1
Other	3
Median no. of HLA mismatches (range)	4 (2–5)

Table 6.1: Donor and Recipient Demographics

Eleven 'Extended Criteria Donor' (ECD) and 'Donation after Circulatory Death' (DCD) kidneys were placed on Life port® (a hypothermic machine perfusion system) prior to transplantation. Donor and recipient demographics are described in the table above.

Ten of the donors were DCD organs and one DBD organ. For the 10 DCD kidneys, the agonal time (withdrawal of treatment to cold perfusion) and WIT1 (cardiac arrest to cold perfusion) were 27mins (range 18 – 155) and 13mins (range 9 – 18) respectively. Details of the donor and recipient demographics are described in Table 6.1. None of the donors were diabetic and 4 had hypertension. None of the recipients were lost to follow up and the median duration of follow up was 33 months (range 12 – 37). None of the patients had a positive cross-match, donor specific antibodies or any previous transplants.

6.2.3 Hypothermic Machine Perfusion Characteristics

The pre-set perfusion pressure on the Life Port® hypothermic machine perfusion system was 30mmHg. Renal flow and resistance rates were measured as follows. Mean (± SEM) start flow rate across the cohort was 72.3 (± 8.6) ml/min. When compared with 1h (127.4 (± 14.9) ml/min) and end of perfusion (117.8 (± 14.5) ml/min) flow rates, the difference was found to be significant ($p=0.005$ and $p=0.022$ respectively) (Figure 6.2 [A]). Mean (± SEM) resistance was 0.432 (± 0.07) mmHg/ml/min at start of flow, when compared with 1h (0.246 (± 0.04) mmHg/ml/min) and end of perfusion (0.215 (± 0.04) ml/min) resistance rates, the difference was found to be significant ($p=0.014$ and $p=0.032$ respectively) (Figure 6.2 [B]). In agreement with previous reports (242), we found increased flow rate and decreased resistance pressure in the first hour of perfusion, with apparent steady state function thereafter. The 1 h time point was therefore selected as the first point at which the flow rate and resistance

pressure were stable, and also represented a time that was relevant in informing organ usage decisions.

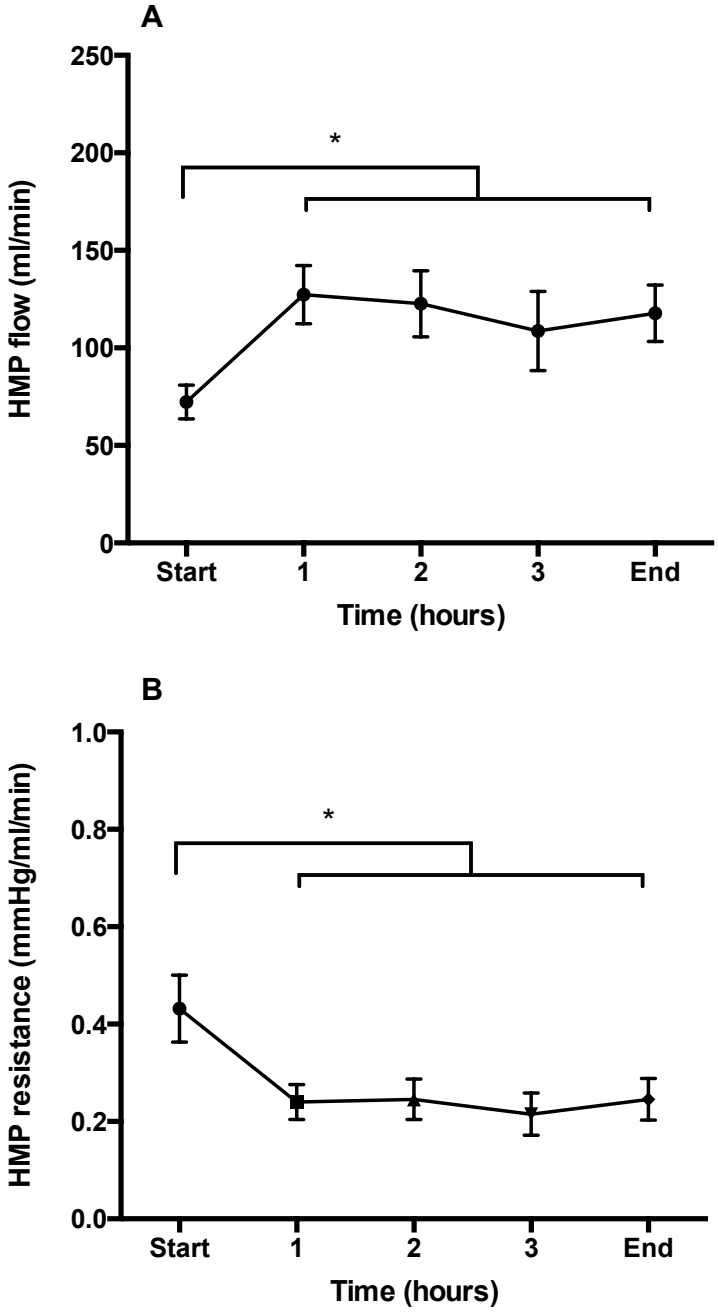

Figure 6.2: Hypothermic Machine Perfusion Flow Rates

Eleven 'Extended Criteria Donor' (ECD) and 'Donation after Circulatory Death' (DCD) kidneys were placed on Life port® (a hypothermic machine perfusion system) prior to transplantation. Flow rates (ml/min) [**A**] and resistance rates (mmHg/ml/min) [**B**] were measured at hourly time points until the end of perfusion. Data were plotted as mean ± SEM.

Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.2.4 Clinical Outcomes

There was 1 graft failure at 33 months and 1 patient death (with a functioning graft) at 21 months. One patient experienced an episode of acute rejection within the first year following transplant. Median eGFR at 6 months and 12 months post-transplantation was 40 (22 – 99) and 41 (22 – 100) ml/min respectively. Nine (82%) patients had DGF.

6.2.5 MiR-21 Expression in HMP

MiR-21 expression levels from HMP samples taken from 11 ECD kidneys placed on the hypothermic machine perfusion system after 1h of perfusion were correlated with eGFR at 6 and 12 months post-transplantation. Individuals in this group exhibited little change in eGFR over this period, consistent with their clinical stability. The expression levels of miR-21 showed significant correlation with eGFR of the kidney graft at 6 and 12 months post-transplantation (R=-0.71, p=0.014; and R=-0.68, p=0.021 respectively (Figure 6.3).

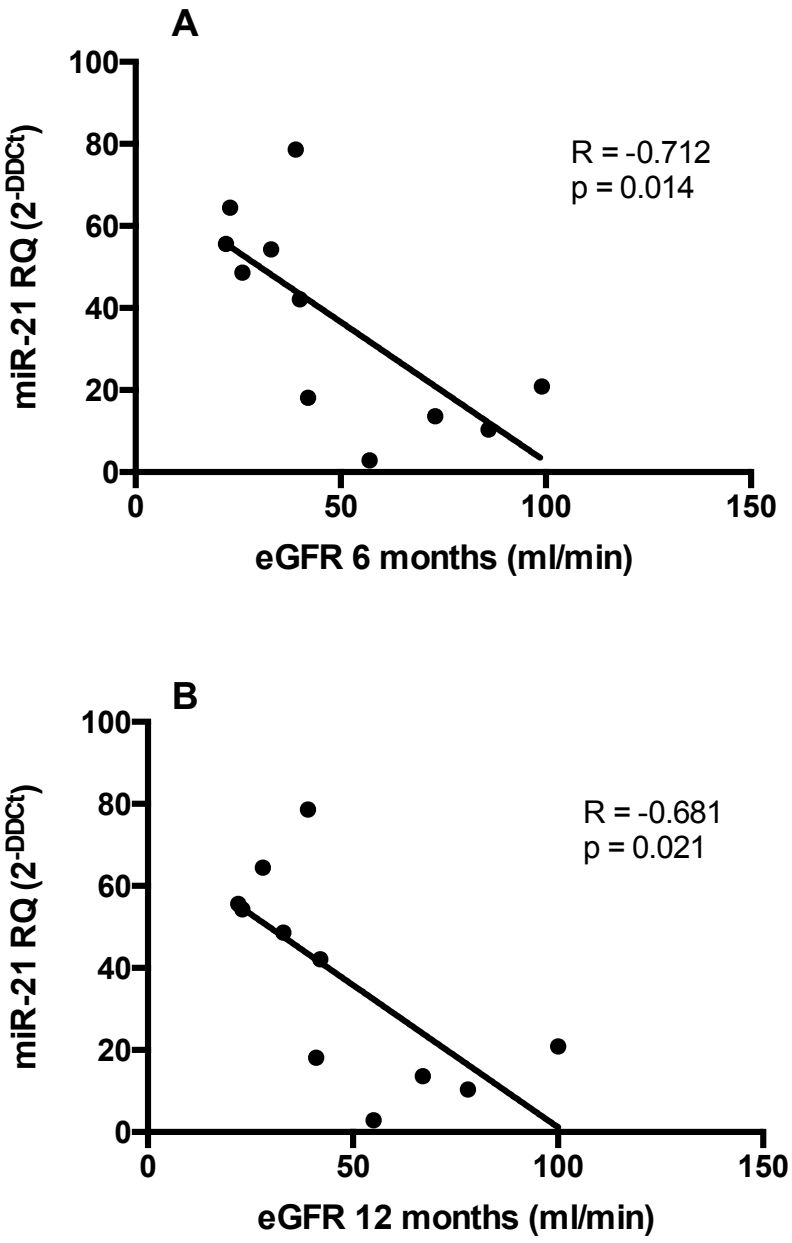

Figure 6.3: Correlation of miR-21 Expression in HMP with eGFR

Following RNA extraction using miRNeasy Mini Kits (Qiagen) from Hypothermic machine Perfusate (HMP) samples from 11 ECD DCD kidneys placed on Life port® (a hypothermic machine perfusion system) at 1 h after perfusion, RT-qPCR analysis was performed for miR-21. The expression levels of miR-21 were normalised to cel-miR-39 and correlated with eGFR at 6 months [**A**] and 12 months [**B**] post-transplantation.

6.3 Discussion

The data presented in this chapter show that microRNAs can be extracted and measured reproducibly in HMP fluid samples, and that the expression of miR-21 in HMP at 1h after perfusion of kidneys placed on the hypothermic machine perfusion system correlates significantly with graft function at 6 and 12 months. This is a novel approach to pre-transplant organ assessment. Previous studies have shown that hypothermic machine perfusion significantly improves outcomes when compared with static cold storage (76, 77), and the data presented here also confirm good outcomes from hypothermic machine-perfused kidneys. The Life Port® hypothermic machine perfusion system is one such approach in common current usage across transplant centres.

This study analysed 10 DCD and one DBD kidneys. Increased use of ECD organs has increased risk of poor outcome due to extensive damage, and an increased discard rate that exacerbates the problem of donor shortage. A reliable method to objectively assess organ viability is therefore needed, particularly for DCD and ECD donors, prior to transplantation. The ideal pre-transplant organ assessment test would be a simple, sensitive and reliable screening method to measure organ viability that accurately predicts graft function. I believe that such a biomarker (e.g. miR-21) would be extremely informative to the transplant surgeon in the aiding of the decision-making process for recipient selection and guidance of appropriate post-transplant management. This is timely at present, because in the UK, the number of ECD donors is increasing, with one-third of all UK donors being 60 years or older (10). In addition to this, identifying a non-

invasive biomarker of the extent of IRI may enable stratified management approaches for transplant patients, and testing of potential treatments to attenuate IRI and reduce the risk of DGF and PNF.

To the best of my knowledge, this is the first study that has evaluated the microRNA expression in HMP samples in kidney transplantation and therefore represents a novel approach to pre-transplant organ assessment. One additional study has evaluated the profile of microRNAs in liver transplantation (243). In this study, microRNA evaluation was performed in perfusate flushes from cadaveric donors, and the investigators found that microRNAs were differentially expressed between DBD and DCD liver transplants, and that the expression levels were predictive of ischemic-type biliary tract lesions post-transplantation. Organ preservation or perfusion fluid, whether HMP or perfusate flushes, may thus provide a valuable pre-transplantation source with which to identify biomarkers of organ viability. The findings described in this chapter demonstrate that microRNA analysis of machine perfusate may have value in predicting outcomes following kidney transplantation.

The primary limitation of this study is the small sample size restricted to older ECD kidneys from a single centre, using the same tightly defined perfusion protocol for the kidney and sample acquisition and profiling approach for all samples. I have demonstrated the stability and reproducibility of microRNA analysis in machine perfusate measured at 1h post-perfusion, together with the capability of miR-21 in this study to act as a sentinel for graft function. In order for these findings to be more generally applicable, it will be important for them

to be tested in larger cohorts collected from multiple centres, reflecting the full range of clinical practice in terms of kidneys selected for hypothermic machine perfusion.

This data clearly supports the fact that microRNAs are becoming increasingly important as biomarkers in multiple disease processes including kidney injury and transplantation. A larger cohort of HMP will be required to validate these findings, and further define the capability of perfusate miR-21 to predict significant AKI prior to transplantation.

In conclusion, in the era of ECD kidneys a reliable measure of organ quality is urgently needed and this chapter suggests that miR-21 may be such a marker.

Chapter 7 – General Discussion

The main aspect of this thesis was to study kidney ischaemia reperfusion injury (IRI) in the context of transplantation. At present there is a real shortage in the supply of organ donors, leading to the increased use of DCD and ECD donors, which have increased IRI. IRI is arguably the most important cause of delayed graft function (DGF), a form of acute kidney injury (AKI) in the transplanted kidney. DGF can lead to many problems, including a higher risk of graft failure. Understanding the underlying mechanisms of IRI and its potential treatments, such as ischaemic preconditioning (IPC), is key. In addition to this, microRNAs, recently described post-transcriptional regulators of gene expression, have been shown to be important in many physiological and pathophysiological situations, and as potential biomarkers. Therefore, with this background in mind, the aims and objectives of this thesis were firstly, to understand the role of microRNAs in IRI and IPC by using an *in vivo* model of AKI, and secondly, to utilise this knowledge in order to determine the utility of microRNAs as non-invasive biomarkers of IRI in the context of clinical kidney transplantation.

At the time of starting my thesis, this department had an established unilateral IRI model of AKI in the rat. I used this model to test one IPC regime, which did not reduce injury to the kidney (Chapter 3). One of the major limitations of this set of experiments was that there was that, given the unilateral injury, there was no alteration in measured excretory kidney function. Therefore, for the next set of experiments (Chapter 4), I adapted this model to bilateral kidney IRI. I used this bilateral IRI model to test various IPC regimes, with the aim of identifying an IPC regime that would be beneficial. I elected to test varying continuous and pulsatile regimes systematically because of the variety of outcomes (beneficial

and not) reported by other investigators. Having tested 6 different regimes, I showed that pulsatile IPC regimes were beneficial and that continuous regimes were not (similar to the one utilised in the unilateral IRI model). Moreover I showed that one pulsatile regime was the most beneficial in reducing injury to the kidney. I tested IPC regimes that were localised to the kidney and 'early' i.e. the IPC was conducted only minutes prior to the IRI. There are clearly more studies that can be undertaken in this model. One of them, currently being undertaken by a PhD student following on from this work, is to test the role of remote IPC (hind limb ischaemia prior to IRI). Other future studies could evaluate the effects of 'late' IPC, i.e. perform the IPC days before the IRI.

The rat was used as the animal model, as it was already established as the *in vivo* model of choice with robust results from previous studies and the work of a previous MD student. Rats have been successfully used in many animal studies investigating kidney IRI. There are obvious advantages to using a rat over other animals such as mice, as well as limitations. One of the advantages is that it is easier to operate on, as its larger, and more blood volume can be taken. However, an important limitation is that it is relatively more difficult to genetically manipulate within the rat. Whereas, with mice, it is much easier to manipulate their underlying genetics and there is more variety of genetically modified mice than rats. Therefore a useful follow on project from this thesis would be to develop a mouse model of AKI, allowing for genetic manipulation of the microRNAs identified from this thesis. Other alternative animals that could be used can be pigs for example, their obvious advantage being increased similarity

to humans in terms of the pathology and response to treatments, however with the big limitation of needing additional infrastructure and resources.

One of the most important factors when selecting a model of IRI is length of ischaemia and reperfusion. In this thesis, I chose to use a relatively acute model of AKI, and it showed to be an easily reproducible, robust and reliable *in vivo* model of AKI. However, there is evidence that IRI can cause kidney fibrosis in the long-term, and therefore a model of AKI with a longer-term follow up would be useful in our understanding of the role of microRNAs in this context, in particular miR-21, which has been implicated in fibrosis.

Having identified an IPC regime that was beneficial, within a robust and easily reproducible *in vivo* model of AKI, the next objective was to profile the microRNAs within the kidney in this model. I did this by using a hybridisation microarray and NGS, the 2 most well established methods of small RNA profiling currently available. These experiments demonstrated that kidney IRI in the rat has a unique microRNA signature, which is diminished by IPC. Both of these profiling methods showed overlap between key microRNAs, which I confirmed in all of the samples using RT-qPCR. Nevertheless, a lot of microRNAs that were identified by these techniques did not overlap between the two. A possible explanation for this is that the two techniques are biased towards different pools of microRNAs, for example, there may be a preferential hybridisation of sequences or microRNAs of certain GC content to the arrays. It is possible that these microRNAs, which were not tested further in this thesis, may well play an

extremely important part in IRI and IPC, and therefore a potential future project would be to determine their role in IRI.

By showing that IRI has a unique microRNA signature that is diminished by IPC, it is likely that it represents an injury signature. It is also most likely the case that these microRNAs are functionally very important in IRI. In order to determine this question, further detailed analyses needs to be undertaken, that would identify potential targets of these microRNAs. A provisional target prediction analysis of microRNAs identified from this thesis (miR-21, miR-221, and miR-222) in Ingenuity Pathway Analysis (IPA) software (performed by Professor Donald Fraser) has identified a complex network of intracellular pathways and targets that overlap, suggesting an underlying functional network that merits further investigation (Appendix 4). However, such microRNA target and functional prediction remains inexact and much work will be needed to determine the functional importance of these microRNAs in the context of IRI and IPC. On-going work in this laboratory to address this includes a deeper IPA analysis and proteomics studies.

Another aspect to consider is that the kidney is a complex organ with different cells. In order to address this, laser capture micro dissection (LCM) was used. It was shown that the microRNAs were found in various tissue components of the renal cortex, with a higher concentration within tubular cells, in keeping with the fact that they may be markers released following injury and that because tubular cells are more prone to damage following IRI, more microRNAs are released by

them. Therefore, I chose to investigate the role of microRNAs as biomarkers in the context of human kidney transplantation (Chapters 5 and 6).

I also showed that microRNAs measured in urine are highly predictive of DGF in kidney transplant patients (Chapter 5). I profiled microRNAs in urine samples one day post-kidney transplantation, in patients subsequently developing DGF and in those not developing DGF, using a TLDA array. I found 7 microRNAs were significantly up regulated in DGF and therefore potentially useful biomarkers, in particular miR-21, which remained significantly up regulated in DGF within the first 5 days post-transplantation. This is the first study that has evaluated the urinary microRNA profile in kidney transplant patients in the context of DGF. More work needs to be done in order to differentiate the microRNA profile between DGF and other forms of AKI post-transplantation, such as rejection, as development of these microRNAs into non-invasive biomarkers of DGF may avoid the need for invasive biopsy of the kidney graft. This is a future project that I am planning to undertake.

One limitation of Chapter 5's work was the relatively small sample size within the groups, although previous studies that have investigated microRNAs as biomarkers in kidney transplantation have also had a relatively small number of patients. The substantial fold-changes and high level of statistical significance that I have found in my dataset are suggestive that these findings represent real and important differences in the groups that that were studied. It is important to evaluate these further in larger sample cohorts, and to enable this next phase of the work, we have been in discussion with the tissue banks led by Prof Neil

Sheerin in Newcastle University and Prof Rutger Ploeg in Oxford University. Larger cohorts may also allow us to appreciate a better correlation of eGFR with microRNA expression levels.

The identification and refinement of a biomarker that is specific to kidney injury in the context of transplantation would allow for its potential use in determining outcomes prior to transplantation. Such a thing would so advantageous to the transplant surgeon, as given the fact that the organs used are increasingly from ECD, DCD, marginal and older donors, any methodology that allows for accurate prediction of outcomes prior to their implantation, would aid in the decision making process of usage or discard and selection of most suitable recipient. Therefore, I measured miR-21 in hypothermic machine perfusate samples and correlated it to the eGFR at 6 and 12 months post-transplantation (Chapter 6). Interestingly, I showed that microRNAs are stable, can be extracted and measured reproducibly in HMP fluid samples, and that the expression of miR-21 in HMP at 1 hour after perfusion of kidneys placed on the hypothermic machine perfusion system correlated significantly with graft function at 6 and 12 months. This is the first study that has evaluated microRNAs in HMP, representing a novel approach to pre-transplant organ assessment, and this work is timely, with the number of ECD donors increasing in the UK, and one-third of all UK donors being 60 years or older. Clearly, a larger cohort of HMP will be required to validate my findings, and to further define the capability of perfusate miR-21 to predict significant ischaemic injury prior to transplantation. We are in the process of collecting more HMP samples from kidneys that are placed on Lifeport® at Cardiff Transplant Unit.

Chapters 5 and 6 clearly support the fact that microRNAs are becoming increasingly important as biomarkers of kidney injury in the clinical setting of kidney transplantation.

Overall Conclusions

Kidney IRI has a unique microRNA signature that is attenuated by beneficial protective IPC in the rat, suggesting that it is an injury signature with functional importance. MicroRNAs are readily detectable in the urine of human kidney transplant recipients, and show a promising capability to predict DGF. MicroRNAs are also detectable in HMP solution and show significant promise as biomarkers and potential therapeutic targets in this context.

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Appendices

Appendix 1 – Wales Kidney Research Tissue Bank patient consent form

Wales Kidney Tissue Bank Patient Consent Form

GIG CYMRU NHS WALES | Bwrdd Iechyd Prifysgol Caerdydd a'r Fro
Cardiff and Vale University Health Board

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

Surname:	Unit No:
First Name:	
Date of Birth:	Sex:
Address:	Telephone Number:

To confirm agreement with each of the statements below, please initial in the box

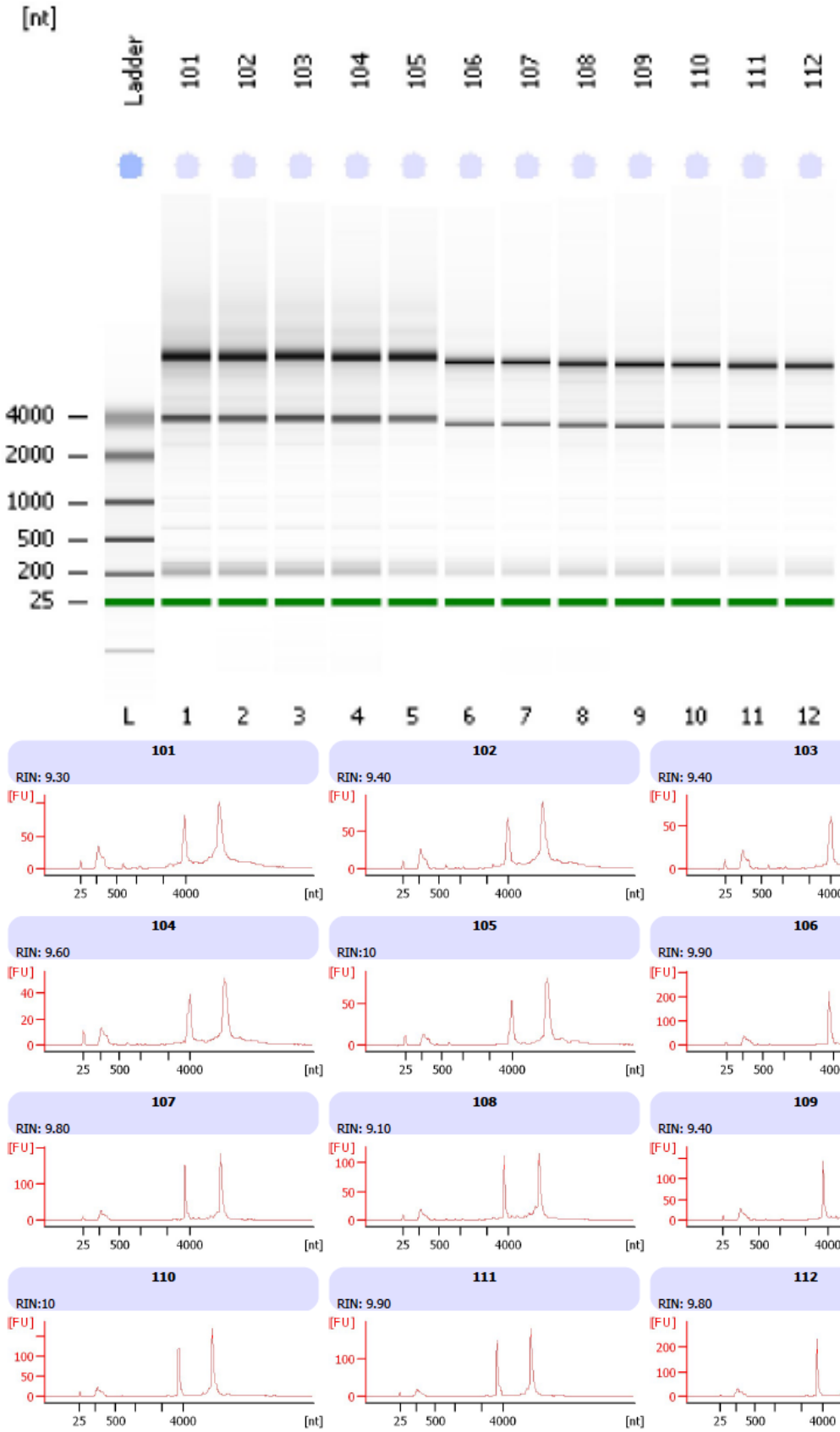
1. I have read and understood the information sheet version 2, dated December 2010. I have had the opportunity to consider the information and ask questions. ☐
2. I understand that my participation is voluntary and that there will be no financial reward for taking part. I am also free to withdraw at any time without giving any reason, and without my care or legal rights being affected. ☐
3. I agree to donate my residual kidney tissue as a "gift" to the tissue bank according to the conditions in the patient information sheet ☐
4. I give permission for 10-20ml of my blood to be collected. ☐
5. I give permission for a sample of my urine to be collected. ☐
6. I give permission for my residual dialysis fluid to be collected. ☐
7. I understand that researchers from other institutions may access my samples, that research may take many years, and that the information gained will not benefit me or my family directly. ☐
8. I give permission for my samples to be used for studies on DNA, that might identify genes or diseases that run in families, and can be passed on to blood relatives. ☐
9. I give permission for health information to be collected from my doctor or medical records ☐
10. You may contact me in the future to take part in other research projects or surveys ☐

Participant's Name (print) Date Signature

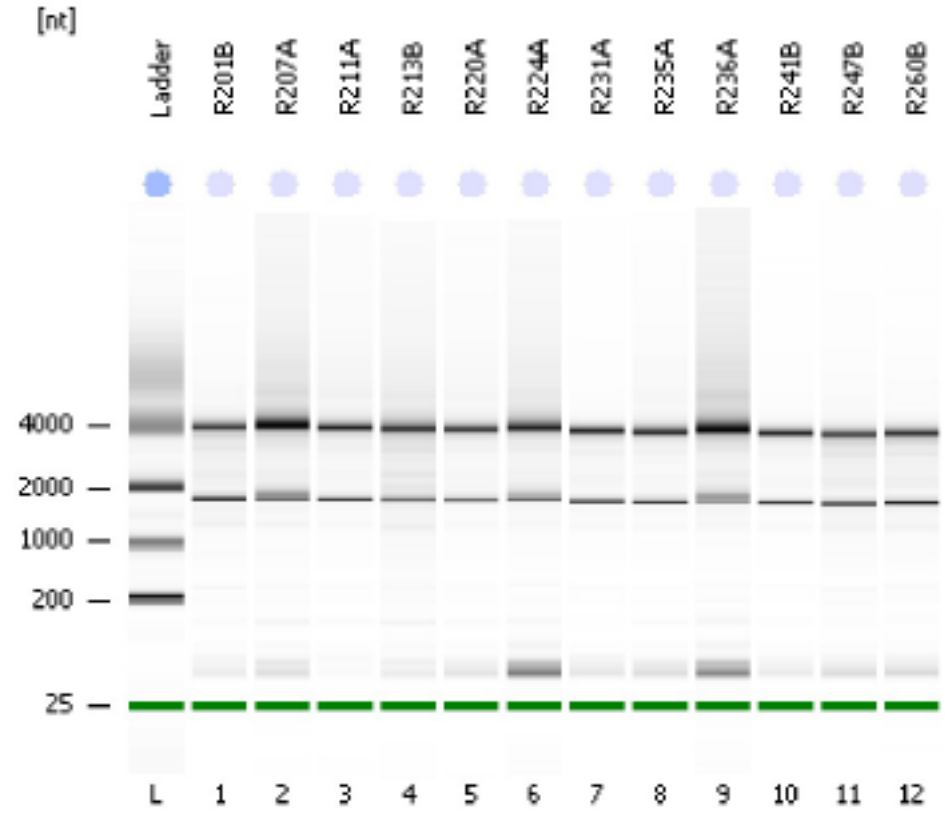
Name of person taking consent (print) Date Signature

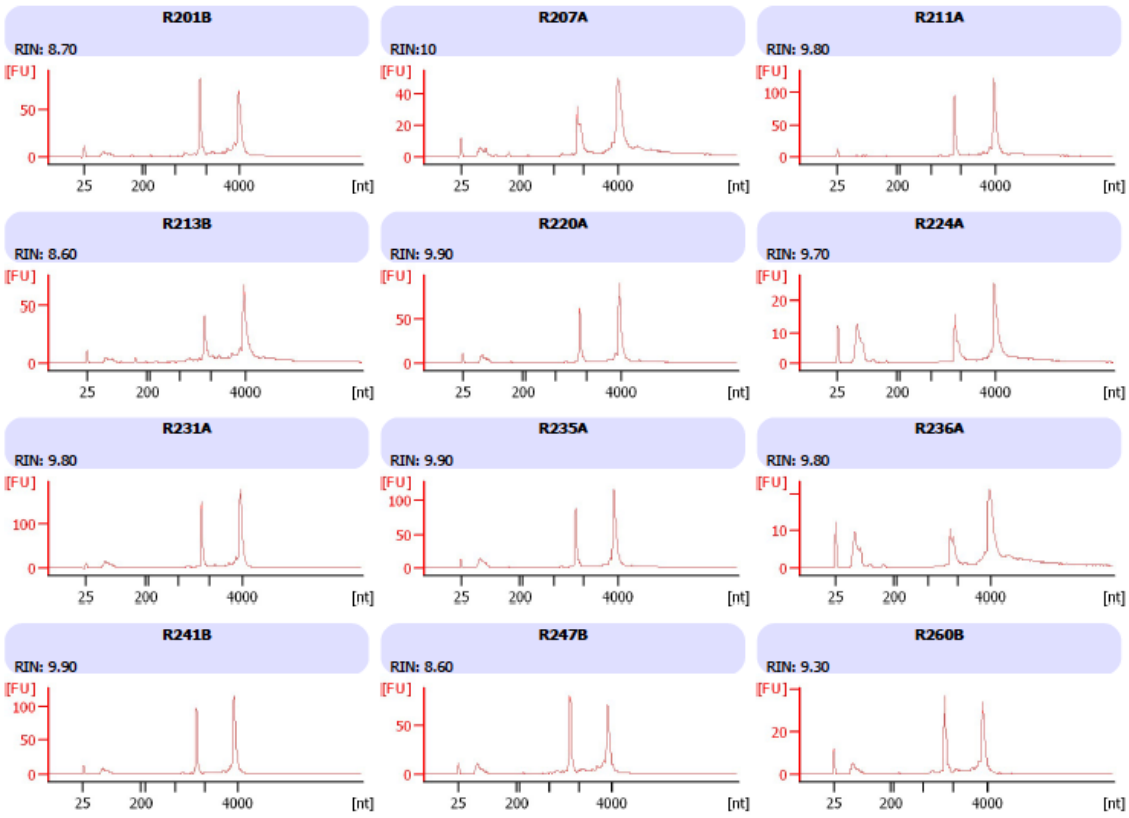
Version 2, December 2010

Appendix 2 – Agilent bio-analyser results from Chapter 3 experiments



Appendix 3 – Agilent bio-analyser results from Chapter 4 experiments





Appendix 4 – Ingenuity Pathway analysis for miR-21, -221 and -222