MicroRNA levels, hypoxia and cardiopulmonary bypass in the development of acute kidney injury

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Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Annette Lisa Mazzone

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Abbreviations

ACEi	angiotensin-converting enzyme inhibitor
ACT	activated clotting time
ADQI	acute dialysis quality initiative
AF	atrial fibrillation
AGO2	argonaute-2
AKI	acute kidney injury
AKIN	Acute Kidney Injury Network
AMPK	AMP-activated protein kinase
ARB	angiotensin receptor blocker
Ago	argonaute
ANOVA	analysis of variance
ATACAS	aspirin and tranexamic acid for coronary artery surgery
BSA	body surface area
cDNA	complementary DNA
CABG	coronary artery bypass grafting
CAD	coronary artery disease
C. elegans	*Caenorrhabdidits elegans*
CI	confidence interval
CKD	chronic kidney disease
CKMB	creatine kinase muscle/brain
CORONARY	CABG Off or On Pump Revascularisation study
CPB	cardiopulmonary bypass
CRF	chronic renal failure
CSA-AKI	cardiac surgery associated acute kidney injury
Cq	quantification cycle
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
DO_2	oxygen delivery
EDTA	ethylenediamine tetraacetic acid

endo-siRNAs	endogenous small interfering RNAs
ESRD	end stage renal disease
EuroScore	European system for cardiac operative risk evaluation
FDR	false discovery rate
FMC	Flinders Medical Centre
GDP	goal directed perfusion
GFR	glomerular filtration rate
Hb	haemoglobin
Hct	haematocrit
HEK	human embryonic kidney
HIF	hypoxia inducible factor
ICGC	International Cancer Genome Consortium
ICU	intensive care unit
IGFBP-7	insulin-like growth factor-binding protein 7
IL	interleukin
IRI	ischaemia-reperfusion injury
KDIGO	kidney disease improving global outcomes
KEGG	Kyoto encyclopedia of genes and genomes
KIM-1	kidney injury marker 1
lnc RNA	long non-coding ribonucleic acid
LV	left ventricular
MAP	mean arterial pressure
mRNA	messenger RNAs
MI	myocardial infarction
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines
MMC	Monash Medical Centre
miRNA	micro ribonucleic acid
myomiR	myocardial associated miRNAs
NCBI	National Centre for Biotechnology Information
NGAL	neutrophil gelatinase associated lipocalin
NGS	next generation sequencing

NYHA	New York Heart Association
O_2	oxygen
OPCABG	off pump coronary artery bypass grafting
OR	odds ratio
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinases
piRNA	PIWI-interacting RNA
PCO_2	partial pressure of carbon dioxide
PO_2	partial pressure of oxygen
PRE	preoperative
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
POST	postoperative
PTCA	percutaneous transluminal coronary angioplasty
RBC	red blood cell
RBF	renal blood flow
RCT	randomised controlled trial
RIFLE	Risk-Injury-Failure-Loss-End stage
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNase	ribonuclease
ROOBY	Randomised On/Off Bypass trial
RQ	relative quantification
RR	relative risk
rRNA	ribosomal RNA
RRT	renal replacement therapy
RT	reverse transcriptase
qRT-PCR	quantitative real time reverse transcription PCR
sCr	serum creatinine
siRNA	small interfering ribonucleic acid
snRNA	small nuclear RNA

snoRNA	small nucleolar RNA
SIRS	systemic inflammatory response syndrome
TIMP-2	tissue inhibitor of metalloproteinases 2
TRICS	transfusion requirements in cardiac surgery
tRNA	transfer RNA
TSI	tissue specific index
uPO2	urinary oxygen content
VHL	von hippel-lindau

Standard International units of measurement

°C	degrees Celsius
dL	decilitre
fmol	femtomole
g	gram
h	hour
IU	international units
kg	kilogram
L	litre
m	metre
m^2	square metre
M	molar
mg	milligram
min	minute
mL	millilitre
mmHg	millimetre of mercury
ng	nanogram
nt	nucleotide
pmol	picomole
s	second
µL	microlitre
µmol	micromole
v/v	volume/volume

Summary

Acute kidney injury (AKI) is a frequent and important complication of cardiac surgery and is associated with increased complications, length of hospital stay and mortality. During cardiac surgery procedures, cardiopulmonary bypass (CPB) is used to support the function of the heart and lungs. A number of modifiable factors of CPB, resulting in oxygen supply/demand mismatch have been investigated in playing a potential role in the development AKI. The challenge with managing AKI following cardiac surgery is that there is no effective intervention once AKI has been diagnosed.

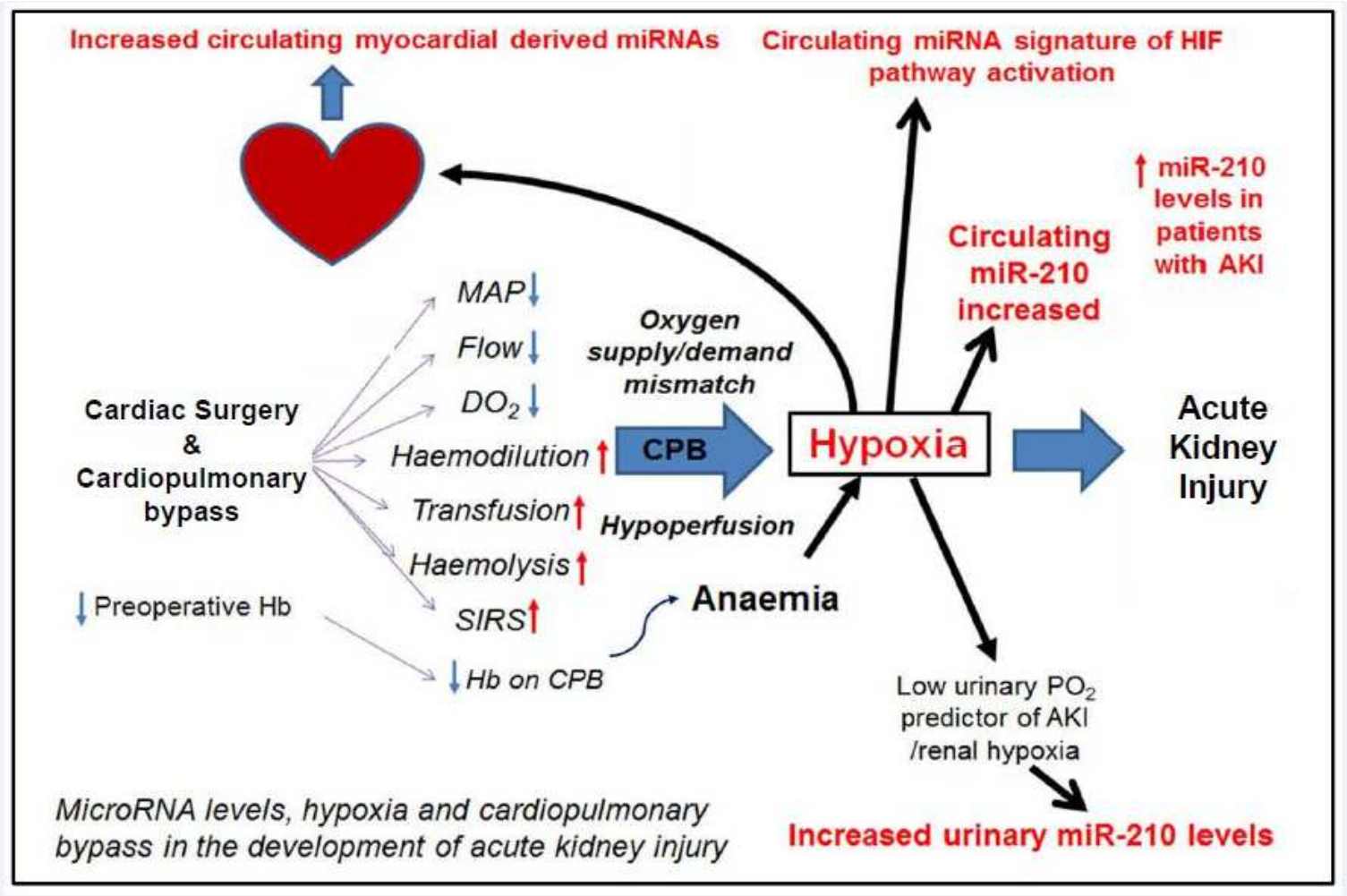
Efforts to prevent cardiac surgery associated AKI are limited by the ability to predict which patients will develop AKI due to the inability to monitor the risk of its development intraoperatively. Traditional biomarkers, including serum creatinine, provide prognostic information that damage has occurred and do not reflect real time change in renal function. Due to the number of potential known modifiable risk factors of CPB, the time on CPB provides an ideal opportunity to investigate the development of AKI as intervention during this known period of insult is feasible.

The potential application of microRNAs (miRNAs) as disease biomarkers has been of considerable recent interest due to the vital role miRNAs play in biological process, with altered expression associated with altered physiological states including hypoxia. The expanding roles of miRNAs, not only as biomarkers, but their involvement in the control and progression of disease processes, including AKI, makes them ideal to study.

The specific aims of this thesis were to successfully isolate and measure circulating and urinary miRNAs, such as miRNAs associated with hypoxia and haemolysis to gain a greater understanding of the molecular response to CPB; and to elucidate the molecular mechanisms involved in the development of AKI following CPB potentially identifying a predictive marker of AKI during cardiac surgery. The results of this thesis present the first comprehensive investigation into miRNAs expression levels during cardiac surgery with CPB, with differential patterns of miRNA release observed. Hypoxically regulated miR-210 levels were found to be increased during CPB and into the early postoperative period. Investigation in a cohort of patients who developed AKI found

levels of miR-210 to be increased compared to patients who did not develop AKI. Levels of miR-210 measured during CPB also significantly correlated with levels of urinary PO_2, a predictive marker of AKI, suggesting a hypoxic release of miR-210 release during CPB.

This thesis provides evidence of hypoxically regulated miRNA release, dysregulation of hypoxia related molecular pathways and provide evidence of a potential hypoxia response to CPB which has not be previously shown. The results of this thesis provide ground work for a greater understanding of the molecular effects of CPB and may help guide future directions of CPB conduct and management serving as a tool for examining effects of changes in management, leading to improved outcomes.



Graphical abstract of the major findings in this thesis: MicroRNA levels, hypoxia and cardiopulmonary bypass in the development of acute kidney injury. Circulating and urinary miRNAs were increased during and following CPB. Levels of HIF-regulated miR-210 were increased during and following CPB with higher levels in patients who developed CSA-AKI. A decrease in urinary PO_2, a predictor of AKI, was associated with increased miR-210 levels. Highly expressed miRNAs released during and following CPB activate and regulate hypoxia pathway. *Mean Arterial Pressure (MAP), Oxygen Delivery (DO_2), Systemic inflammatory response syndrome (SIRS), Haemoglobin (Hb), Cardiopulmonary Bypass (CPB), micro-Ribonucleic acid (miR), Hypoxia Inducible Factor (HIF), Acute Kidney Injury (AKI).*

Publications, presentations and awards arising during thesis

Publications

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Gleadle, J. M., & Mazzone, A. (2016). Remote ischaemic preconditioning: closer to the mechanism? *F1000Res, 5*, 2846. doi:10.12688/f1000research.9633.1

Mazzone, A. L., Baker, R. A., & Gleadle, J. M. (2016). Mending a broken heart but breaking the kidney. *Nephrology (Carlton), 21*(10), 812-820. doi:10.1111/nep.12799

Oral Presentations

Mazzone A.L Serum microRNA expression profiles and activation of the HIF-1 signalling pathway indicate the upregulation of a hypoxic response during CPB. Presented at the 35th Annual Scientific Meeting, Australia and New Zealand College of Perfusionists, Adelaide, Australia Nov 2018. Awarded Medtronic Encouragement Award

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- Medtronic Encouragement Award - Awarded at Annual Scientific Meeting, Australia and New Zealand College of Perfusionists, Adelaide, Australia Nov 2018.
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Chapter 1: INTRODUCTION

1.1 Cardiac Surgery and Acute Kidney Injury

Acute kidney injury (AKI) is one of the most serious in-hospital postoperative complications for surgical patients. Acute kidney injury covers an entire spectrum of damage to the kidney ranging from minor changes in kidney function to renal failure requiring dialysis (Mehta *et al.*, 2007). Acute kidney injury is a complex diagnosis and has been described using several definitions and diagnostic criteria. Regardless of the definition used, the incidence ranges from 5% of all hospital admission to 42% of patients undergoing cardiac surgery (Wang & Bellomo, 2017). Surgical patients are particularly predisposed to AKI due to the physiological insults induced by major surgery especially in patients with pre-existing comorbidities (Goren & Matot, 2015). Despite advances in care in the intensive care setting, overall in-hospital mortality rates in patients suffering severe AKI requiring dialysis remains as high as 70% (Palevsky, 2013). Traditional biomarkers, including serum creatinine, have been markers of AKI, providing retrospective information that damage has occurred and do not reflect real time change in renal function. An ideal biomarker would provide information about the physiological status of the kidney, in as close to real time as possible, in individual patients so that conditions likely to promote development of AKI can be avoided.

Approximately 2 million cardiac surgery procedures are carried out worldwide, with over 20 000 adult patients undergoing cardiac surgery in Australia and New Zealand each year (Hu *et al.*, 2016). The operations vary in complexity from coronary artery bypass grafting (CABG) procedures to enhance coronary artery blood flow to the repair or replacement of stenotic or regurgitant valves and correction of complex congenital abnormalities. During these procedures cardiopulmonary bypass (CPB) is used to support the function of the heart and lungs. Cardiac surgical procedures expose the kidney to a significant risk of acute injury, often being undertaken on patients with pre-existing chronic kidney disease (CKD) and have a poorly defined risk of subsequent longer term reductions in renal function and development of CKD.

Cardiopulmonary bypass has been used for cardiac surgery since the 1950's and is used thousands of times daily throughout the world. The 2017 Report of the Australian and New Zealand Society

of Cardiothoracic Surgeons National Database reported outcomes in over 10 000 adult patients undergoing cardiac surgical procedures in 2017 with over 91% of these procedures performed using CPB. In 2017 off-pump coronary artery bypass grafting (OPCABG) only comprised 7% of the procedures reported, whilst transcatheter valve replacement accounted for 2%. Although most patients tolerate cardiac surgery well, subtle as well as clinically apparent complications occur including neurological dysfunction, bleeding and renal impairment.

Cardiopulmonary bypass uses an extracorporeal circulation to temporarily replace the function of the heart and lungs during surgery, thereby maintaining perfusion, oxygenation and carbon dioxide removal. It provides the surgeon with a bloodless surgical field and, with cardioplegic arrest, a motionless heart. Briefly CPB involves systemic heparinisation of the patient, cannulation to drain deoxygenated blood from the right side of the heart into a reservoir from where blood is actively pumped into the oxygenator. Oxygenators provide gas exchange to blood through a microporous membrane allowing oxygen to diffuse across the membrane into blood and carbon dioxide to be removed. Prior to gas exchange blood passes through a heat exchanger, an integral component of the oxygenator.

Heat exchangers function in combination with an external heater-cooler unit that pumps temperature controlled water into the water phase of the heat exchanger, which is separated from the blood phase by a highly conductive material, including stainless steel and polyurethane. The function of the heat exchanger is to regulate the temperature of the blood perfusing the patient allowing patient temperature to be varied over a wide range depending on the surgical procedure being performed. Following passage through the oxygenator, blood is then returned to the patient's arterial system via a cannula positioned in the ascending aorta, or less frequently in the axillary or femoral artery. Prior to stopping the heart beating, a clamp is placed across the aorta, isolating the heart from the circulation and cardioplegia, most commonly hyperkalemic, is delivered to the coronary arteries via the aortic root, or the coronary sinus or directly into the coronary ostia to protect the heart. Cardioplegia provides myocardial protection by reducing myocardial oxygen requirements to reduce the effects of ischaemia due to isolation of the coronary circulation.

The technique of OPCABG was developed in an attempt ameliorate some of the perioperative complications associated with CPB and aortic manipulation. (Jansen *et al.*, 1998; van Dijk *et al.*, 2001). The OPCABG technique allows the heart to continue beating throughout the operation, and by using a stabiliser to minimise the movement of the heart, the surgeon is able to perform the graft procedures while physiological pulsatile flow is delivered to the body. Haemodynamic changes occur in OPCABG due to mobilisation of the heart during the operation, compromising cardiac output due to a reduction in stroke volume and systolic and diastolic perfusion pressures (Couture *et al.*, 2002).

Diagnosis of acute kidney injury

The current diagnosis of AKI is challenging since it is highly reliant upon measurements of serum creatinine, which suffers from major limitations as a reliable biomarker for measurement of kidney function (Moran & Myers, 1985). Although serum creatinine is not a perfect marker of renal function, it continues to be the standard method for classification and diagnosis of AKI. Other biomarkers have been studied but there is still no superior measure than serum creatinine measurement in clinical practice (Najafi, 2014). Creatinine is an end product of creatine metabolism which is exported into the blood from skeletal muscle. Creatinine is freely filtered by the glomerulus and is not reabsorbed; hence creatinine clearance is calculated by determining the ratio between the serum concentration of creatinine and the urine concentration of creatinine, a technique discussed by Bull et al. (1950). The rate of creatinine excretion by the kidney is proportional to skeletal muscle mass, which is presumed constant. Although creatinine clearance is a valuable measure of the glomerular filtration rate (GFR), clinically it is more common to measure plasma or serum creatinine alone and to use this as an indicator of the GFR. Hence serum creatinine levels have an inverse relationship to GFR (Bagshaw & Gibney, 2008).

Serum creatinine has its limitations as it does not reflect real time changes in GFR or reflect acute injury process within the kidney. The time relationship between changes in serum creatinine and concomitant changes in GFR does not allow accurate estimation regarding timing and reversibility of renal injury and the severity of kidney dysfunction, thus delaying diagnosis and intervention. However, measurement of serum creatinine still constitutes the main measure for assessment of renal function due to the simplicity and availability of its measurement. This delay in recognition may delay appropriate supportive and therapeutic interventions. In practice, the diagnosis of AKI

depends on observing an increase in plasma creatinine which may not become readily apparent until 24 to 72 hours after a decrease in GFR (Endre *et al.*, 2011).

Classification of acute kidney injury

Measurement of serum creatinine is also the foundation of grading of severity of AKI (Najafi, 2014). The Acute Dialysis Quality Initiative Group developed a consensus definition for AKI, the Risk-Injury-Failure-Loss-End Stage kidney disease (RIFLE) classification. The RIFLE classification defines three grades of severity of AKI (Risk, Injury and Failure) based on serum creatinine and urine output and two clinical outcomes (Loss, End-stage) (Bellomo *et al.*, 2004). In 2007, the Acute Kidney Injury Network (AKIN) group, an international collaboration of nephrologists and intensivists, proposed refinements and modification to the RIFLE criteria. The AKIN group sought to increase the sensitivity of the RIFLE criteria by recommending that a smaller change in serum creatinine be used as a threshold to define the presence of AKI and identify patients with AKI stage 1. Secondly a time constraint, requiring at least two values of serum creatinine obtained within a period of 48 h for the diagnosis of AKI, was recommended. Thirdly, any patient that received RRT were classified as Stage 3 AKI. (Mehta *et al.*, 2007). Both definitions also incorporate severity and duration of oliguria as alternative criteria. It is currently unknown whether discernible advantages exist with one approach to definition and classification versus the other, with both criteria found to have clinical relevance for the diagnosis of AKI, classifying the severity of AKI and monitoring the progression of AKI (Bagshaw *et al.*, 2008)(Table 1.1.1).

The delay between an insult to the kidney and measurement of increased creatinine concentration required for diagnosis creates uncertainty regarding the timing of the renal insult causing injury. The current RIFLE consensus requires at least a 33% decline in GFR resulting in at least a 50% increase in serum creatinine. Since there is an inverse relationship between serum creatinine and GFR, a small increase in serum creatinine represents a substantial decline in GFR. Alterations in serum creatinine lag several days behind actual changes in GFR. Even if an increase in serum creatinine is observed, the delay required for diagnosis creates uncertainty. Due to the drawbacks of glomerular filtration markers such as creatinine the ideal diagnosis of AKI may rest with other urinary and/or serum tissue injury markers. Since current therapy for AKI is limited to supportive measures and preventative strategies, none of which have been definitively shown to alter

morbidity or mortality, an ideal biomarker of AKI would allow the early detection of kidney injury before an increase in serum creatinine concentration (Bonventre & Weinberg, 2003).

A biomarker that is released into the blood or urine by the injured kidney and is analogous to the troponin release by injured myocardial cells after myocardial ischaemia, could be a more sensitive and specific marker of AKI than serum creatinine (Edelstein, 2008). In addition, earlier detection of AKI with a kidney specific biomarker may result in earlier intervention to repair the damaged kidney or allow intervention to prevent AKI if the timing of the insult is known. Acute kidney injury following cardiac surgery with the use of CPB is a common and problematic complication following cardiac surgery. Cardiac surgery and the use of CPB provide a unique opportunity to prospectively study the effects of a predictable known insult to the kidney. With alterations in renal blood flow (RBF), hypotension, ischaemia/reperfusion injury and potential reduction in oxygen delivery during CPB, cardiac surgery with CPB provides an ideal controlled setting to observe the effects of multiple known insults to the kidney and the development of AKI.

Table 1.1.1. RIFLE and AKIN classification of acute kidney injury

RIFLE	Creatinine/ GFR criteria	Urine output criteria	AKIN	Creatinine/ GFR criteria	Urine output criteria
Risk	↑ serum creatinine 150% or ↓ GFR > 25%	< 0.5 mL/kg/h for 6 h	Stage 1	↑ serum creatinine ≥ 0.3 mg/dL or ↑serum creatinine 150-200%	**< 0.5 mL/kg/h for 6 h**
Injury	↑serum creatinine 200% or ↓ GFR > 50%	< 0.5 mL/kg/h for 12 h	Stage 2	↑ serum creatinine 200-300%	**< 0.5 mL/kg/h for 12 h**
Failure	↑ serum creatinine 300% or > 4mg/dL ↓ GFR 75%	< 0.3mL/kg/h for 24 h or anuria for 12 h	Stage 3	↑ serum creatinine > 300% or > 4mg/dL ↓ GFR 75%	**< 0.3 mL/kg/h for 24 h or anuria for 12 h**
Loss	**Persistent acute renal failure – complete loss of kidney function > 4 weeks**				
ESKD	**End stage kidney disease > 3 months**				

Incidence of cardiac surgery-associated acute kidney injury

Acute kidney injury is a frequent and serious complication of cardiac surgery with an incidence variably reported from < 3% to > 43%, depending in large part on the definition of AKI used. Noyez (2011) examined 995 consecutive patients undergoing cardiac surgery at a single centre, identifying AKI using six different definitions. There were marked differences in the incidence of AKI depending upon the definition used (range 4.9% to 38.1%, p <0.001). A similar variation was seen in the meta-analysis by Pickering et al. (2015) of 46 studies and over 240 000 patients. The use of broadly accepted definitions of AKI facilitates better analysis and comparison of studies examining AKI following cardiac surgery.

RIFLE and AKIN classification in cardiac surgery - in hospital outcomes

The RIFLE and AKIN classifications have been used extensively to document the incidence of AKI following cardiac surgery. The broad range of incidence of AKI following cardiac surgery reflecting not only differences in defining AKI but potentially highlighting differences in patient management. Regardless of the definition used or the incidence of AKI reported, it is clear that deterioration of renal function post cardiac surgery results in increased in-hospital mortality. A meta-analysis by Pickering showed that AKI following CPB was associated with a 4-fold increase in early mortality (relative risk; 4.0, CI 3.1-5.2) (Pickering *et al.*, 2015).

Table 1.1.2. Early incidence of acute kidney injury following cardiac surgery

Author	Number of cases	Cohort	Retrospective/ Prospective	Sites	AKI Definition	AKI Incidence	% requiring dialysis
(Loef *et al.*, 2005)	843	CABG Valve Other	Retrospective	Single	↑ in serum creatinine level of > 25% in first 7 days from preoperative level	17.2%	0.7%
(Kuitunen *et al.*, 2006)	808	CABG Valve Other Transplant	Retrospective	Single	RIFLE	19.3%	3.2%
(Lassnigg *et al.*, 2008)	7241	CABG Valve Other	Prospective	Multiple	RIFLE (48 hr window) AKIN	3% 8.2%	1.8%
(Hobson *et al.*, 2009)	2973	CABG Valve Aortic Transplant Thoracic	Retrospective	Multiple	RIFLE	43%	2.5%
(Brown *et al.*, 2010)	4837	CABG Valve Other	Retrospective	Single	AKIN	39%	3.1%
(Englberger *et al.*, 2011)	4836	CABG Valve Other	Retrospective	Single	RIFLE AKIN	26% 18.9%	2.0%
(Lopez-Delgado *et al.*, 2013)	2940	CABG Valve Other	Retrospective	Single	Modified RIFLE	14%	3.2%
(Ryden *et al.*, 2014)	27929	CABG Only	Retrospective	Multiple	AKIN	13%	Not reported
(Hansen *et al.*, 2015)	4742	CABG Valve Other	Retrospective	Multiple	KDIGO	30.7%	1.8%

Long term outcomes of acute kidney injury

In addition to early AKI following cardiac surgery, longer term adverse effects of postoperative AKI on subsequent renal function and mortality have been described. Lopez-Delgado et al. (2013) evaluated the longer term mortality risk associated with renal impairment, using a modified RIFLE classification, following cardiac surgery with CPB. Two thousand nine hundred and forty patients with no prior kidney disease were included in the single centre study. Fourteen percent (n = 409) were diagnosed with RIFLE class AKI. Patients with RIFLE class AKI had significantly worse long-term survival over the 7-year follow up period, with 10.6% mortality in those patients who did not develop AKI and 21.4% in patients who developed AKI, with 15.9% in RIFLE-R, 25% in RIFLE-I and 44.7% in RIFLE –F.

These findings are in agreement with those of Hobson and others (2009) who reported a relationship between long term mortality and AKI with small changes in serum creatinine after cardiothoracic surgery. In their retrospective study of 2973 patients with no history of CKD, 1265 (43%) had an episode of AKI (RIFLE criteria). Of the 1265 patients 637 (22%) were identified as RIFLE-R, 386 (13%) were classified as RIFLE-I, and 242 (8%) were classified RIFLE-F. Patients with complete or partial renal recovery after AKI had a 10-year survival of 44% compared with 63% for patients with no episode of AKI following cardiothoracic surgery. Of the patients who had no renal recovery, very few survived to 10 years to calculate a survival rate.

In a five year follow up study by Brown et al. (2010), not only was the incidence of AKI and its impact on long term survival investigated but also the impact of the duration of the AKI episode. Of the 4987 patients who underwent cardiac surgery, 39% were identified as developing AKI as defined by AKIN. Duration of AKI was defined by the number of days AKI was present and categorised as AKI for 1 to 2, 3 to 6, and at least 7 days. Long term survival was significantly associated with duration of AKI. Eight hundred and ninety-six (18.5%) patients developed AKI for 1-2 days, 552 (10.8%) for 3 to 6 days and 438 (9.1%) for at least 7 days. The adjusted hazard ratios for mortality were 1.66 (95% CI 1.32-2.09), 1.94 (95% CI 1.51-2.49) and 3.40 (95% CI 2.73 to 4.25) respectively.

Aside from mortality there is a paucity of reports of other long-term outcomes following an episode of AKI after cardiac surgery. Ryden et al. (2014) carried out a long term study of AKI defined by AKIN after isolated CABG and determined the long term risk of myocardial infarction (MI) or death. The investigators found that AKI post CABG was associated with an elevated risk of MI or death in the short and long term. In their population-based cohort study of 27 929 patients undergoing isolated CABG, 13% developed AKI following surgery. During a mean follow up of 5 years, there were 2119 (7.6%) patients who suffered an MI and 4679 (17%) deaths. For AKIN stages 1, 2 and 3 multivariable adjusted hazard ratios were 1.35 (1.15-1.57), 1.80 (1.53- 2.13) and 1.63 (1.29-2.07) for MI, with hazard ratios for all-cause mortality of 1.30 (1.17-1.44), 1.65 (1.48-1.83) and 2.68 (2.37–3.03) respectively. This study identified both that AKI after CABG is associated with an increased risk for MI and death at five years and highlighted that the increased risk is significant even for small increases in serum creatinine.

These results were supported by the findings of Hansen et al (2015), who reported that early and late onset AKI within 30 days of elective cardiac surgery was associated with a similar increase in 5 year risk of mortality, MI, heart failure and stroke. Of the 4742 patients, 1457 (30.7%) developed AKI within 30 days of surgery as defined by an increase in baseline creatinine according to the KDIGO criteria. The five year hazard ratio for mortality was 1.37 (95% CI 1.05-1.80) and 1.41 (95% CI 1.11-1.80) for the composite cardiovascular endpoints of MI, heart failure or stroke.

A meta-analysis by Coca et al. (2012) found AKI may increase the risk for CKD and end-stage renal failure. In a systematic review comparing risk of CKD, end-stage renal disease and death in patients with and without AKI, 13 cohort studies were selected, evaluating long term renal and non-renal outcomes of AKI. The study found patients with AKI, defined by various definitions, had higher risks of developing CKD (pooled adjusted hazard ratio 8.8, 95% CI 3.1-25.5), end stage renal disease (ESRD) (pooled adjusted HR 3.1, 95% CI 1.9-5.0) or mortality (pooled adjusted HR 2.0, 95% CI 1.3-3.1) than patients without AKI. The authors identified AKI as an independent risk factor for CKD, ESRD, and death and other important non-renal outcomes.

A second study by Brown et al. (2016) in 1610 patients undergoing isolated CABG over 8 institutions found severity of AKI using the AKIN stage criteria was associated with a significantly increased risk of 5-year readmission and mortality. The authors reported AKIN stage 1 and stages 2-3 were associated with a significant increased hazard of 5-year readmission. In addition, AKIN stage 1 and stages 2-3 were associated with a significant 3.5-fold increased hazard of 5-year mortality. The authors suggested current and novel therapeutic interventions to reduce AKI may have a significant long-term impact for patients, and the health care system, in reducing readmission and mortality up-to 5 years.

Risk factors for cardiac surgery-associated acute kidney injury

It is unlikely that a single etiologic factor causes perioperative AKI. Cardiac surgery associated-acute kidney injury (CSA-AKI) is the consequence of multiple interactive pathways influenced by the preoperative state of the patient, perioperative administration of drugs known to increase the likelihood of CSA-AKI, CPB and the differing severity of perioperative insults and the complexity and nature of the surgery. Numerous factors have been attributed to increase the risk of CSA-AKI in susceptible patients including prolonged duration of CPB, prolonged aortic cross clamping with increased cardiac ischaemia reperfusion injury, activation of the systemic inflammatory response reduced oxygen delivery and low cardiac output syndrome compromising RBF (Gaffney & Sladen, 2015).

Preoperative risk factors

Acute kidney injury following cardiac surgery is frequent yet incompletely understood. At least five validated risk-predictive models of AKI following CPB have been developed. The major risk factors identified are age, female gender, preoperative renal insufficiency, low ejection fraction, emergency surgery and diabetes (Kumar & Suneja, 2011). Preoperative renal function may be further compromised by diuresis and the use of non-steroidal anti-inflammatory drugs (Carmichael & Carmichael, 2003). Many patients undergoing cardiac surgery are on angiotensin-converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARB), statin and/or aspirin therapy. However, whether such medications have a beneficial protective role or put patients at an increased risk for AKI following surgery remains unclear (Lewicki *et al.*, 2015; Myles *et al.*, 2016; Zou *et al.*, 2016).

Perioperative risk factors – cardiopulmonary bypass considerations

The perioperative period and the use of CPB provides an ideal time to investigate the development of AKI as intervention is still feasible. A number of factors during CPB have been investigated in playing a potential role in the development of AKI including, the mean arterial pressure (MAP) maintained during CPB, arterial pump flow, haematocrit (Hct), transfusion, oxygen delivery (DO_2) and RBF.

Mean arterial pressure and acute kidney injury

An inadequate MAP during CPB can result in inadequate perfusion and oxygen delivery. A central consideration in the use of CPB is the level of arterial pressure to be achieved. However, there is inconclusive evidence on the relationship between the MAP targeted during CPB and the incidence of AKI following cardiac surgical procedures. Mean arterial pressure on CPB is determined by arterial pump flow and patient systemic vascular tone. A number of early prospective observational studies examined the association between hypotension during CPB and adverse outcomes. The predominant outcome assessed in these trials was neurological dysfunction, which formed the evidence base for maintenance of a target MAP of between 50 to 60 mmHg in keeping with the lower limits of cerebral autoregulation (Stockard *et al.*, 1973).

Several small-scale studies have been carried out to determine the optimal MAP during CPB to reduce the risk of CSA-AKI. In 2012 Sirvinskas et al. prospectively randomised 122 patients greater than 70 years of age into 3 groups. Group 1 was maintained at 60 $\leq$ 70 mmHg, group 2 at 45 $\leq$ 60 mmHg and group 3 at 70 - 95 mmHg of MAP, maintained with the use of vasoconstrictors. They found no significant difference among the groups with regard to increases in serum creatinine, need for diuretics or length of hospital stay. The authors concluded there is no significant relationship between MAP and postoperative renal function. The study was limited by not having continuous pressure measurements recorded throughout CPB nor did it report RIFLE criteria creatinine based renal outcomes as proposed in the methods.

In 2002 Fischer et al. carried out a retrospective study investigating renal outcomes in 143 patients undergoing cardiac surgery with CPB with normal preoperative renal function. Three patient groups were selected, those who developed AKI requiring dialysis within the first 48 h post-surgery (n = 44, acute renal failure (ARF) group), those who developed AKI as determined by an increase in serum creatinine within 48 h post-surgery (n=51, CREA group) and those with normal postoperative renal function determined by creatinine levels (n=48, control group). Cardiopulmonary bypass duration was longer and average mean arterial pump flow was lower in the ARF group compared to the control group. While MAP on CPB was similar between the 3 groups, duration of CPB at a MAP of < 60mmHg was longer in the ARF group (77.9 $\pm$ 60 min) compared to the CREA (61.5 $\pm$ 36.4 min) and control group (45.8 $\pm$ 25.7 min). This retrospective study is limited as it does not describe how pressure measurements were recorded throughout CPB.

Other studies have been carried out to determine whether changes in individual patient preoperative MAP relative to intraoperative MAP predisposes to AKI rather than a target a generalised MAP on CPB. Kanji et al. (2010) hypothesised that a greater difference in intraoperative MAP relative to preoperative MAP would be an important predisposing factor for AKI. Of the 157 consecutive high risk patients observed, 65 (41%) developed AKI within the first 24 h post-surgery according to RIFLE criteria. They found a drop in MAP of > 26 mmHg was independently associated with the development of AKI following cardiac surgery. They concluded that whilst there was not a universal pressure threshold that related to the development of AKI, change in MAP is a critical risk factor.

A recent study by Kander et al. (2019) of 90 patients found increased MAP during CPB did not decrease the incidence of acute or chronic kidney injury after cardiac surgery. In the study patients were randomised either to a control group or a high pressure group (arterial pressure > 60 mmHg). Mean arterial pressure was firstly maintained by increased arterial pump flow with the vasoconstrictor noradrenaline used as for intervention in the high pressure group to maintain a MAP > 60 mmHg. Mean arterial pressure in the control group was 47 $\pm$ 5 mmHg and MAP in the high pressure group was 61 $\pm$ 4 mmHg. The authors found there were no significant differences in GFR or incidence of AKI (RIFLE criteria).

Postoperative urinary NGAL/creatinine ration was also comparable between the groups. They concluded that an increased MAP during CPB did not decrease the risk of acute or chronic AKI in patients undergoing cardiac surgery with CPB. There remains insufficient evidence to guide the optimal MAP for patients undergoing CPB to avoid the development of AKI.

Arterial pump flow and acute kidney injury

Arterial pump flow is a major determinant of oxygen delivery during CPB. Data defining the optimal flow required to provide adequate tissue perfusion during CPB are limited. Flow rates are primarily based upon a patient's body surface area (BSA) with a flow rate during CPB of 2.2 - 2.4 L/min/m^2 commonly used. The figure, 2.2-2.4 L/min/m^2, being comparable to the cardiac index of a normothermic anaesthetised patient (Cook *et al.*, 1997). While the effect of arterial pump flow rate on cerebral blood flow and cerebral metabolism has been examined, investigations into its effect on other organs including the kidney are limited.

Andersson et al. (1994) studied the effect of pump flow rate on renal perfusion measuring RBF in the left renal vein. They observed that during CPB RBF was primarily determined by pump flow rate and that renal autoregulation was not maintained during CPB. However, Slogoff et al. (1990) found no relationship between low arterial pump flow rates during CPB (< 1.6 L/min/m^2) and new postoperative renal dysfunction. Renal dysfunction was defined as a 24 hr postoperative creatinine level greater than 130 μmol/L (1.5 mg/dL) and 25% greater than the preoperative levels.

A recent study by Lannemyr et al. (2019) evaluated the renal effects of three different CPB flow rates applied in a randomised order in each patient during CPB. Seventeen patients with normal serum creatinine undergoing normothermic CPB were included. Following insertion of a pulmonary artery catheter and renal vein catheter, renal oxygen extraction, a direct measure of the renal oxygen delivery/renal oxygen consumption ratio, and renal filtration fraction were measured during CPB. Renal filtration fraction was measured by renal extraction of ^{51}chromium-ethylenediamine tetraacetic acid. Following initiation of CPB and aortic cross-clamping, the pump flow rate was randomly varied between 2.4, 2.7, and

3.0 L/ min/m^2, with haemodynamic variable measured after 10 min at each flow rate. The main finding of the study was that increased arterial pump flow improved renal oxygenation, expressed as reduced renal oxygen extraction, a direct measure of an improvement of the renal oxygen supply/demand relationship. By increasing pump flow from 2.4 to 3.0 L/min/m^2, the oxygen supply/demand relationship was improved by 30% and renal oxygen extraction was restored to levels measured prior to CPB.

The authors speculate that this increase in global renal oxygenation could potentially improve the oxygenation of the renal medulla and reduce the tubular hypoxic injury. However, RBF was not directly measured and oxygen consumption could therefore not be assessed. The authors concluded that higher CPB flow rates than those conventionally used improve renal oxygenation, most likely due to increased renal oxygen delivery with maintained or reduced oxygen consumption.

Haematocrit and acute kidney injury

Adequate red cell mass as determined by Hct is vital for oxygen transfer and delivery. Progressive haemodilution causes a proportional decrease in the oxygen-carrying capacity of the blood. As blood becomes more dilute, however, it also becomes less viscous, leading to increased blood flow in the macrocirculation and microcirculation. This increased flow partially compensates for the decreased oxygen-carrying capacity of blood, but only to an as-yet-undefined "critical" Hct concentration beyond which further haemodilution results in reduced tissue oxygen delivery (Chen *et al.*, 2000).

The current practice of CPB involves the addition of 500-2000 mL of non-hematic fluids (crystalloid and colloid fluids used to prime the CPB circuit) to the patient's blood volume, frequently results in marked haemodilution. Other factors contributing to haemodilutional anaemia during cardiac surgery include patient characteristics including baseline haemoglobin concentration, anaesthetic factors including volume management prior to initiation and during CPB, and other CPB related factors including the use of retrograde autologous priming, acute normovolaemic haemodilution and cell salvage (Ranucci *et al.*, 2006; Schwann *et al.*, 2001). The extent to which haemodilution can compromise renal oxygenation and contribute to AKI is unclear. Anaemia-induced tissue hypoxia due to acute

haemodilution may be a central mechanism of organ injury, including in the development of AKI (Shander *et al.*, 2011). Multiple large studies have found a significant relationship between lower Hct and increased incidence of AKI (Table 1.1.3)

Table 1.1.3 Relationship between lower haematocrit on cardiopulmonary bypass and incidence of acute kidney injury

Author	Number of cases	Retrospective/ Prospective	Sites	Nadir Hct	Observation	AKI Incidence	% requiring dialysis
(Karkouti *et al.*, 2005)	9080	Prospective	Single	< 21%	Adjusted odds ratio for AKI requiring dialysis with nadir Hct < 21% was 2.34	AKI requiring dialysis only reported	1.5%
(Ranucci *et al.*, 2006)	1766	Retrospective	Multi	< 23%	Risk of renal injury requiring dialysis doubled in patients with nadir Hct < 23% and who received blood transfusion	AKI requiring dialysis only reported	0.7%
(Haase *et al.*, 2012)	920	Retrospective	Single	< 27%	Lower nadir Hct was associated with an increased risk of AKI	RIFLE 19.5%	5.2%
(Mehta *et al.*, 2013)	13734	Retrospective	Single	< 22%	Lowest Hct was inversely related to AKIN stage 2 and 3	AKIN (Stage 2 and 3) 5.2%	Not reported
(Ellis, 2015)	15221	Prospective	Multi	< 23%	Lower nadir Hct was associated with an increased risk of AKI	AKIN (Stage 2 or 3) 2.7%	Not reported

Many improvements to CPB technology have been introduced over the past decade to limit the risk associated with haemodilution with a focus on redesign of the circuit to reduce volume, the use of retrograde autologous priming and management of intraoperative fluid administration. Observational studies have suggested some improvement in the incidence of AKI following such changes (Ranucci *et al.*, 2015).

Transfusion and acute kidney injury

The adverse influence of low Hct on the development of AKI is complicated by the transfusion of packed red blood cells (RBC) to increase Hct levels. Findings from observational and randomised studies have raised concerns about the effects of transfusion strategies on longer-term outcomes, because both anaemia and allogeneic transfusion can have effects that persist beyond hospitalisation for surgery. Anaemia can lead to tissue hypoxia in patients who are at high risk for perioperative complications. Allogeneic transfusion has short-term and long-lasting effects on the innate and adaptive immune system, which can potentially lead to poor clinical outcomes (Mazer *et al.*, 2017). The risk of transfusion must be balanced against the risks of untreated anaemia, which can compromise tissue oxygenation and also increase the risk of MI, stroke and AKI (Karkouti *et al.*, 2005).

Karkouti (2012) published a systematic review focusing on the relationship between perioperative transfusion and AKI after cardiac surgery with CPB. The majority of studies reported an independent relationship between perioperative blood transfusion and AKI with each unit of perioperative blood transfusion independently associated with a 10 - 20% increase in risk of AKI after cardiac surgery with CPB.

The Transfusion Requirements in Cardiac Surgery (TRICS) III trial was a large multicentre trial carried out in a wide range of hospitals and countries to compare a restrictive transfusion strategy with a liberal strategy in patients undergoing cardiac surgery with CPB. Five thousand, two hundred and forty-three adults undergoing cardiac surgery who had an European System for Cardiac Operative Risk Evaluation (EuroScore) I of 6 or more (on a scale from 0 to 47, with higher scores indicating a higher risk of death after cardiac surgery) were randomised to a restrictive red cell transfusion strategy (transfusion if the haemoglobin (Hb)

concentration was < 7.5 g/dL intraoperatively or postoperatively) or a liberal transfusion strategy (transfusion if the Hb was < 9.5 g/dL intraoperatively or postoperatively when the patient was in the ICU or was < 8.5 g/dL when the patient was in the non-ICU ward). The primary composite outcome was death from any cause, myocardial infarction, stroke, or new-onset renal failure with dialysis by hospital discharge or 28 days after surgery, whichever came first. The authors reported that in patients undergoing cardiac surgery who were at moderate-to-high risk for death, a restrictive strategy regarding red-cell transfusion was noninferior to a liberal strategy with respect to outcomes of death, MI, stroke or new-onset renal failure with dialysis or less transfusion (Mazer *et al.*, 2017).

A six-month follow up of the TRICS III trial continued to show there were no significant differences between the restrictive strategy and liberal strategy in the individual components of the primary composite outcome including death from any cause, MI, stroke, or new-onset renal failure with dialysis, or secondary outcomes including red cell transfusion (Mazer *et al.*, 2018).

Duration of cardiopulmonary bypass and acute kidney injury

In a meta-analysis by Yi et al. (2016), 12 studies were identified in which the association between CPB time and RIFLE defined AKI was examined. Longer duration of CPB was found to be associated with a higher risk of developing AKI, which in turn significantly affected overall mortality. In a subsequent prospective study, of 2940 consecutive operations, Lopez-Delgado et al. (2013) identified longer CPB duration as in independent intraoperative predictor of AKI. The average time on CPB was 109 $\pm$ 37 min for patients without AKI compared with 135 $\pm$ 55 min in patients who developed AKI.

Renal blood flow and acute kidney injury

Renal ischaemia has been considered an important pathway in the development of CSA-AKI. In studies by Lannemyr et al. (2017), the effects of normothermic CPB on RBF, GFR, renal oxygen consumption and the renal oxygen supply/demand relationship were investigated. With the use of a renal vein catheter, RBF and GFR were measured before, during and after CPB. Arterial and renal blood samples were taken for measurement of renal oxygen delivery

and consumption as determined by para-aminohippurate clearance. They reported CPB induced renal vasoconstriction and redistribution of blood flow away from the kidneys, which in combination with haemodilution during CPB, decreased renal oxygen delivery by 20%. While renal oxygen delivery was reduced, GFR and renal oxygen consumption remained unchanged. This resulted in an oxygen supply/demand mismatch during CPB which continued postoperatively following termination of CBP.

Oxygen delivery and acute kidney injury

Retrospective studies have confirmed the association between nadir DO_2 on CPB and AKI with the identification of a "critical DO_2". In 2005 Ranucci et al. published an important study examining the importance of DO_2 on renal outcomes, investigating the role of the lowest DO_2, lowest Hct and pump flow during CPB as possible risk factors for the development of AKI and renal dysfunction. Lowest DO_2 was calculated on the basis of indexed pump flow (mL/min/m^2) using the mean indexed value during 30 min of CPB around the time when lowest Hct was recorded), arterial oxygen tension, Hb value (mg/dL) and Hb saturation, according to the equation:

$$DO_2 = \text{pump flow} \times (\text{Hb} \times 1.36 \times \text{Hb saturation} + 0.003 \times \text{arterial oxygen tension})$$

From the results of this retrospective single centre study of 1048 consecutive patients undergoing CPB, the authors determined the best predictor for AKI and peak postoperative serum creatinine levels was the lowest DO_2, with a critical DO_2 value at 272 mL/min/m^2. The study demonstrated the association between lowest Hct on CPB and adverse renal outcomes and introduced the lowest DO_2 indexed on CPB as potentially a more reliable predictor of AKI requiring dialysis. A second retrospective study by de Somer et al. (2011) in 359 patients over two institutions, found nadir DO_2 levels of < 262 mL/min/m^2 were independently associated with the development of postoperative AKI stage 2 according to the AKIN criteria and was significantly associated with prolonged ICU and postoperative hospital lengths of stay. This led to the authors to conclude that the maintenance of adequate DO_2 during CPB may limit the risk of postoperative AKI and proposed "the concept of goal-directed CPB perfusion merits further investigations that are based on clinically

significant quality indicators such as DO_2". A number of studies have supported these findings.

Magruder et al. (2015), using propensity scoring, matched 85 patients who developed AKI after CPB with 85 control patients who did not. Univariate analysis revealed patients who developed AKI had lower nadir DO_2 on CPB (208 vs 230 mL O_2/min/m^2). These patients experienced prolonged ICU stay (213 hr vs 88 hr) and total length of stay (19 days vs 12 days). Mortality was 35.3% in patients who developed AKI compared with 5.9% of patients who did not develop AKI.

In a follow-up study Magruder et al. (2017) retrospectively determined whether a protocol based upon a goal directed perfusion (GDP) management approach, including a conservative targeted DO_2 of 300 mL/min/m^2 primarily achieved via higher CPB flow rate, was associated with reduced AKI incidence when compared with patients undergoing surgery using conventional CPB management (pump flow rates based on body surface area (BSA), to achieve a cardiac index of 2.0-2.2 L/min/m^2) (Magruder *et al.*, 2017). They included 88 patients in the GDP group and 88 matched patients. The authors reported patients in the conventional CPB group received more vasoconstrictor therapy (2.1 mg vs 1.4 mg phenylephrine, $p < 0.001$) and had lower nadir DO_2 (mean 241 vs 301 mL O_2/min/m^2), with the incidence of AKI in the conventional CPB group of 23.9% and 9.1% in the GDP group. The authors concluded the difference to perfusion management and implementation of a GDP initiative was associated with a reduced incidence of AKI following cardiac surgery.

Ranucci et al. (2018) recently reported a randomised controlled multicentre study to determine whether a GDP strategy aimed at maintaining DO_2 at $\geq$ 280 mL/min/m^2 reduced the incidence of AKI (primary endpoint any AKI by AKIN classification). The trial enrolled 350 patients undergoing cardiac surgery at 9 institutions. A total of 326 patients completed the study. Patients in the treatment arm were managed with a GDP strategy during CPB aimed to maintain DO_2 at $\geq$ 280 mL/min/m^2. The perfusion strategy for patients in the control arm was factored on institutional protocols on body surface area and temperature. The results

of the study showed that GDP maintain DO_2 at $\geq$ 280 mL/min/m^2 is effective in reducing AKIN stage 1 AKI, with no benefits demonstrated on secondary endpoints of ICU stay, major morbidity, RBC transfusions and operative mortality. The authors commented that "given the efficacy of GDP in preventing only minor degrees of AKI in low-risk patients, our results do not definitively suggest a change in clinical practice". Further studies are needed to define perfusion interventions that may reduce more severe levels of renal injury (AKIN stage 2 or 3). Most recently, a large cohort study of 19 410 patients from the Australian and New Zealand Collaborative Perfusion Registry (ANZCPR) registry has reported that minimum DO_2 during CPB is associated with RIFLE class R and I with an optimal threshold of 270 mL/min/m^2 with a 7% increase in risk of AKI for every 10 mL/min/2 reduction in DO_2 (Newland *et al.*, 2019).

While many of the risk factors associated with AKI following cardiac surgery are intrinsic to the patient and are unmodifiable, nadir DO_2 has been identified as a modifiable risk factor. GDP challenges the way perfusion is conducted with the aim of goal directed perfusion being to optimise DO_2 and tissue perfusion (Dijoy *et al.*, 2015). The concept that arterial pump flow should be based on DO_2, rather than body surface area, continues to challenge the conduct of CPB.

Haemolysis, acute kidney injury and cardiopulmonary bypass

Haemolysis, resulting in increased plasma free Hb, is an important consequence of CPB and may be an important contributor to postoperative kidney injury. The potential mechanisms of Hb mediated AKI include intratubular Hb precipitation causing obstruction, iron-facilitated oxidant damage to tubular epithelial cells and induction of vasoconstriction due to reduced nitric oxide bioavailability (Vermeulen Windsant *et al.*, 2011). Cardiopulmonary bypass related haemolysis is caused by mechanical shear stress within the extracorporeal circuit including roller pumps, turbulent passage through the oxygenator, reservoir, filters, arterial and venous cannulae, cardiotomy suction, the air to blood interface, cell salvage and transfusion (Ricci *et al.*, 2014). There is also an increase in erythrocyte fragility and aggregability due to contact with the foreign extracorporeal circuit (Vercaemst, 2008).

Vermuelen Windsant et al. (2014) found that haemolysis indicated by plasma levels of free Hb and nitric oxide consumption significantly increased in patients undergoing cardiac surgery requiring CPB. The extent of haemolysis and nitric oxide consumption correlated significantly. Patients undergoing OPCABG procedures did not show increased plasma free Hb or nitric oxide consumption. Patients suffering from AKI, as defined by AKIN, displayed significantly greater concentrations of plasma free Hb during surgery than did patients who did not develop AKI.

In paediatric patients haemolysis induced by CPB has also been associated with AKI, with the reported incidence of AKI in paediatric patients undergoing cardiac surgery ranging from 20-86% depending on the definition used (Toda & Sugimoto, 2017). Mamikonian et al. (2014) also found that changes in plasma free Hb and duration of CPB were significantly associated with the development of AKI postoperatively. In 40 paediatric patients, plasma free Hb levels increased markedly on CPB with concurrent decreases in haptoglobin.

Off pump coronary artery bypass grafting and incidence of acute kidney injury

Many of the risk factors reviewed for the development of kidney injury following cardiac surgery are linked to the use of CPB. Cardiopulmonary bypass and the lack of pulsatile flow, the haemodilutional effects of anaemia, the atheroembolism from manipulation of the aorta, the haemolysis generated by components of the CPB circuit, the systemic inflammatory response syndrome (SIRS) induced and low cardiac output syndrome, and global hypoperfusion, attributed to the use of CPB have been indicated as contributing factors for the development of CSA-AKI.

The negative sequelae of the use of CPB has been one of the drivers to the development of cardiac surgery for coronary artery disease without the use of CPB, with the procedure termed OPCABG. The development of mechanical stabilisers allows CABG surgery to be performed on a beating heart. Two large randomised trials have attempted to address the relative benefits and risks of performing CABG with or without CPB.

The Randomised On/Off Bypass (ROOBY) trial was a controlled, single-blinded, randomised trial conducted at 18 veteran affairs medical centres in the US Over 2000 patients

undergoing CABG were randomly assigned to either on or off pump procedures. Primary short-term end points were a composite of death or complications including stroke or AKI requiring dialysis before discharge or within 30-days following surgery. The primary long-term end points were death, repeat revascularisation or non-fatal infarct within one year. There was no significant difference in 30-day outcomes between patients undergoing OPCABG and patients undergoing CABG with CPB. Outcomes for new AKI not requiring dialysis were not reported (Shroyer *et al.*, 2009).

The multicenter CABG Off or On Pump Revascularisation study (CORONARY) trial also compared outcomes following OPCABG and CABG with CPB. Four thousand seven hundred and fifty-two patients were enrolled with 2375 randomised to undergo OPCABG and 2377 to undergo CABG with CPB. Again, there was no significant difference in the rate of primary composite outcomes including death, stroke, MI and new renal failure requiring dialysis between patients undergoing OPCABG and patients undergoing CABG. The CORONARY trial also analysed renal outcomes by RIFLE criteria. While there were no statistically significant differences between groups in the RIFLE injury class, there was a statistically significant difference in outcome between the groups in the RIFLE risk group. Three hundred and eighty two (17%) patients undergoing OPCABG compared with 443 (19.5%) of patients undergoing CABG with CPB had an increased risk of AKI as defined by the RIFLE criteria (Lamy *et al.*, 2012).

Garg et al. (2014) reported on the risk of AKI in the CORONARY trial to determine if there was a difference between the two treatment groups in kidney function one-year later, as determined by a 20% or greater loss in estimated GFR. The main outcomes were AKI within 30 days of surgery (> 50% increase in serum creatinine concentration from pre randomised concentration) and loss of kidney function at one year. There was no significant difference between the two groups in loss of kidney function at one year

Patients with CKD, however, have been found to benefit from OPCABG surgery. In a meta-analysis carried out by Wang et al. (2018), that included 17 studies with 201 889 patients, OPCABG utilised for patients with CKD was associated with significantly lower early

mortality as compared to CABG with CPB. Off pump CABG was also associated with decreased risk of atrial fibrillation, cerebrovascular accident, red-cell transfusion, pneumonia, prolonged ventilation and shorter hospital stay. No difference was found regarding long-term survival or MI.

In regards to the development of AKI, the benefit of OPCABG for a reduction in the development of CSA-AKI remains controversial. The technique of OPCABG may be associated with a reduced risk of mild to moderate AKI in high-risk patients immediately following surgery when compared with CABG with CPB. However, there is no direct evidence the improved kidney outcomes extend further than the immediate postoperative period with no significant differences in primary outcomes reported at 12 months between the two groups. Off pump CABG, while avoiding the use of CPB, still has detrimental effects. Intraoperative haemodynamic instability and global hypoperfusion due to heart displacement during anastomosis of grafts and atheroembolism from side clamping may explanations the lack of difference observed in outcomes including AKI between patients undergoing OPCABG and CABG with CPB.

Management of cardiopulmonary bypass and the development of acute kidney injury

Acute kidney injury is a frequent and important complication of cardiac surgery and is associated with increased complications, length of hospital stay and mortality. There is currently limited evidence to make strong recommendations regarding how to conduct optimal CPB with regard to MAP, adequate arterial pump flow, lowest Hct, risk of perioperative transfusion and the role of haemolysis in the development of AKI. Despite the enthusiasm for OPCABG and the avoidance of CPB, off-pump surgery has not conclusively shown any benefit in the reduction of AKI, AKI requiring dialysis or a long-term survival advantage. While the effects of AKI on long-term mortality are known, uncertainty remains as to the long term effects of AKI on renal function. In patients with CRF, outcomes post cardiac surgery remains poor with a significantly higher morbidity and mortality.

The challenge with managing AKI following cardiac surgery is that there is no effective intervention once AKI has been diagnosed. Efforts to prevent AKI associated with cardiac surgery are limited by:

1) the ability to predict which patients will develop AKI following cardiac surgery, with available preoperative risk scoring systems available having limited predictive efficacy and

2) the inability to monitor the risk of AKI developing intraoperatively, prior to development of kidney injury.

Mechanisms of acute kidney injury

The major causes of AKI are typically classified into three broad descriptive categories: prerenal, post renal and intrinsic renal. Prerenal causes of AKI may complicate any situation characterised by true hypovolemia including third space volume shifting into the interstitium. During prerenal kidney injury, perfusion to the brain and myocardium is maintained by the complex interaction of the sympathetic nervous system, stimulation of the renin- angiotensin-aldosterone system and vasopressin release. Activation of these systems is associated with a compensatory increase in intra renal activity of several vasodilatory agents that can often maintain an adequate blood supply to the kidney. Renin is secreted by juxtaglomerular cells converting angiotensin to angiotensin I. Angiotensin-converting enzyme, produced by the lungs, converts angiotensin I to angiotensin II. Angiotensin II is a potent stimulator of aldosterone secretion and constitutes a major input to the adrenal cortex controlling its production and release. Aldosterone causes an increase in sodium reabsorption and potassium excretion at the distal tubule and collecting duct of the nephron. The net effect is an increased level of sodium reabsorption resulting in an increase in circulating blood volume and maintenance of perfusion pressure.

When a patient suffers perioperative AKI due to prerenal factors including, hypovolemia, haemorrhage, over diuresis, anaesthesia induced hypotension due to low systemic resistance, low cardiac output induced by anaesthesia and CPB and increased intra-abdominal pressure, the kidneys may have not yet have sustained cellular injury (Goren &

Matot, 2015). The resultant decline in GFR is often recoverable upon reversal of the systemic disturbance that has led to the decline in RBF (Brady & Singer, 1995). Post renal kidney injury results from urinary tract obstruction and accounts for less than 5% of cases complicating hospital admission (Brady & Singer, 1995). Similarly to pre-renal injury, in post renal failure the decline in GFR is often rapidly reversible upon removal of obstruction to urinary drainage (Kaufman *et al.*, 1991).

The causes of AKI due to intrinsic renal damage can be classified according to the primary site of the lesion; tubular, glomerular, vascular or interstitial. Intrinsic AKI can be associated with ischaemia, toxins or primary interstitial or glomerular disease (Goren & Matot, 2015). Acute tubular necrosis is the most common cause of intrinsic AKI (Mindell, 1997). Tubular injury most commonly occurs during the perioperative period and is associated with ischaemia resulting in acute tubular necrosis in the postoperative period (Carmichael & Carmichael, 2003). Ischaemia is characterised predominately by injury to the proximal tubule (Bonventre, 1993). The increased susceptibility of the tubules to ischaemia is due to the high oxygen consumption by extensive adenosine triphosphate-dependent transporters in tubular cells. Renal sodium transport is the main oxygen consuming function of the kidney and is closely linked to RBF for sodium transport, particularly in the thick ascending limbs of the loop of Henle and the proximal tubules (Brezis & Rosen, 1995)

Ischaemic kidney injury differs from pre renal kidney injury in that renal hypoperfusion may be severe enough to injure tubular epithelium and if prolonged enough may lead to death of tubular cells. Acute kidney injury, however, does not always strictly obey these definitions as pre renal AKI, if profound and lasting, can lead to intrinsic AKI.

Cellular pathophysiology of acute kidney injury

The endothelial and smooth muscle cells of the microcirculation play a critical role in the pathophysiology of AKI. Endothelial cells are important determinants of vascular tone, activation of the immune system and smooth muscle responsiveness (Sprague, 2009). Small arterioles in post ischaemic AKI vasoconstrict more than arterioles in the normal kidney. The vasoconstriction is a result of increased tissue levels of angiotensin II, thromboxane A2,

prostaglandins, leukotrienes and stimulation of the sympathetic nervous system (Bonventre & Yang, 2011).

The tubulo-glomerular feedback mechanism is an adaptive mechanism that links the rate of glomerular filtration to the concentration of sodium, potassium and chloride in the tubule fluid at the macula densa. The tubulo-glomerular feedback mechanism contributes to vascular vasoconstriction in a manner that coordinates RBF and GFR with tubular reabsorption (Peti-Peterdi *et al.*, 2002). The feedback mechanism contributes to a functional pre-glomerular arteriolar vasoconstrictive response resulting from the macula densa sensing more solute delivery to the distal nephron, potentially due to inadequate sodium reabsorption in the injured more proximal part of the tubule. The macula densa are specialised cells in the thick ascending limb of the loop of Henle closest to the Bowman's capsule, which mark the end of the thick ascending limb and beginning of the distal convoluted tubule and pass between the afferent and efferent arterioles. The macula densa detect the luminal content of the nephron and ultimately contribute to control of GFR and regulation of blood pressure via the secretion of the hormone renin (Blantz *et al.*, 2007; Evans *et al.*, 2013).

Regulation of the blood supply to the kidneys is influenced by humoral and neural factors. Alteration to this supply to the kidney impacts regional blood flow. Putative mechanisms include local oedema, activation of the cytokine system causing leukocyte sequestration and activation of the coagulation system resulting in congestion of medullary blood vessels and compromise of the microcirculation further reducing local blood flow to the medulla. The reduction in blood flow results in interference of flow to the proximal tubule and thick ascending limb, the most vulnerable nephron segments which are already on the border of hypoxia due to high metabolic demands of active adenosine triphosphate-dependent solute transport and oxygen consumption (Brezis & Rosen, 1995).

Renal oxygenation and acute kidney injury

The vulnerability of the kidney to injury is largely due to its predisposition to hypoxia under stress. The kidney receives 25% of cardiac output and renal oxygen consumption is

relatively high, second only to the heart (Ricksten *et al.*, 2013). Cortical blood flow is approximately 300 mL/min/100 g of tissue and the partial pressure of oxygen (PO_2) of the cortex is about 50 mmHg as measured by oxygen microelectrodes in the cortex and medulla of anaesthetized rats and healthy human subjects (Brezis *et al.*, 1984; Zhang *et al.*, 2014). In contrast blood flow in the medulla is significantly less, with blood flow in the outer medulla around 200 mL/min/100 g of tissue and inner medulla from 50 to 100 mL/min/100 g (Mattson, 2003).

The renal parenchymal oxygenation profile is non-homogenous with most cases of AKI reflecting imbalanced regional renal oxygen supply and consumption, especially in regions which are prone to hypoxia. The PO_2 of the medulla can be as low at 20 mmHg under normal physiologic conditions, which makes the medulla vulnerable to hypoxia if flow is reduced further (Brezis & Rosen, 1995). Complex mechanisms are designed to maintain safe oxygen levels in the medulla by the regulation and matching of regional blood flow, pressure and oxygen consumption for tubular transport.

Medullary pO_2 has been directly quantified in animal models of renal failure induced by radiocontrast administration and renal ischaemia-reperfusion injury, confirming the tissue in the medulla is readily subjected to hypoxic conditions (Brezis *et al.*, 1984; Legrand *et al.*, 2008). Animal studies by Brezis et al. (1994) reported that medullary oxygenation declined when MAP fell below 60-65 mmHg, as vasa recta blood flow and medullary oxygen delivery eventually decline.

Hypoxia and acute kidney injury

Oxygen content in the kidneys is maintained at relatively stable levels by a unique and complex functional interplay between RBF, GFR, O_2 consumption and arteriovenous O_2 shunting in the vasa recta (Haase, 2013). Hypoxia inducible factors (HIFs) play a vital role in oxygen supply and adaptation to hypoxic conditions. HIFs are oxygen sensitive transcription factors involved in oxygen dependent gene regulation that mediate cellular adaption to oxygen deprivation and tissue protection under hypoxic conditions in the kidney (Rosenberger *et al.*, 2006). HIF is a heterodimer composed of an oxygen regulated α-

subunit (HIF-1α or HIF-2α) and a constitutive β-subunit. In the presence of oxygen, HIF-α is hydroxylated by prolyl hydroxylases, which require molecular oxygen as a substrate (Safran & Kaelin, 2003). The hydroxylated prolyl residues are recognized by the von Hippel-Lindau protein as a component of an ubiquitin ligase, which targets HIFα for destruction through the ubiquitin-proteasome pathway. Under hypoxic conditions, HIF is not degraded, but instead accumulates in the cell and induces transcription of target genes (Haase, 2006). HIF-1α upregulates a number of factors implicated in cytoprotection, including angiogenic growth factors such as vascular endothelial growth factor and erythropoietin (Mansfield *et al.*, 2005). In the kidney, HIF-1α is expressed in tubular cells, whereas HIF-2α is expressed in peritubular cells, renal interstitial fibroblasts and endothelial cells. Differences in HIF expression among tubular cell types have been proposed to underlie their disparity in vulnerability to hypoxic injury (Rosenberger *et al.*, 2006). While renal hypoxia has been demonstrated both in animal models *in vivo* and isolated perfused kidneys, real-time identification and timely diagnosis of AKI and specifically of hypoxic renal injury are unfortunately lacking.

Current biomarkers for acute kidney injury

While the prevention of AKI is the ultimate goal, numerous novel urinary and plasma biomarkers have been proposed as a faster and more accurate way for early identification of AKI. For the clinical application of a new biomarker it should provide earlier detectability and clinical accuracy than the current diagnostic test of the measurement serum creatinine concentration (de Geus *et al.*, 2012). Serum creatinine is a suboptimal marker of AKI as it is not a marker reflecting tubular injury but rather a functional marker of glomerular filtration. (Waikar *et al.*, 2012). The use of serum creatinine may also lead to delays in diagnosis because of the slow kinetics of the rise in serum creatinine after injury (Waikar *et al.*, 2009).

Current definitions including RIFLE, AKIN and KDIGO are reliant on serum creatinine levels and urine output rates as determinants of AKI, which can often lag behind the actual occurrence of tubular or other intrarenal cellular injury. Serum creatinine is also limited in that its concentrations are affected by factors such as muscle mass, intravascular volume and drug interactions. Thus, changes in serum creatinine do not accurately or consistently

reflect real time change in renal function. Similarly, damage to renal tubular epithelia may not be reflected in serum creatinine or urine output until the damage progresses to a critical threshold (Teo & Endre, 2017).

The shortcomings of serum creatinine levels and urine output have led to the expansion of the AKI biomarker field. Renal tubular damage-specific biomarkers could offer an opportunity to diagnose AKI at an earlier time point. The potential role of novel biomarkers such as neutrophil gelatinase-associated lipocalin (NGAL), kidney injury marker 1 (KIM-1), urinary interleukin 18 (IL-18) and cystatin-C have been extensively reported. However, there are insufficient data to support their prognostic use for the detection of AKI. In a review of AKI biomarkers by Vanmassenhove et al. (2013) of 74 studies, no consistent conclusions could be drawn on the prognostic value of such serum or urinary biomarkers. Ho et al. (2015) examined 28 studies in the context of cardiac surgery and concluded that, known urinary, plasma and serum biomarkers of AKI possessed only modest discrimination at best when measured within 24 hours of surgery.

Urine tissue inhibitor metalloproteinase-2 and insulin like growth factor binding protein-7, both inducers of G1 cell cycle arrest, a key mechanism implicated in AKI, have been identified as biomarkers for predicting the development of moderate or severe AKI (KIDGO stage 2 or 3) (Kashani *et al.*, 2013). Husain-Syed et al. (2019) also investigated the use of the urinary cell cycle arrest biomarkers and their association with decreased renal functional reserve. Renal functional reserve describes the capacity of the kidney to increase GFR in response to stimuli and is defined by difference between peak “stress” GFR and resting GFR (Sharma *et al.*, 2014). The authors reported elevated postoperative cell cycle arrest biomarkers, tissue inhibitor metalloproteinase-2 and insulin like growth factor binding protein-7, were associated with decreased renal functional reserve 3 months following cardiac surgery with CPB despite normalisation of serum creatinine prior to discharge (Husain-Syed *et al.*, 2019).

The use of CPB during cardiac surgery and activation of blood components by the non-physiological components of the extracorporeal circuit initiates a systemic inflammatory response contributing to the development of AKI (Rosner *et al.*, 2008). Novel biomarkers

including cell-free DNA have received increasing attention as a danger-associated molecular pattern and have been used as clinical markers in cancer, trauma and sepsis. Cell-free DNA becomes released from necrotic and apoptotic cells as well as by activated neutrophils. Studies have demonstrated that circulating cell free DNA concentrations reflect the amount of neutrophil extracellular traps, extracellular chromatin fibers decorated with neutrophil granular proteins potentially inducing endothelial damage and organ dysfunction (Garnacho-Montero *et al.*, 2014). Serum cell-free DNA/neutrophil extracellular traps have been found to be markedly elevated after cardiac surgery and correlation with perioperative renal dysfunction, defined by the RIFLE, has been reported (Likhvantsev *et al.*, 2017).

In a study carried out by Merkle et al., (2019) cell free DNA levels were measured to predict AKI following CPB. Plasma levels of cell-free DNA, NGAL and creatinine were measured in 58 patients undergoing cardiac surgery with CPB. Blood samples were taken preoperatively, immediately after surgery, and at day 1, 2, 3 postoperatively. The authors reported cell-free DNA levels were significantly elevated in patients who developed late AKI (24 hr) but not in those with AKI development during the first 24 hr (early AKI). The authors suggested monitoring of cell free DNA cells from the first postoperatively day might represent a valuable tool to predict late AKI after CPB.

Despite the expectations of novel biomarkers to be sensitive, practical and accurate in predicting AKI, results are uncertain. The circulatory concentration profile of most biomarkers of AKI are adversely affected by the fluid balance and diuretic therapy. This concern regarding urinary biomarkers could be overcome by correcting their excretion to urinary creatinine, however creatinine kinetics may be dramatically altered in patients during CPB. Additional concern for biomarkers is that most of the tested biomarkers are present in the circulation and their excretion in the urine does not necessarily reflect renal origin or damage (Abassi *et al.*, 2019). A promising technology for the early real-time detection of evolving medullary hypoxia and for assessing risk of hypoxic AKI is a continuous determination of urinary oxygen content (uPO_2) (Zhu *et al.*, 2018).

In a consensus statement published by the Acute Dialysis Quality Initiative (ADQI) working group it was stated that while biomarkers are useful to complement the ability of RIFLE/AKIN to define AKI, there is currently insufficient data to support their use for AKI staging (McCullough *et al.*, 2013). Large-scale validation studies measuring association between novel biomarkers and clinically relevant outcomes are required before biomarkers can be introduced into clinical practice in order to facilitate early diagnosis, guide intervention and monitor disease progression. An ideal biomarker is one that helps to understand the pathophysiology and epidemiology of AKI and can thus be used to alter clinical management and improve outcomes.

Urinary oxygen content, cardiopulmonary bypass and acute kidney injury

Urinary oxygen content provides an estimate of medullary oxygenation whereby the PO_2 of urine in the collecting ducts would be expected to equilibrate with the tissue PO_2 of the inner medulla. In 2013, Evans et al. described the determinants of urinary oxygen tension and the potential for use of uPO_2 as a "physiological biomarker" of risk of AKI in the cardiac surgical setting (Evans *et al.*, 2013).

In 2018, Zhu et al. tested the hypothesis that urinary hypoxia during cardiac surgery requiring CPB predicts later development of AKI. Using a fiber optic probe placed in the bladder catheter of 65 patients undergoing cardiac surgery with CPB, bladder uPO_2 was continuously measured throughout the perioperative period and into the postoperative period, until catheter removal. The results showed that uPO_2 decreased during CPB, often reaching its nadir during rewarming or after weaning from CPB. Nadir uPO_2 was lower in the 26 patients who developed AKI than in the 39 patients that did not. The authors concluded that low uPO_2 during adult cardiac surgery requiring CPB predicts AKI, so may identify patients in which intervention, to improve renal oxygenation, might reduce the risk of AKI. This result supports the role of uPO_2 having a prognostic value during CPB which may be valuable to identify patients at risk of developing AKI.

Could markers of renal hypoxia act as predictors of acute kidney injury?

Biomarker development in nephrology is crucial to help develop therapeutic strategies for the prevention and treatment of AKI. Current biomarkers, including NGAL and cystatin C are useful to complement the ability of RIFLE/AKIN to define AKI, however there is currently insufficient data to support their use for AKI staging nor do they act as predictors of AKI. Other potential biomarkers of renal hypoxia include erythropoietin which has been found to increase following CPB and is being investigated as a potential biomarker of anaemia-induced tissue hypoxia following cardiac surgery with CPB (Hare *et al.*, 2018). There is still an urgent clinical need to identify new noninvasive diagnostic and prognostic biomarkers for the development of AKI. Accordingly, a biomarker that can detect AKI early may facilitate intervention within this narrow window of reversibility. Ideally, such a biomarker would identify injury as it occurs intraoperatively or at least within a few hours following surgery. The period of CPB provides a unique opportunity to potentially identify a marker of renal hypoxia to predict and possibly intervene to reduce the incidence of CSA-AKI.

The expression of many microRNAs (miRNAs), small non-coding 19-25 nucleotide RNA sequences, are tissue specific and their dysregulation has been associated with various diseases including many cancers, heart disease and kidney diseases including AKI (Ikeda & Pu, 2010; Lorenzen *et al.*, 2011; Lu *et al.*, 2005). Thus, circulating miRNAs are attractive biomarker candidates as conceptually their expression profile in blood or urine can potentially mirror changes in tissues. Some of the innate properties of miRNAs make them highly attractive as potential biomarkers. MiRNAs are readily detected in small volume samples using specific and sensitive quantitative real-time PCR, and their levels in plasma, serum and urine are stable (Arroyo *et al.*, 2011; Mitchell *et al.*, 2008). Moreover, blood and urine collection is a common and easy collection process. The expression of circulating and urinary miRNAs in patients undergoing cardiac surgery utilising CPB has not previously been examined in detail. The expression of specific miRNAs during CPB may shed light on alterations in pathways associated with the perioperative development of AKI including hypoxia (Hüttenhofer & Mayer, 2017).

1.2 MicroRNAs, Acute Kidney Injury and Cardiac Surgery

Definition and discovery of microRNAs

MicroRNAs are small non-coding 19-25 nucleotide RNA sequences that post-transcriptionally regulate the expression of target genes by binding to complementary target messenger RNAs (mRNA) and preventing the translation of mRNA into protein (Mendell, 2005). They represent one component of a larger collection of non-coding RNAs with regulatory functions, which includes endogenous small interfering RNAs (endo-siRNAs), PIWI-interacting RNAs (piRNAs), tRNA fragments and other long non-coding RNAs (lnc RNAs) (Esteller, 2011; Kim *et al.*, 2009; Mendell & Olson, 2012).

MiRNAs were first discovered in the nematode *Caenorrhabdidits elegans (C. elegans)*. The *C. elegans* gene lin-4 was found to encode a small RNA that regulated translation of lin-4 and lin-28 (Lee *et al.*, 1993; Olson & Ambros, 1999; Wightman *et al.*, 1993). Following the discovery of lin-4, let-7 was also shown to control expression of target genes (Pasquinelli *et al.*, 2000; Reinhart *et al.*, 2000). Deletions of these genes in loss-of-function studies led to mutants that failed to develop and differentiate at appropriate larval stages (Lee *et al.*, 1993; Reinhart *et al.*, 2000). Further investigations showed that lin-4 and let-7 are evolutionarily conserved in multiple species, implying a universal role for these genes in animals (Lagos-Qunitana *et al.*, 2002; Pasquinelli *et al.*, 2000).

Since the discovery of miRNAs in *C. elegans*, over two thousand miRNAs have been identified in animals, plants, viruses and other organisms. MiRBase is an online repository of microRNA sequences and annotations (Griffiths-Jones *et al.*, 2006). Established in 2002, miRBase provides a consistent naming system for miRNAs and provides a central site collecting all known miRNA sequences. It provides links to primary evidence for each miRNA in regards to function and predicted gene targets. MiRBase version 22, released in October

2018, contains miRNA sequences from 271 organisms including 48 860 distinct mature miRNA sequences (Kozomara *et al.*, 2019). One miRNA can target hundreds of genes and a single gene can be regulated by multiple RNAs. It is estimated that one to two thirds of human protein coding genes are regulated by miRNAs (Esteller, 2011).

MicroRNA biogenesis and mechanism of actions

Primary miRNAs (pri-miRNA) are transcribed from the genome and are rapidly processed into precursor hairpins in the nucleus (Bartel, 2004). Protein, exportin-5, transports the hairpin pri-miRNA out of the nucleus into the cytoplasm. Following transport to the cytoplasm the pri-miRNA is further processed into its mature form by Dicer. The miRNA is then recruited to the RNA-induced silencing complex (RISC) and regulates the output of protein-coding genes through diverse mechanisms, including translational repression, translational activation or messenger RNA degradation (Baek *et al.*, 2008; Friedman *et al.*, 2009; Landgraf *et al.*, 2007)(Figure 1.2.1).

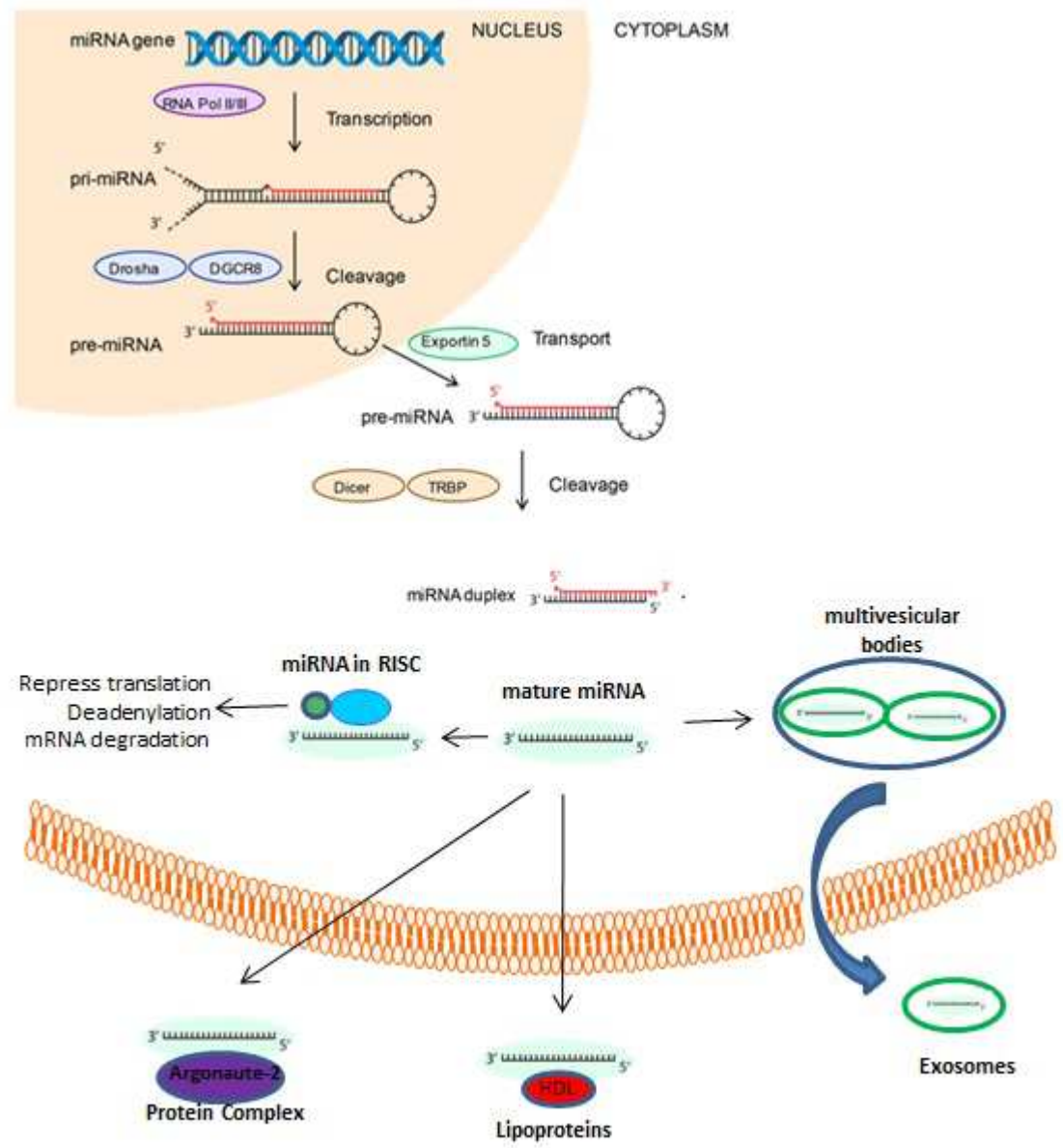


Figure 1.2.1 Mechanisms regulating miRNA processing and release.
Transcribed primary miRNAs (pri-miRNAs) are cleaved in the nucleus by Drosha to form a precursor miRNA (pre-miRNA) which is then exported to the cytoplasm by Exportin-5 and further cleaved by Dicer to produce 21-23 nucleotide duplexes. One strand of the miRNA duplex (mature miRNA) can either associate to the RISC complex and guide translation, deadenylation or mRNA degradation or be released by the cell. Mature miRNAs released by the cell can be bound to RNA-binding proteins, such as Argonaute or to high density lipoproteins. Alternatively, miRNAs can be loaded in microvesicles or in exosomes. Abbreviations: miRNA, microRNA; pre-miRNA, miRNA precursor; pri-miRNA, primary miRNA transcript; RISC, RNA-induced silencing complex.

MiRNAs are not confined to within the cell. During processing miRNAs are potentially shuttled from the RNA-induced silencing complex, prepared for export and secreted from cells into the extracellular environment and thus have been found in most biological fluids (Vickers *et al.*, 2011). MiRNAs exist in the circulation in a stable extracellular form despite the highly destructive ribonuclease (RNase) -rich environment of the bloodstream. RNase is a nuclease that catalyses the degradation of RNA. Multiple strategies exist to protect miRNAs against the destructive nature of RNases. While many circulating miRNAs are protected from degradation by RNases by encapsulation in membrane bound vesicles, such as exosomes, the majority of extracellular circulating miRNAs are stabilised and protected by binding to high-density lipoproteins or bound to RNA-binding protein complexes, including Argonaute2 (Ago2), the key effector protein of miRNA-mediated silencing (Arroyo *et al.*, 2011; Vickers *et al.*, 2011). Utilising ultracentrifugation and size-exclusion chromatography methods, Arroyo and et al (2011) found that circulating miRNAs including miR-210 and miR-16 are predominantly associated with a ribonucleoprotein complex, such as Ago2, while miRNAs such as let-7a are primarily encapsulated in membrane-bound vesicles. Vesicle encapsulated miRNAs, such as exosomes, are hypothesised to originate from cell types known to generate vesicles, such as platelets, and are released upon their activation (Arroyo *et al.*, 2011).

Extracellular miRNAs, whether encapsulated in membrane bound vesicles or bound to protein complexes, are taken up by cells where they act as physiologically functional molecules exerting gene silencing through the same regulatory mechanism as cellular miRNAs (Kosaka *et al.*, 2010). By controlling gene expression and phenotype, circulating miRNAs, similarly to hormones and neurotransmitters, may play roles as signalling molecules in physiological and pathological events providing vital cell to cell communication (Vickers *et al.*, 2011).

In 2008 Lawrie et al. first identified miRNAs in human blood. Since the discovery of miRNA in blood, extracellular miRNAs have been found in sera, plasma, tears, breast milk, urine and saliva (Weber *et al.*, 2010). Due to their stability in biological fluids circulating miRNAs have potential as disease biomarkers with differential abundances of specific miRNAs being

reported in many disorders including cardiovascular disease, cancer, kidney disease, neurological disorders such as Alzheimer's disease and inflammatory diseases. Hence, research has focused on the use of extracellular miRNAs for early detection, prevention and treatment of disease.

MicroRNAs in development and disease

MiRNAs have been shown to play important roles in vital biological process such as cell proliferation, metabolism, differentiation and apoptosis and are important regulatory molecules in normal development and disease progression (Mendell & Olson, 2012). Due to the significant regulatory functions of miRNAs in different pathologies and altered expression on miRNAs reported in many disease states, the potential application of miRNAs as disease biomarkers has been of considerable recent interest.

The presence of circulating miRNAs and the vital role they play into biological processes has also led to investigations into their function in cell to cell communication, with studies revealing that miRNAs are selectively targeted for secretion in specific cell-types and are taken up by distant target cells to possibly regulate gene expression. In 2009, Zernecke and others were one of the first to highlight the function of miRNAs as cell to cell communicators. They reported the delivery of miR-126 by apoptotic bodies induced vascular protection by recruiting progenitor cells for tissue repair and homeostasis thus conferring features of plaque stability on different mouse models of atherosclerosis. Further, *in vitro* and animal studies by Zhang et al. (2010) showed secreted monocytic miR-150 enhanced targeted endothelial cell migration. This study demonstrated that cells can secrete miRNAs, and deliver them into recipient cells, where they can regulate target gene expression and recipient cell function.

Some miRNAs are exclusively expressed in certain tissues or cell types. Thus, it is not surprising that specific miRNAs expression pattern could be identified for a wide spectrum of human diseases including cancer, cardiovascular disease and AKI to pathological conditions caused by dysfunction of the immune system or hypoxia (Kreth *et al.*, 2018). Although the RNA biomarker approach is promising, it is not yet clear which tissue sources

or types of RNAs will give optimal biological information and optimal predictive value in any specific clinical setting (Lugli *et al.*, 2015). Determining the altered expression of specific miRNAs in relation to their regulatory function may help to understand the pathophysiology of disease processes (Fichtlscherer *et al.*, 2010).

MicroRNAs and hypoxia

Hypoxia occurs during several physio-pathological circumstances such as rapid tissue growth, organ and tumour development and acute and chronic ischaemia (Giaccia *et al.*, 2004). Diminished oxygen concentration induces a cascade of responses for cellular adaption to a low-oxygen environment. Induction of protein coding genes by low-oxygen has dominated the focal point of hypoxia research specifically in cancer research with tissue hypoxia a dynamic feature of virtually all solid tumours (Semenza, 2010a). The study of gene regulation promoted by a low-oxygen microenvironment has identified a number of miRNAs, including miR-210, miR-21, miR-23, miR-24, miR-26, miR-103, miR-107 and miR-181 to be up-regulated under hypoxic conditions, while a large number have been found to be down regulated in hypoxia (Chan *et al.*, 2012; Fasanaro *et al.*, 2008).

MiR-210, is known as the master hypoxamir. It plays a unique and complex role in the cellular responses to hypoxia (Fasanaro *et al.*, 2008; Ivan & Huang, 2014). Hale and others have identified miR-210 as a communicable effector of hypoxic adaption by preparing recipient tissues for incipient hypoxic stress and accelerating adaptation to hypoxia, through pathways such as altering mitochondrial metabolism, regulating angiogenesis and apoptosis (Hale *et al.*, 2014; Ivan & Huang, 2014).

MicroRNAs and haemolysis

Numerous studies have identified miR-16 as being a highly abundant miRNA in erythrocytes and found levels of miR-16 to be affected by haemolysis generated by blood collection and sample processing (Kirschner *et al.*, 2011; Pizzamiglio *et al.*, 2017; Pritchard *et al.*, 2012). As previously discussed, haemolysis is an important consequence of CPB and may be an

important contributor to postoperative kidney injury. To date levels of miR-16 have not been investigated during CPB where haemolysis occurs *in vivo*.

MicroRNA expression measurement

Three principle methods are used to measure the expression levels of miRNAs

1. quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)
2. microarray and
3. next generation sequencing (NGS)

While qRT-PCR is the gold standard in detection and quantitation of gene expression, methods are laborious and with a rapid increase in number of miRNAs reported, renders qRT-PCR inefficient on a genomic scale. Real time reverse transcription quantitative PCR is central to biomarker validation where potential biomarkers need to be measured with greater accuracy and precision in a larger sample set, thus qRT-PCR is better used for validation rather than discovery (Git *et al.*, 2010).

Next generation sequencing provides the opportunity to examine all miRNAs at one time including variations in expressed sequences and the potential to identify novel miRNAs associated with disease (Burgos *et al.*, 2013). The advantage of this technique is that it is not dependent on any prior sequencing information, it provides information on all RNA species, it can determine the absolute abundance in expression and allows for discovery of novel miRNAs (Bernardo *et al.*, 2011)

Microarrays are used to measure the expression levels of large numbers of miRNAs simultaneously. The main purpose of microarrays is to determine the relative change in expression between states. A limitation of microarray profiling is the requirement of prior sequence information to be used for probe design. Microarray is a well-established technique, which has been used to screen for multiple potential gene expression biomarkers and drug targets, and microarray gene expression data continues to be a useful source for exploration of potential biomarkers (Devonshire *et al.*, 2013). Microarray however is limited by the miRNAs selected for study (Git *et al.*, 2010).

MiRNAs regulate gene expression with one miRNA having multiple potential target genes, potentially several hundred, and conversely each target gene may be regulated by multiple miRNAs. An insight into target genes and potentially functionality of miRNAs can be determined using target prediction programs. The computational bioinformatics target predication programs assist with defining a target, examining the effects on gene expression or functional role in signaling cascades (Bernardo *et al.*, 2011).

MicroRNAs and acute kidney injury

The role of circulating and urinary miRNAs has been investigated in AKI in a variety of clinical disease states including following cardiac surgery and in the intensive care unit (ICU) setting. Many miRNAs have been shown to be involved in the amplification or reduction of acute injury processes (Kreth *et al.*, 2018). Lorenzen et al. (2011) tested the hypothesis that expression of circulating miRNAs detected in the circulation of critically ill patients with AKI requiring renal replacement therapy (RRT) are dysregulated and may serve as biomarkers predicting survival. Circulating plasma miRNAs were measured by qRT-PCR in a mixed cohort of 77 patients admitted to the ICU under medicine, general surgery and cardiac surgery, 30 age-matched healthy controls and 18 critically ill patients with acute MI. While circulating levels of miR-16 and miR-320 were found to be down regulated in the plasma of AKI patients, the hypoxically regulated miRNA, miR-210, was upregulated compared to healthy disease controls. Cox regression and Kaplan-Meier curve analysis revealed miR-210 was an independent and powerful predictor of 28-day survival. The authors concluded that circulating miRNAs were altered in patients with AKI and that miR-210 predicts mortality in the patient cohort. MiR-210 potentially serves as a novel biomarker for AKI and may be a reflection of cellular hypoxia.

Investigations into the role of miR-210 and hypoxia have found that miR-210 may play a protective role, protecting renal cells against hypoxia-induced apoptosis by targeting HIF-1α (Liu *et al.*, 2017). Several HIF-1α regulated target genes have been identified that play essential roles in cellular and systemic physiological responses to hypoxia including glycolysis, erythropoiesis, angiogenesis and vascular remodeling (Semenza, 2001). Liu et al.

(2017) reported that in a rat model of systemic hypoxia, with a hypobaric chamber, and local kidney hypoxia, using a microvascular clamp to induce hypoxic injury, that miR-210 levels were upregulated significantly in both models. Further *in vitro* studies verified that miR-210 is involved in the molecular response to hypoxia with miR-210 suppressing HIF-1α expression by targeting the 3' untranslated region of HIF-1α mRNA in an immortalized proximal tubule epithelial cell line.

Urine represents a noninvasive source in which miRNAs can be investigated. Studies of miRNAs in urine have mainly focused on their roles as noninvasive biomarkers with little experimental demonstration of the origins of the miRNAs found in urine. Urinary miRNAs can be present either in cells originating from the kidney or in cells that infiltrated the renal tissue and thereafter were shed in the urine. Free miRNAs either bound to extracellular proteins or in exosomes may be released from kidney cells or enter the urine from the circulation by glomerular filtration (van de Vrie *et al.*, 2017). MiRNAs are stable in urine, with around 50% of urinary miRNAs remaining after 5 days of storage at 4°C as assessed by qRT-PCR (Mall *et al.*, 2013). Because of their stability and accessible nature, urinary biomarkers have a potential as noninvasive biomarkers for AKI.

Ramachandran et al. (2013) profiled 1809 miRNAs in pooled urine samples from 6 healthy controls and 6 patients with AKI, either critically ill patients admitted to ICU or from patients following cardiac surgery with CPB. Acute kidney injury was defined by a serum creatinine increase of at least 50% over baseline values. Of the 1809 miRNAs profiled, 378 stably selected miRNAs were measured in the 12 samples individually with 7 highly expressed miRNAs detected in the urine of patients with AKI selected for further investigation in a larger cohort of patients. The 7 highly expressed miRNAs were assessed in 98 patients with AKI and 97 patients without AKI. The authors identified 4 miRNAs capable of significantly differentiating patients with AKI from individuals without AKI including miR-21, miR-200c, miR-423 and miR-4640.

MicroRNAs and cardiac surgery

There remains a paucity of literature on the expression levels of miRNAs during cardiac surgery and importantly during CPB. In 2016 Emanueli et al. investigated whether exosomal miRNAs were released by cardiac cells and increase in the circulation after cardiac surgery. They also investigated if circulating exosomes and exosomal cardiac miRNAs correlate with cardiac troponin, a biomarker of myocardial damage. From samples taken preoperatively, prior to CPB, 24 hr and 48 hr postoperatively, they found plasma concentrations of exosomes and their cargo of miRNAs including cardiac specific miR-1 and miRNAs, miR-24, miR-133a, miR-133b, miR-210 and miR-122 increase in patients undergoing CABG. The increase in exosomal miRNAs positively correlated with troponin levels. This study provides evidence that miRNAs of possible cardiac origin circulate as part of exosomes and in non-exosomal plasma fractions.

Summary

Acute kidney injury following cardiac surgery is a frequent and important complication of cardiac surgery and is associated with increased complications, length of hospital stay and mortality. There are a number of unmodifiable preoperative risk factors for an increased risk of CSA-AKI including age, gender, diabetes, the use of preoperative medications and preoperative kidney disease.

Cardiopulmonary bypass provides an ideal time to investigate the development of AKI as intervention during this period is still feasible. A number of factors of CPB and surgery itself have been investigated in playing a potential role in the development of CSA-AKI including haemolysis, atheroembolism, SIRS induced by the CPB circuit and factors associated with CPB management including MAP, arterial pump flow, DO_2, Hct, degree of haemodilution and avoidance of anaemia.

Traditional biomarkers, including serum creatinine, are markers of injury providing prognostic information that damage has occurred but do not reflect real time change in renal function. An ideal biomarker would provide information about the physiological status of the kidney, in as close to real time as possible, in individual patients so that

conditions likely to promote development of AKI can be avoided. The measurement of uPO_2 is currently being investigated, as it has prognostic value during CPB, helping to identify patients at risk of developing CSA-AKI. Urinary oxygen content aids in the identification of patients at risk of developing AKI due to changes in oxygen content in urine in the bladder., This is based on the assumption that changes in urinary oxygen content reflect changes in medullary PO_2. However, uPO_2 measurement does not identify the pathological changes that are occurring during AKI.

The expanding roles of miRNAs, not only as biomarkers, but their involvement in the control and progression of disease processes, including AKI, makes them ideal to study. Investigations into whether changes in miRNA expression can aid in quantifying changes to the modifiable factors of perfusion have not been investigated. Establishing if unique miRNAs are released during CPB may help to gain a greater understanding of the molecular mechanisms induced by CPB. Quantification of the expression of specific miRNAs, such as miRNAs associated with hypoxia and haemolysis, may elucidate the molecular mechanisms involved in the development of AKI following CPB. Investigation into the release of hypoxically regulated miR-210 during the insult of CPB may indicate renal hypoxia revealing miR-210 as a predictive marker of AKI during cardiac surgery. In turn, this knowledge may improve perfusion management, and serve as a tool for examining effects of changes in management, leading to improved outcomes.

1.4 Research objectives

- To examine levels of miRNAs during cardiac surgery with CPB
- To explore if differential patterns of miRNAs are expressed in AKI
- To explore if miR-210 expression varies with other markers of AKI
- To examine release of small RNAs during CPB, to determine if release is global or specific and tissue of origin of release
- To examine molecular pathways dysregulated during CPB
- To determine if specific miRNAs predicting renal hypoxia and predicting AKI are released during CPB

The specific aims of this thesis are:

- To gain a greater understanding of the molecular response to CPB and to explore the potential of microRNAs as predictive markers of AKI during cardiac surgery.

Chapter 2: MATERIALS AND METHODS

2.1 Study populations

Two separate study populations of patients undergoing cardiac surgery have been analysed for this project. The study populations discussed in Chapter 3 (Cohort A) and Chapter 6 (Cohort C) were recruited from Flinders Medical Centre (FMC), South Australia. The studies were approved by the Southern Adelaide Clinical Human Research Ethics Committee (202.13), and written informed consent was obtained. The study population (Cohort B) discussed in Chapters 4 and 5 were recruited from Monash Medical Centre (MMC), Victoria. The study was approved by the Human Research Ethics Committee of Monash Health (Reference number 12375B). Each patient had blood and urine collected preoperatively, intraoperatively and postoperatively. All biological samples were processed and frozen at -80°C until required for analysis.

2.2 General methods

For the following methods, details and suppliers of chemicals and reagents, equipment, oligonucleotides, buffers and solutions are listed in tables in APPENDICE A-D.

Cell line preparation

In accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines (MIQE), a technical control was used in all qRT-PCR experiments to ensure the primers used for reverse transcription and amplification were targeting the miRNA of interest (Bustin *et al.*, 2009). The RNA for these controls was prepared from the following cell lines:

Cell line RCC4 VHL$^-$

The original renal carcinoma cell line RCC4 is von Hippel-Lindau (VHL) deficient (Sigma-Aldrich, St Louis, MO). RNA extracted from RCC4 VHL- was used as a technical control for the assay detecting hsa-miR-210 by qRT-PCR.

Cell line RCC4 VHL^{+}

The renal carcinoma cell line with the restored wild-type VHL gene (Sigma-Aldrich, St Louis, MO) and RNA extracted was used as a technical control for the assay detecting hsa-miR-16 by qRT-PCR.

Cell line HEK 293

The human embryonic kidney (HEK) cell line (ATCC, Manassas, VA, USA) was spiked with synthetic cel-miR-54 and RNA extracted was used as a technical control for the assay detecting cel-miR-54 by qRT-PCR.

Cell Culture

VHL and VHL plus cell lines were cultured in Dulbecco's modified eagle medium (ThermoFisher Scientific, Waltham, MA). HEK-293 cell line was cultured in RPMI 1640 medium (ATCC, Manasssas, VA, USA) at 37° C and 5% CO_2, and were shown by routine testing to be mycoplasma free. Addition of antibiotics to the culture medium was not required. Cells were maintained at less than 80% confluence, with media renewal two to three times per week. Cells were sub-cultured (passaged) approximately once per week, with a sub-cultivation ratio of 1:3 to 1:8. To passage cells, culture medium was removed and discarded, and the cell layer briefly rinsed with 1× phosphate buffered saline (PBS) to remove traces of serum which naturally contains trypsin inhibitor. PBS was removed, and 1× trypsin-ethylenediamine tetraacetic acid (EDTA) solution was added to the flask. Cells were incubated at 37°C for approximately 5 min, until observation of the cells under an inverted microscope showed the cell layer to be dispersed. To deactivate trypsin, growth medium containing serum which naturally contains trypsin inhibitor, was added and cells were mixed by gently pipetting. Aliquots of the cell suspension were added to new culture flasks with additional growth medium for continued culture, or were removed for cell counting and seeding of plates at the commencement of a new experiment. Cells were discarded after a maximum of 10 passages.

Cells were stored in foetal bovine serum and 10% dimethyl sulfoxide, in cryovials at -80°C (for short term storage). Cells were frozen slowly by placing cryovials in a Mr Frosty™

freezing container (ThermoFisher Scientific, Waltham, MA) filled with 100% isopropanol. Cells were removed from -80°C and thawed in a 37 °C water bath when required.

RNA extraction from cell lines and quantification

For cells cultured in 6-well 9.6 cm^2 plates (Corning, NY, USA) TRIzol Reagent (ThermoFisher Scientific, Waltham, MA) was used to extract RNA from samples. Following media removal from the plates, 1 mL of TRIzol Reagent was added directly to the cells in the culture dish. Cells were lysed directly in the culture dish by pipetting up and down several times. Total RNA was extracted according to the manufacturer instructions as detailed below.

Homogenised samples were incubated for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex. For phase separation, 200 µL of chloroform was added to a sample, and the tube shaken vigorously by hand for 15 sec. The samples were then incubated for 2 to 3 min at room temperature and centrifuged at 12000 x g for 15 min at 4°C. This process separated the mixture into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase with RNA remaining exclusively in the aqueous phase. Once centrifuged, the aqueous phase of the sample was removed, avoiding any withdrawal of the interphase or organic layer. The aqueous phase was transferred to a new tube.

For RNA precipitation, 0.5 mL of 100% isopropanol was added to the aqueous phase. The sample was incubated at room temperature for 10 min, and centrifuged at 12000 x g for 20 min at 4°C. The supernatant was carefully removed from the tube leaving only the RNA pellet. The pellet was washed by adding 1 mL of chilled 75% (v/v) ethanol. The sample was vortexed briefly, then centrifuged at 7500 × g for 5 min at 4°C. The ethanol wash was then discarded, and the remaining RNA pellet air-dried on ice for 10 min. The RNA pellet was then resuspended in 50 µL of RNase-free water, ready for quantitation. RNA concentration was assessed using a Nanodrop 8000 spectrophotometer and sample was prepared to a final concentration of 8 ng/µL.

2.2 Preparation of patient samples for qRT-PCR

RNA extraction from serum and plasma for qRT-PCR

TRIzol LS Reagent (ThermoFisher Scientific, Waltham, MA, USA) was used to obtain RNA from serum and plasma samples spiked with 5 fmol of *Caenorhabditis elegans* cel-miR-54 mimic (annealed oligo duplex active sequence 5' UACCCGUAAUCUUCAUAAUCCGAG 3'). Cel-miR-54 miRNA was used as a synthetic exogenous spike-in for subsequent data normalisation and analysis.

750 µL of TRIzol LS was added to 250 µL of serum or plasma spiked with 5 fmol of *C. elegans.* Homogenised samples were incubated for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex. For phase separation, 200 µL of chloroform was added to a sample, and the tube shaken vigorously by hand for 15 sec. The samples were then incubated 3 min at room temperature and centrifuged at 12000 x g for 15 min at 4°C. This process separated the mixture into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase with RNA remaining exclusively in the aqueous phase. Once centrifuged, the aqueous phase of the sample was removed, avoiding any withdrawal of the interphase or organic layer. The aqueous phase was transferred to a new tube.

For RNA precipitation, 0.5 mL of 100% isopropanol and 20 µg of RNase free glycogen, to assist with co-precipitation, was added to the aqueous phase. The sample was incubated overnight at 4°C. Following overnight precipitation, the sample was centrifuged at 12000 x g for 10 min at 4°C with the RNA/glycogen pellet forming a gel-like pellet on the side and bottom of the tube. The supernatant was carefully removed from the tube leaving only the RNA pellet. The pellet was washed by adding 1 mL of chilled 75% (v/v) ethanol. The sample was vortexed briefly, then centrifuged at 7500 × g for 5 min at 4°C. The ethanol wash was then discarded, and the remaining RNA pellet air-dried on ice for 10 min. The pellet was re-suspended in 20 µL of RNase free water and stored at -80°C.

The patients in each of the three cohorts were administered the anticoagulant drug heparin a known inhibitor of PCR. Patients undergoing cardiac surgery with CPB at both FMC and MMC were administered 300 IU/kg of heparin prior to the establishment of CPB with additional heparin administered during CPB if required to maintain an activated clotting time (ACT) of greater than 400 s. Patients undergoing OPCABG at FMC were administered a bolus of 10000 IU prior to positioning of the tissue stabiliser and commencement of coronary artery grafting. All patient samples were treated with heparinase I, neutralising the anticoagulation action of heparin, allowing successful PCR amplification to occur (See APPENDIX E).

Following extraction of RNA, 10 µL aliquot of sample was pre-treated with 0.5 µL of RNase Inhibitor (Cat No M0307L, New England BioLabs® Inc, Ipswich, MA) and 2 units of heparinase I (Sigma-Aldrich®, St Louis, MO) in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 4mM $CaCl_2$) for 1 h at 25°C to remove heparin (Wang *et al.*, 2009). Heparinase acts by cleaving the α-glycoside linkage at antithrombin III binding site in heparin. This results in small oligosaccharide fragments with heparinase I neutralizing the proteins action of anticoagulation. Samples were stored at -80°C until analysis. Preliminary experiments showed that heparinase does not affect qRT-PCR amplification in serum, plasma or urine from non-heparinised patients, while allowing unaffected PCR amplification in samples potentially contaminated with heparin (results shown in APPENDIX E1 and APPENDIX E2).

RNA extraction from urine for qRT-PCR

A Urine Exosome RNA Isolation Kit (Norgen Biotek Corp, Thorold, ON, Canada) was used to extract RNA from 1 mL of native urine spiked with 5 fmol of *Caenorhabditis elegans* (as above). Following the manufacturer's protocol 1 mL of urine was centrifuged at 2500 rpm for 10 min at room temperature. Following transfer of the cell-free urine to a new tube, 300 µL of Slurry B1 was added and the sample vortexed. The sample was centrifuged at 2500 rpm for 2 min to pellet the resin. Following centrifugation the supernatant was discarded. The pellet was resuspended in 300 µL of Lysis Buffer A by pipetting up and down and the sample incubated for 15 min at room temperature. Following the 15 min incubation, 300 µL of 67% (v/v) Isopropanol was added and the sample vortexed. The sample was transferred

to a mini filter spin column with a collection tube and centrifuged at 14000 rpm for 1 min and the flow through discarded. To wash the column 400 µL of Wash Solution A was added and the sample centrifuged at 1 000 rpm for 1 min. The flow through was discarded and the step was repeated two more times for a total of 3 washes. To dry the column, the spin column was centrifuged at 14000 rpm for 3 min. The spin column was transferred to an elution tube. Fifty µL of Elution Solution A was added to the column and the column was centrifuged for 2 min at 2000 rpm followed by 2 min at 14000 rpm. To increase the yield of RNA, the eluted sample was transferred back on to the column for a further 2 min centrifugation at 2000 rpm followed by 2 min at 14000 rpm. For qRT-PCR analysis, a 10 µL aliquot of the eluted 50 µL urine RNA sample was pretreated with 0.5 µL of RNase Inhibitor (Cat No M0307L, New England BioLabs® Inc, Ipswich, MA) and 2 units of heparinase I (Cat No H2519, Sigma-Aldrich®, St Louis, MO) (in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 4mM $CaCl_2$) for 1 h at 25°C to remove heparin. Samples were stored at -80°C until analysis. RNA extracted from urine was pre-treated with heparinase as preliminary experiments showed heparin, metabolised by the kidney and eliminated in urine, prevents amplification of the PCR reaction.

MicroRNA qRT-PCR

TaqMan miRNA assays (ThermoFisher Scientific, Waltham, MA, USA) were used for expression profiling. Complementary DNA (cDNA) was synthesised using 2.5 µL total RNA heparinase-treated serum or 2.5 µL total RNA heparinase-treated urine, 1.5 µL miRNA-specific primers and 3.5 µL master mix. MiRNA-specific RT primers included hsa-miR-210 (assay ID 000512), hsa-miR-16 (assay ID 000391) and, cel-miR-54 (assay ID 001361) (ThermoFisher Scientific, Waltham, MA, USA). For each reverse transcription (RT) reaction, the master mix contained 0.075 µL 100 millimolar (mM) deoxynucleoside triphosphate (dNTP), 0.5 µL multiscribe RT enzyme, 0.75 µL 10 x RT buffer, 0.095 µL RNase inhibitor, and 2.08 µL water (TaqMan™ MicroRNA Reverse Transcription Kit, ThermoFisher Scientific, Waltham, MA, USA). Once the master mix, miRNA specific primer, and RNA were added to the wells of 0.2 mL 8-tube strips (Bio-Rad, Hercules, CA, USA) samples were incubated on ice for 5 min and loaded into a thermal cycler (Veriti®, Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). The RT program on the thermal cycler consisted of a 30 min

incubation at 16°C, a 30 min incubation at 42°C, a 5 min incubation at 85°C, and finally an incubation at 4°C until ready for use in real-time PCR.

Real-time PCR was carried out according to the TaqMan® Gene Expression Assays protocol (ThermoFisher Scientific, Waltham, MA, USA) using triplicate 10 µL reactions for each biological replicate including 1 µL of RT product, 0.5 µL miRNA-specific primer and probe assay mix, 5 µL 1X TaqMan universal PCR Master Mix No AmpErase UNG (Applied Biosystems, ThermoFisher Scientific, Waltham, MA) and 3.84 µL water. Once all reactions were loaded into wells of 0.1 mL 4-tube strip PCR tubes (Qiagen®, Foster City, CA, USA), samples were loaded into a thermal cycler. Thermal cycling was performed using a Rotorgene Q (Qiagen®, Foster City, CA, USA) and consisted of a 10 min incubation step at 95°C, 50 cycles of a 15 sec denaturing step at 95°C and a 60 sec annealing/extension step at 60°C.

MiRNA levels were normalised relative to the levels of the external spike cel-miR-54. Expression levels were calculated from quantification cycle (Cq) values using Q-gene (Muller *et al.*, 2002). The Cq (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold, exceeding background levels. Cq levels are a relative measure of the concentration of target in the PCR reaction. Cq levels are inversely proportional to the amount of target miRNA in the sample, the lower the Cq level the greater amount of target miRNA in the sample. $Cq \leq 29$ are strong positive reactions indicative of abundant amounts of target miRNA in the sample. A Cq of 30 - 35 are positive reactions indicative of moderate amounts of target miRNA. A Cq of 38 - 40 are weak reactions indicative of minimal amounts of target miRNA or represent environmental contamination.

A 'No Template Control' was included in every qRT-PCR experiment to determine if the RNA was sample contaminated with DNA during preparation. The no amplification control is a mock reverse transcription containing all the qRT-PCR reagents except the reverse transcriptase.

Isolation of small RNA from serum for Bioanalyzer analysis and high-throughput real time PCR

Analysis with the Aligent 2100 Bioanalyzer was carried out to provide information on sizing, quantification and quality control of RNA extracted from serum. High-throughput real time PCR, with nanolitre-scale platforms, enabled the characterisation of a larger number of miRNA sequences simultaneously.

For Bioanalyzer and high-throughput real time PCR analysis, RNA was extracted from patient serum with the miRCURY™ RNA Isolation Kits for Biofluids (Exiqon, Copenhagen, Denmark). For high-throughput real time PCR analysis serum samples were spiked with 5 fmol of plant *Arabidopsis thaliana* derived synthetic miRNA mimic ath-miR-159a (sequence 5' UUUGGAUUGAAGGGAGCUCUA 3') (ThermoFisher Scientific, Waltham, MA, USA). RNA column-based isolation kits were used for RNA extraction for Bioanalyzer and high-throughput real time PCR analysis as studies have shown RNA exosomal and cellular miRNA yield are purer in column based extraction (Eldh *et al.*, 2012). RNA isolation using spin columns is costly, when compared to phenol based extraction such as TRIzol LS, hence this method of isolation was used for Bioanalyzer and OpenArray high-throughput real time PCR analysis where a smaller number of samples were analysed.

According to the manufacturers protocol for sample preparation, to pellet any debris and insoluble components, involved centrifuging the serum samples at 3000 x g for 5 min. An input volume of 250 µL of serum was used for RNA isolation. To lyse membranised particles/cells, 75 µL of Lysis Solution BF was added to 250 µL of serum. The sample was vortexed for 5 s and incubated at room temperature for 3 min. Proteins were then precipitated by adding 25 µL Protein Precipitation Solution BF. The sample was vortexed for 5 s and incubated at room temperature for 1 min. Following incubation, the sample was centrifuged for 3 min at 11000 x g. The supernatant was transferred into a 2 mL collection tube and mixed with 338 µL of Isopropanol. The sample was then loaded into a miRNA Mini Spin Column BF. After centrifugation for 30 s at 11000 x g at room temperature, 100 µL of Wash Solution 1 BF was added to the column and centrifuged for 30 seconds at 11000 x g at room temperature. The flow through was discarded and 700 µL of Wash Solution II BF was

then added to the spin column and centrifuged for 30 s at 11000 x g at room temperature. To completely dry the membrane, 250 µL of Wash Solution II BF was added and the column was centrifuged for 2 min at 11000 x g. Twenty µL of RNase free water was added to the column. After a 1 min incubation period at room temperature the sample was centrifuged for 1 min at 11000 x g. The final purified RNA was eluted in 20 µL of RNase free water.

To further precipitate and concentrate RNA, samples underwent a further precipitation by adding 20 µg glycogen, 25 µL of 3M sodium acetate (pH 5.2) and 750 µL of 100% ethanol. After vortexing samples were incubated at -80°C overnight. The following day, samples were centrifuged at 16000 x g for 30 min at 4°C. After removing supernatant, 200 µl of 75% (v/v) ethanol was added to the pellet, vortexed and centrifuged at 16000 x g for 10 min at 4°C. Following removal of the supernatant the pellet was resuspended in 2.5 µL of RNase free water for Bioanalyzer analysis or 6 µL of RNase free water for high-throughput real time PCR analysis and stored at -80°C.

Bioanalyzer - measurement and quality control of small RNA

The quantity and quality of the RNA extracted from serum, using the miRCURY™ RNA Isolation Kits for Biofluids (Exiqon, Copenhagen, Denmark), was determined using an Agilent Bioanalyzer (Aligent Technologies, Santa Clara, CA, USA) with a Small RNA Analysis Kit (Aligent Technologies, Santa Clara, CA, USA). The small RNA chips were run on an Aligent 2100 Bioanalyzer (Aligent Technologies, Santa Clara, CA, USA), at the ACRF Cancer Genomics/Adelaide Microarray Centre, Adelaide.

High-throughput real time PCR

The Taqman™ OpenArray™ Human Advanced MicroRNA Panel was used for high-throughput miRNA profiling (ThermoFisher Scientific, Waltham, MA, USA). The fixed-content panel comprised 754 human TaqMan miRNA probes derived from Sanger miRBase release v14 (current miRBase v22 as at December 2018). The OpenArray reverse transcription reaction was performed according to the manufacturer's protocol using 3 µL of total RNA in a 4.5 µL mix of 0.75 µL Megaplex RT primer pools (Human Pools A and B), 1.5 µM dNTP's with dTTPs, 75U Multiscribe Reverse Transcriptase, 1X RT buffer, 1.5 µM MgCl2, 1.8U

RNAase Inhibitor (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). Reverse transcription reaction was performed in a thermal cycler (Veriti®, Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) under the following conditions:

Reverse transcription conditions:

Stage	Temp	Time
Cycles (40 cycles)	16°C 42°C 50°C	2 min 1 min 1 sec
Hold	85°C	5 min
Hold	4°C	∝

To increase the quantity of desired cDNA before performing PCR and to significantly increase the ability to detect low abundance transcripts, a pre-amplification step was performed according to the manufacturer's recommendations. 2.5 µL RT product was mixed with 1X Megaplex PreAmp primers (10X Human Pool A and B,), 1X TaqMan PreAmp master mix (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) to a final volume of 25 µL. Pre-amplification reaction was performed in a in a thermal cycler (Veriti®, Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) under the following conditions.

Pre-amplification cycle conditions:

Stage	Temp	Time
Hold	95°C	10 min
Hold	55°C	2 min
Hold	72°C	2 min
Cycle (12 cycles)	95°C 60°C	15 s 4 min
Hold	99.9°C	10 min
Hold	4°C	∝

The preamplification product was diluted with RNase free water to a ratio of 1:40. Twenty-two and a half μL of diluted preamplification product was added to 22.5 μL of 2X TaqMan OpenArray Real time PCR Master Mix (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) in the 384-well OpenArray sample loading plate. The manufacturer's protocol was followed and the Taqman OpenArray Human miRNA panels were automatically loaded by the OpenArray AccuFill System. Arrays were run on the QuantStudio 12K Flex Real-Time PCR System at Flinders Genomics Facility, Flinders University. Seven hundred and fifty-four miRNAs were profiled in each sample including the exogenous control ath-miR-159a. All miRNA results were normalised to ath-miR-159a and data was analysed using Expression Suite Software v.1.0.1 (ThermoFisher Scientific, Waltham, MA, USA). Cq's of >35 were not included in analysis. A low amplification threshold of $\leq$ 1.2 indicated poor efficiency of the PCR reaction and these reactions were labelled as 'undetermined' and excluded from analysis by the software.

Measurement of plasma-free haemoglobin

Plasma levels of free Hb for all patient cohorts were routinely measured as part of a Serum Indices Test to detect haemolysis, bilirubin and lipaemia on a Roche/Hitachi Modular Analyser (Hitachi High-Technologies Corp. Tokyo, for Roche Diagnostics GmbH, Germany) in the SA Pathology Biochemistry Laboratory, Flinders Medical Centre. Levels of haemolysis were assessed by spectrophotometry with scanning wavelengths from 350 to 650 nm.

Measurement of troponin T, creatine kinase, serum creatinine, urine creatinine

As part of the Flinders Medical Centre Department of Cardiothoracic Surgical Unit protocol, routine bloods are taken preoperatively, 6 hr, 12 hr and 72 hr postoperatively to measure creatine kinase and troponin T. Serum creatinine levels were determined when clinically assessed. Urine creatinine levels were used to correct for dilution of CPB when analysing urinary miRNAs. Creatine kinase muscle/brain (CKMB), troponin T, serum creatinine and urine creatinine levels were measured using a Roche/Hitachi Modular Analyser (Hitachi High-Technologies Corp. Tokyo, for Roche Diagnostics GmbH, Germany) in the SA Pathology Biochemistry Laboratory, FMC.

2.3 Statistical analysis

Expression of miR-16 and miR-210 for each individual was described using the mean of the 3 values as determined by qRT-PCR. In order to adjust for urinary creatinine, each individual value was expressed relative to levels of exogenous cel-miR-54 using quantification cycle (Cq) values from Q-gene. Repeated measures analysis of variance (ANOVA) was performed with group as a between-subjects factor and time as a within subjects factor. Between group differences at each time point were considered significantly different when the overall time X group effect based on 9 degrees of freedom was significant ($p < 0.05$) in addition to a p-value < 0.05 at each specific time point. Mann Whitney U test was used to determine differences in preoperative baseline values between the 2 groups. Statistical analyses performed with (Stata V15.1, StataCorp LLC, USA).

The Spearman correlation coefficient was used for assessing correlations between haemoglobin and miRNA expression. Statistical analyses and graph preparation were performed with GraphPad Prism 8 (GraphPad Software Inc, La Jolla, CA). Global and multiple comparisons adjusted P-values of < 0.05 (95% CI) were considered significant. Descriptive data was presented using Box-plots with the whiskers defined using the 25^{th} and 75^{th} percentile ± 1.5 times the inter-quartile range (IQR). Outliers, numbers less than the 25^{th} percentile, or greater than the 75^{th} percentile by more than 1.5 times the interquartile range, are shown as separately plotted points.

To determine associations between levels of circulating miRNAs, preoperative and perioperative risk factors and AKI, univariate logistic regression was performed (Stata V15.1, StataCorp LLC, USA). Preoperative risk factors (9 variables) and intraoperative factors (7 variables) were chosen based on either reported influence on AKI or biologic plausibility. MiRNA's included miR-210 and miR-16 levels at 5 time points. Data was checked for normality by testing for skewness and kurtosis. A number of transformations were evaluated (cubic, square, square root, log, inverse, Blom) to determine the optimal approximation to normality for the miRNA data. Blom transformation was applied to the miRNA values to satisfy the assumption of normality required for logistic regression. Statistical significance was considered to be having a p value of < 0.05.

High-throughput data generated by OpenArray was analysed by Expression Suite Software version 1.0.1 (ThermoFisher Scientific, Waltham, MA, USA). Assays with Amplification score > 1.2 and Cq Confidence > 0.8 were included in analysis. In brief for each sample the average cycle threshold (Ct) was calculated from the mean of technical replicates. A delta Ct (ΔCt) was calculated by normalising the average Ct to an exogenous control, ath-miR-159a. Fold change was determined by $2^{(-\Delta Ct)}$. A fold change $\geq$ 2 was considered significant. To determine the fold change between serum collected preoperatively (the reference group), during CPB and postoperatively the mean of the samples in each biological group was calculated.

Fold change (relative quantification RQ) was calculated using the following equation:

$$\text{Fold Change (RQ)} = \text{mean } 2^{(-\Delta Ct)} / \text{mean } 2^{(-\Delta Ct \text{ reference})}$$

A t-test was then performed and a p-value calculated. Significant miRNA expression differences were determined with multiple comparisons carried out between the three time-points using the Benjamini-Hochberg False Discovery Rate (FDR) with a nominal significance threshold of 0.05 (Benjamini & Hochberg, 1995).

Chapter 3: ISOLATION AND EXPRESSION OF CIRCULATING AND URINARY microRNAs DURING CARDIAC SURGERY

3.1 Introduction

MiRNAs are small RNA molecules that can regulate hundreds of genes. MiRNAs are involved in diverse biological processes including cellular differentiation, proliferation, angiogenesis and apoptosis (Bartel, 2004). They are resilient to degradation, stable in blood, urine and other bodily fluids and are emerging as novel biomarkers reflecting disease states (Chen *et al.*, 2008).

Differential abundances of specific miRNAs have been reported in many diseases. The results of several studies have described changes in miRNA expression in myocardial ischaemia in relation to coronary artery disease (CAD) and MI. Specific cardiac expressed circulating miRNAs, including miR-1 and miR-133, increase in patients with CAD and following MI (D' Alessandra *et al.*, 2010; Fichtlscherer *et al.*, 2010; Long *et al.*, 2012).

Altered expression of miRNAs is also associated with altered physiological states including hypoxia related to ischaemic reperfusion injury. In an animal model of renal ischaemia reperfusion injury Liu et al. (2017) reported miR-210 was overexpressed in the kidney and suggested that miR-210 was involved in signaling pathways to regulate angiogenesis to mitigate the effects of hypoxia. Circulating miR-210 levels have also been reported to be increased in patients with moderate to severe aortic stenosis. Rosjo et al. (2014) measured circulating levels of miR-210 in patients with moderate to severe aortic stenosis. MiR-210 levels were found to be increased in patients with low VO_2 max. Of the 720 miRNAs tested, miR-210 had the closest association with aerobic fitness, suggesting miR-210 release due to cellular hypoxia may have merit as a prognostic biomarker for severity of aortic stenosis.

In critically ill patients in ICU with AKI, Lorenzen et al. (2011) reported the hypoxically regulated miRNA, miR-210, was upregulated when compared to healthy disease controls. Cox regression and Kaplan-Meier curve analysis revealed miR-210 was also an independent and powerful predictor of 28-day survival. The authors concluded that circulating miRNAs

were altered in patients with AKI and that miR-210 predicts mortality in the patient cohort. The authors concluded that impairment of renal perfusion and oxygenation during CPB may play a central role in AKI. Circulating biomarkers of renal hypoxia such as miR-210 could provide important insights into the aetiology and prediction of AKI following cardiac surgery.

MicroRNAs and cardiopulmonary bypass

Cardiac surgery and the use of CPB has been associated with a variable degree of myocardial damage and organ ischaemia including AKI. The extent to which operative, ischaemic and hypoxic stresses during cardiac surgery utilising CPB cause alterations in specific or total circulating miRNAs has not been fully explored. Cardiac surgery provides a unique setting to observe levels of circulating miRNAs in response to a known ischaemic and operative insult.

MiRNA-210 is a hypoxia-inducible miRNA. It has numerous physiological roles including arrest of cell proliferation, repression of mitochondrial respiration and angiogenesis (Chan *et al.*, 2012) (Zhang *et al.*, 2009) (Mutharasan *et al.*, 2011). The release of miRNAs including miR-16 from RBC during haemolysis has been reported (Kirschner *et al.*, 2013). During cardiac surgery, haemolysis is generally attributed to CPB and the CPB related stresses applied to red blood cells within the perfusion circuit. Venous, arterial and suction cannulae, roller pumps, oxygenators and the air to blood interface in the reservoir cause haemolysis and the intravascular rupture of RBC with plasmatic release of free haemoglobin with a haemolysis peak soon after weaning from CPB (Ricci *et al.*, 2014). Increasing evidence suggests that CPB-induced haemolysis may exacerbate kidney injury following cardiac surgery (Kowalewski *et al.*, 2016).

There is a paucity of data of the role of miRNAs in cardiac surgery and miRNA expression levels during the non-physiological conditions of CPB. Yang et al. (2015) reported expression changes of myocardium specific miRNAs including miR-1, miR-21, miR-208a and miR-499 in myocardial ischaemic reperfusion injury in 15 patients undergoing combined mitral and aortic valve operations. Levels of circulating miR-1, miR-208a and miR-499 were measured preoperatively, 45 min after aortic cross clamping, 60 min after reperfusion and 24 h after surgery. Significant differences were reported in miR-1, miR-208a and miR-499 levels following 45 min of aortic cross clamping, with levels further increasing 60 min

following reperfusion. Changes in miR-1 and miR-208a exhibited a similar pattern to those observed for biomarkers assisting in the diagnosis of acute MI including Troponin I and CKMB. The authors concluded that miR-1, miR-208a and miR-499 may be sensitive biomarkers for myocardial ischaemia reperfusion injury during cardiac surgery with CPB. Zhou et al. (2013) also reported a significant increase in the miR-1 levels measured in the serum and urine of 20 patients undergoing elective mitral valve surgery. The source of miR-1 release was not confirmed with the authors concluding increased miR-1 levels measured was potentially due to myocardial injury or induced by CPB.

During the course of the work undertaken in this thesis, Emanueli et al. (2016) reported plasma concentrations of membrane bound vesicles, exosomes, and their cargo of cardiac miRNAs, including miR-210, increased in the circulation 30 min following separation from CPB. Comparing plasma samples taken preoperatively prior to sternotomy and a postoperative sample taken within 30 min of reversal of heparin by protamine, it was reported levels of miRNAs expressed by cardiac myocytes and upregulated by ischaemia, including miR-1, miR-133a, miR-133b, miR-208 and miR-210, increased in the immediate postoperative period. Levels of platelet enriched miR-223 were also increased following CPB while levels of haemolysis associated miR-451 were unaffected.

In the same study, cardiac specific miRNAs were investigated in 6 patients at 4 time points measured preoperatively, during surgery prior to establishment of CPB, at 24 hr and 48 hr postoperatively. The authors reported concentrations of the miRNAs primarily released from the heart, miR-1, miR-133a and miR-133b, increased in the plasma 24 h postoperatively, suggesting CABG with CPB induces the trafficking of exosomes from the ischaemic heart to the peripheral circulation (Emanueli *et al.*, 2016). While the cellular source of release of miR-1 remains unknown, animal studies have also shown that miR-1 is most abundant in the heart, with no significant levels determined in lung, liver, brain, small intestine and kidney. *In vitro* studies have also shown that damaged cardiac cells release miR-1 and the extent of release is associated with the extent of cardiac cell damage (Cheng *et al.*, 2010).

MicroRNAs and off pump coronary artery bypass grafting

Off pump CABG was developed in an attempt to avoid some of the perioperative complications associated with CPB. The OPCABG technique allows the heart to continue beating and maintain systemic circulation, using a device to stabilise the heart during coronary grafting, thus offering physiological pulsatile renal perfusion. However, large multi-centre trials and meta-analyses suggest that while there may be benefit in short term outcomes, including reduced cerebrovascular and renal injury, longer term benefits have not been seen (Kowalewski *et al.*, 2016; Puskas *et al.*, 2015).

Ghorbel et al. (2010) investigated the transcriptomic changes in left ventricular tissue of 5 patients undergoing OPCABG and 5 patients undergoing CABG with CPB. Left ventricular biopsies were taken preoperatively and 20 min after completion of all anastomoses. Following gene microarray analysis the authors concluded OPCABG reduces alteration in gene expression associated with inflammation, apoptosis, hypertrophy and remodeling seen after CABG.

Existing literature predominantly reports on the effect of CPB on miRNAs primarily associated with the myocardium including miR-1, miR-133a and miR-133b. To date there is no study reporting circulating hypoxically regulated miR-210 levels in patients undergoing cardiac surgery during the CPB period. Thus, there remains a paucity of data reporting the effect of the unique period of CPB on circulating miRNAs. A comparison between circulating miRNAs in patients undergoing OPCABG compared with patients undergoing cardiac surgery with CPB has not been investigated.

In order to examine whether miRNAs could be measured before, during and following cardiac surgery with CBP, 2 miRNAs were selected for measurement. Hypoxically regulated miR-210 was selected as it may act as a marker of hypoxia. MiR-16 was selected as a possible internal control as it is known to be readily detectable in blood and has been identified as a stable internal reference gene for miRNA studies in CKD patients (Lange *et al.*, 2017). However, the effects of haemolysis on miR-16 levels during CPB remains unknown. Investigation of the levels of miRNAs during and following CPB will determine the extent to which the stresses of CPB cause alterations in miRNAs. The extent to which cardiac surgery

utilising both CPB and OPCABG differentially affect levels of these circulating and urinary miRNAs will also be investigated.

3.2 Hypotheses and Aims

Hypothesis: MicroRNAs can be isolated and levels measured in the serum and urine of patients undergoing cardiac surgery with CPB.

Aim: To examine whether miRNAs can be successfully isolated and measured in the blood and urine of patients before, during and after cardiac surgery in patients undergoing cardiac surgery with CPB.

Hypothesis: Levels of miR-210 are upregulated in the serum and urine of patients undergoing cardiac surgery with CPB.

Aim: To examine whether levels of hypoxically regulated miR-210 increase in the blood and urine of patients before, during and after cardiac surgery in patients undergoing cardiac surgery with CPB.

Hypothesis: In patients undergoing cardiac surgery, CPB will induce greater changes in miRNA expression than OPCABG

Aim: To examine miRNA profiles of patients undergoing cardiac surgery with CPB and OPCABG

3.3 Methods overview

Patient inclusion

Ten patients undergoing elective cardiac surgery with CPB were studied (4 patients isolated CABG, 4 isolated valves and 2 valve and CABG). In order to provide a detailed characterisation of miRNA levels prior to, during and after surgery, blood and urine samples

were obtained at eleven time points including a preoperative sample (prior to skin incision), 10 min following initiation of CPB, during the rewarming phase of CPB (Rewarm), immediately postoperatively at skin closure and at 4, 6, 8, 10, 12, 18 and 24 h after entry into the intensive care unit (ICU). Potential patients were ineligible for the study if they required emergent/salvage surgery, were unable to provide written, informed consent or were unable to have a urinary catheter inserted due to urethral stricture. Patients were also excluded if they had pre-existing renal disease defined by a preoperative serum creatinine of 150 µmol/L or renal disease requiring dialysis.

Five patients undergoing OPCABG were studied with ten blood and urine samples including pre-operative (prior to skin incision), 5 min following application of the Octopus®, postoperatively at skin closure and at 4, 6, 8, 10, 12, 18 and 24 h in ICU.

Cardiopulmonary bypass procedure

Intravenous heparin (300 IU/kg) was administered immediately before cannulation for CPB and additional doses were given to maintain an activated clotting time of 400 sec or greater. The circuit included a 40 µm arterial filter, an arterial roller pump and a hard-shell membrane oxygenator (Capiox RX25; Terumo Corporation, Tokyo, Japan). The circuit was primed with 1100 mL of Plasma-Lyte 148 solution, 100 mL of Albumex® 20, 50 mL of sodium bicarbonate, 10000 IU of heparin and 1 g of cephazolin. Cardiopulmonary bypass was instituted by cannulation of the distal ascending aorta and insertion of a single two-stage cannula into the right atrium for CABG and aortic valve replacement (AVR) procedures. Bicaval venous cannulation utilised for mitral valve procedures. Roller pumps provided non-pulsatile flow rates of 1.8 - 2.4 L/min/m^2. Patient temperature was allowed to drift to maintenance temperature of 34°C and rewarmed to a maximum of 36.5°C. Cardiotomy suction was not used for isolated CABG. When cardiotomy suction was used, its use was limited during the procedure due to known detriments of the return of pericardial shed blood to the circuit. Cardiac arrest was induced and maintained by intermittent anterograde (CABG only) and retrograde (valve, combined valve + CABG) cardioplegia. Myocardial protection utilised blood-cardioplegia administered at ratio of 4:1 (blood:crystalloid) delivered at 34°C. Target nasopharyngeal temperature for separation

from bypass was 36 - 36.5°C with a rewarming rate of 0.5°C per min. Following weaning from CPB and decannulation, heparin was reversed with protamine. Cell salvage was used for collection of pericardial shed blood prior to heparin administration and following reversal with protamine for all cases.

Off pump coronary artery bypass grafting procedure

Intravenous heparin (10000 IU) was administered prior to grafting. The tissue stabiliser (Octopus® Evolution AS TS2500, Medtronic, Minneapolis, MN) was placed to minimise movement in the arterial territory being grafted. Protamine was used to reverse heparin. Cell salvage was used for all cases.

Collection of serum, plasma and urine

Arterial blood (10 mL) was collected from the radial arterial catheter. For serum separation, 6 mL was aliquoted into a silicone coated blood collection tube (BD Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ) for serum separation. For plasma separation, 4 mL was aliquoted into a blood collection tube containing potassium ethylenediaminetetraacetic acid (K3EDTA) (Vacutainer® Plus Becton, Dickinson and Company) for plasma separation. To generate serum, blood was allowed to clot at room temperature for 30 min. Samples were centrifuged at 1200 x g for 10 min at room temperature. After transferring serum and plasma supernatants into a 15 mL tube, samples were centrifuged at 1800 x g for 10 min at room temperature to remove debris. Samples were aliquoted into 1.5 mL microcentrifuge tubes and stored at -80°C until RNA preparation. Urine (10 mL) was collected from the sample port of the urinary catheter at each time point and stored at -80°C until RNA preparation.

RNA extraction from serum and urine

RNA was extracted from serum or urine and treated with heparinase I according to the method outlined in Chapter 2 pg. 50-52. (*RNA extraction from serum and plasma for qRT-PCR; RNA extraction from urine for qRT-PCR).*

RNA extraction for Bioanalyzer analysis

RNA was extracted from serum according to the method outlined in Chapter 2 pg. 54. *(Isolation of small RNA from serum for Bioanalyzer analysis and high-throughput real time PCR).*

MicroRNA qRT-PCR

Quantitative reverse transcription and real time PCR were carried out according to the general methods described in Chapter 2 pg.52-53. Relative expression levels were calculated from quantification cycle (Cq) values using Q-gene (Muller *et al.*, 2002).

Statistical Analysis

Statistical analysis was carried out as described in Chapter 2 pg. 58.

3.4 Results

There were no significant differences in patient demographics between the patients undergoing cardiac surgery with CPB and OPCABG groups (Table 3.4.1). There were significant differences in ICU stay and length of hospital stay in the cohort of patients undergoing cardiac surgery with CPB.

Table 3.4.1 Preoperative, procedural and outcome characteristics of the 10 patients undergoing cardiopulmonary bypass and 5 undergoing off pump coronary artery bypass grafting - ***Cohort A***

	CPB n = 10	**OPCABG n=5**	**P value**
Baseline			
Age, years	71 (47-86)	64 (54-77)	0.904
Female	0	1	
BMI	26 (24-33)	31 (26-38)	0.141
COPD	2	0	
Previous cardiac surgery	0	0	
Diabetes	3	2	0.698
Hypertension	6	4	0.438
Congestive heart failure	7	2	0.263
Smoking history	8	3	0.408
Ejection fraction < 30%	0	0	
Emergency surgery	0	0	
Cerebrovascular disease	1	1	0.591
Myocardial infarction	4	2	1.00
Preoperative Hb, g/L	131 (79-155)	139 (111-164)	0.810
Preoperative creatinine, μmol/L	91 (65-150)	102 (75-126)	0.502
Procedure			
CABG	4	5	
Valve repair/replacement	4		
Valve + CABG	2		
CPB duration, min	94.5 (126-35)	N/A	
X clamp time	78.5 (23-95)	N/A	
CPB Naso temperature, min ^{O}C	34		
CPB MAP, avg mmHg	58		
CPB Hb, min g/L	93		
CPB Hb, max g/L	96		
CPB blood glucose, min mmol/L	6		
CPB blood glucose, max mmol/L	9		
1st Hb on CPB, g/L	95 (69-114)		
Final Hb on CPB, g/L	98 (92-112)		
Fluid Balance (mL)			
Anaesthetic vol. prior to CPB	125 (100-500)		
Volume added during CPB	300 (0 -800)		
Pre CPB urine volume	112 (0-450)		
Post CPB urine volume	175 (0-400)		
Outcome			
AKI	2	0	
Highest post op creatinine (μmol/L)	97 (69-237)	99 (78-152)	0.810
ICU time (hr)*	43 (23-554)	23 (22-29)	*0.016*
Length of stay (days)	7 (5-32)	6 (6-7)	0.667
Mortality	0	0	

*Data expressed as median and range. No significant differences except for ICU time * (p<0.05, unpaired t-test). Baseline characteristics defined by the Australian and New Zealand Society of Cardiac and Thoracic Surgeons national database. Acute Kidney Injury (AK)I defined by RIFLE. All CPB related data accessed from Australian and New Zealand Collaborative Perfusion Registry. Body Mass Index (BMI), Chronic Obstructive Pulmonary Disease (COPD), Haemoglobin (Hb), Coronary Artery Bypass Grafting (CABG), Cardiopulmonary Bypass (CPB), Nasopharngeal (Naso), Mean Arterial Pressure (MAP), Intensive Care Unit (ICU).*

Circulating microRNA-210 and microRNA-16 in patients undergoing cardiac surgery with cardiopulmonary bypass

Following RNA extraction and heparinase treatment both miR-210 and miR-16 were readily detected by qRT-PCR in patients undergoing cardiac surgery with CPB and OPCABG. Baseline levels, expressed as a ratio of the miRNA of interest with the external control, were similar in patients undergoing cardiac surgery with CPB and OPCABG patients (0.00087 ± 0.00078 vs 0.00031 ± 0.00026 for miR-210, p = 0.12 and 0.0970 ± 0.0847 vs 0.069 5± 0.0562, p = 0.42). Interestingly, there was a 70% increase in the levels of miR-210 in the samples obtained shortly after the initiation of CPB (Figure 3.4.1A) ($p < 0.05$), whilst miR-16 increased nearly 200% (Figure 3.4.1B) ($p < 0.05$). These levels remained significantly elevated for both miRNAs during rewarming ($p < 0.05$) and in the immediate post-operative period ($p < 0.05$), returning to pre-operative levels at 4h (p = n.s).

MiR-210 levels also significantly increased by the time of placement of the Octopus® ($p < 0.05$), however the increase was more modest in OPCABG patients with a 35% increase in levels of miR-210 in the samples obtained following placement of the Octopus and returned to baseline after 4 h (Figure 3.4.1C). However, levels of miR-16 measured in serum of patients undergoing OPCABG were not significantly altered during or following surgery (Figure 3.4.1D).

Using repeated measures ANOVA, for miR-210, there was a significant overall effect of time ($p < 0.001$), indicating miR-210 levels differed over time, a significant overall difference between the two groups, indicating there was a significant difference in miR-210 levels between patients undergoing OPCABG and cardiac surgery with CPB (p = 0.017), but there was no group x time interaction effect (p = 0.118). For miR-16, there was a significant overall

effect of time ($p < 0.001$), a significant overall difference between the 2 groups ($p = 0.022$) and a significant group x time interaction effect ($p = 0.023$).

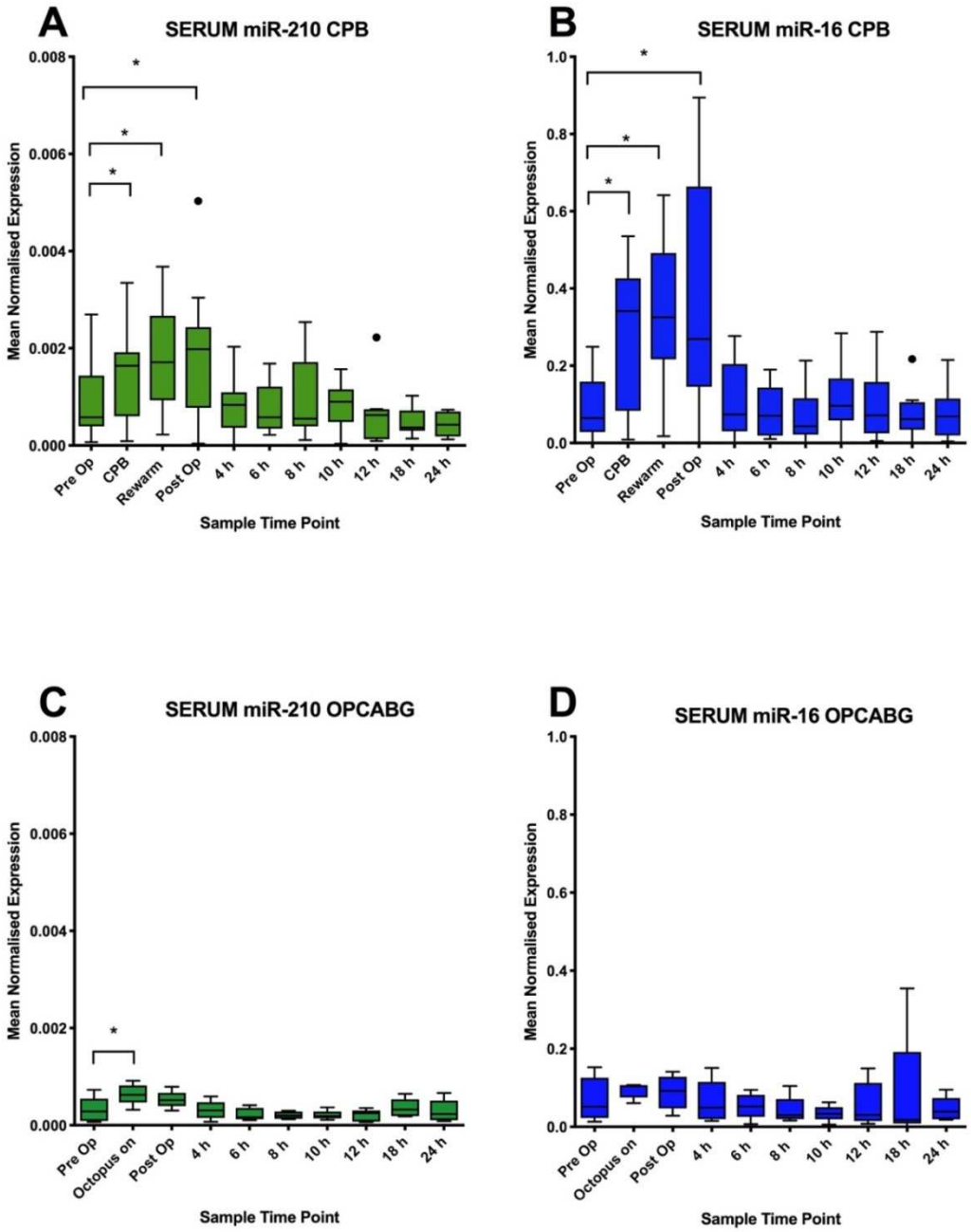


Figure 3.4.1 Expression of circulating miRNA-210 and miRNA-16 in patients undergoing cardiac surgery with cardiopulmonary bypass or off pump coronary artery bypass grafting.

A) Serum miR-210 levels from patients before, during and after cardiac surgery with CPB (*p < 0.05 vs. pre-op). **B)** Serum miR-16 levels from patients before, during and after cardiac surgery with CPB (*p < 0.05 vs. preoperative.). **C)** Serum miR-210 levels from patients before, during and after OPCABG **(***p < 0.05 vs. preoperative. **D)** Serum miR-16 levels from patients before, during and after OPCABG. Data is presented as Box-plots with the whiskers defined using the 25th and 75th percentile ± 1.5 times the inter-quartile range (IQR) (• represent outliers numbers less than the 25th percentile or greater than the 75th percentile by more than 1.5 times the interquartile range). In this and subsequent figures green indicates miR-210 and blue indicates miR-16.

MicroRNA-210 and microRNA-16 in urine of patients undergoing cardiac surgery

Both miR-210 and miR-16 were detectable in the urine of patients undergoing cardiac surgery, but mean concentrations were substantially less than in serum (100-fold less for miR-210 and 700-fold less for miR-16) in patients undergoing cardiac surgery with CPB (Figure 3.4.2A, 3.4.3A). Both miR-210 and miR-16 were detectable in the urine of patients undergoing OPCABG with the mean concentrations less than in serum of patients undergoing CPB (45-fold for miR-210 and 1000-fold less for miR-16) (Figure 3.4.2C, 3.4.3C). No changes in urine miR-210 or miR-16 levels were statistically significant.

The urinary concentration of miRNAs could potentially be adversely affected by fluid balance, diuretic therapy and urine output. This concern was partially overcome by correcting urinary levels to urine creatinine. After correcting for urine concentration using urine creatinine there was a large increase in normalised miR-210 and miR-16 levels in the urine of CPB patients. There was a greater than 200% increase in normalised miR-210 levels in both the rewarming phase of CPB and immediately postoperatively (Figure 3.4.2B). MiR-16 levels increased by 2000% in the urine of patients undergoing cardiac surgery with CPB in the immediate postoperative period (Figure 3.4.3B). There were no changes in patients undergoing OPCABG (Figure 3.4.2D, 3.4.3D).

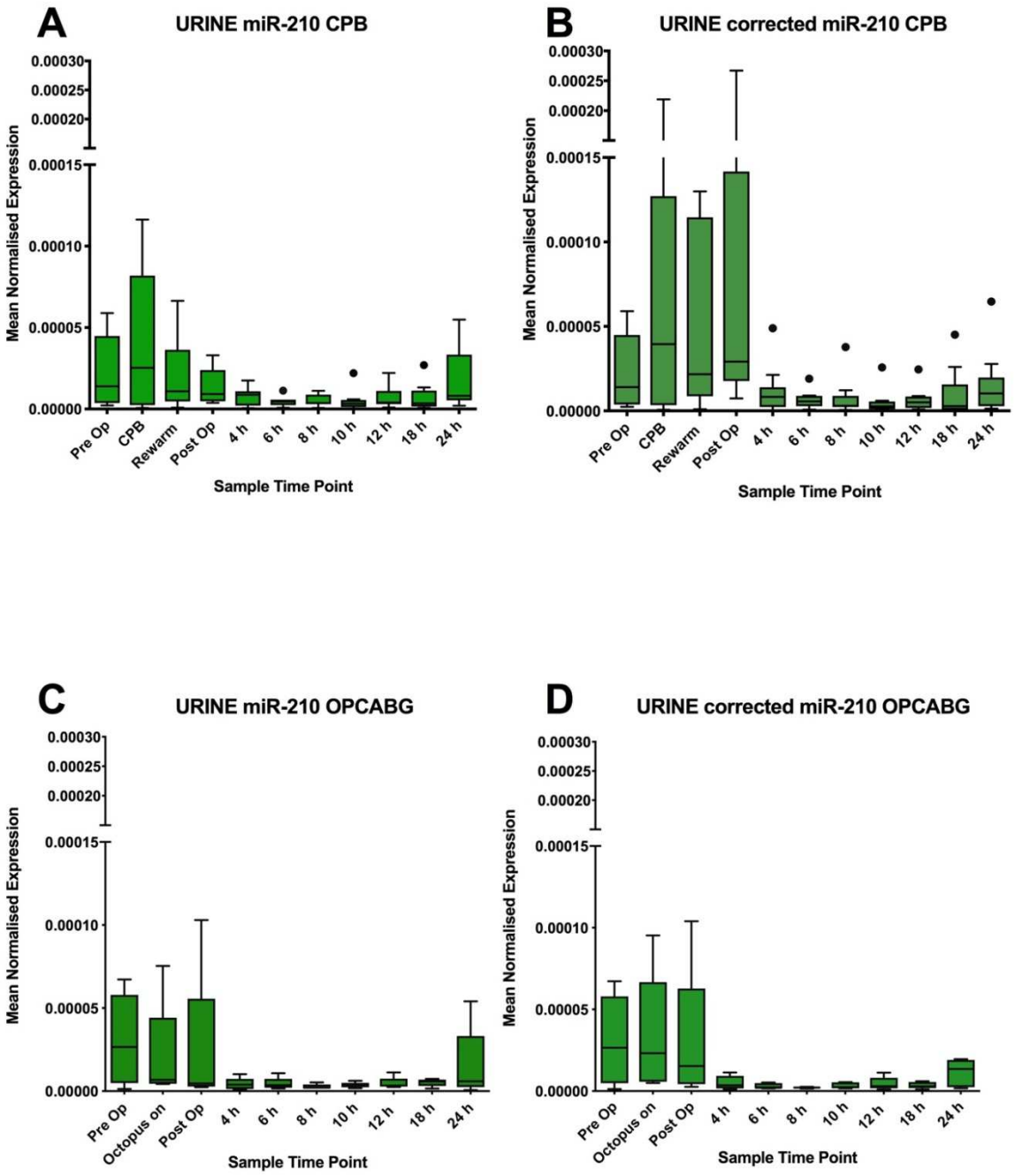


Figure 3.4.2 Expression of miR-210 in urine of patients undergoing cardiac surgery with CPB.

A) miR-210 levels in the urine of patients before, during and after cardiac surgery with CPB. **B)** miR-210 levels in the urine of patients before, during and after cardiac surgery with CPB when corrected for urine concentration using urine creatinine. **C)** miR-210 levels in the urine of patients before, during and after OPCABG. **D)** miR-210 levels in the urine of patients before, during and after OPCABG when corrected for urine concentration using urine creatinine.

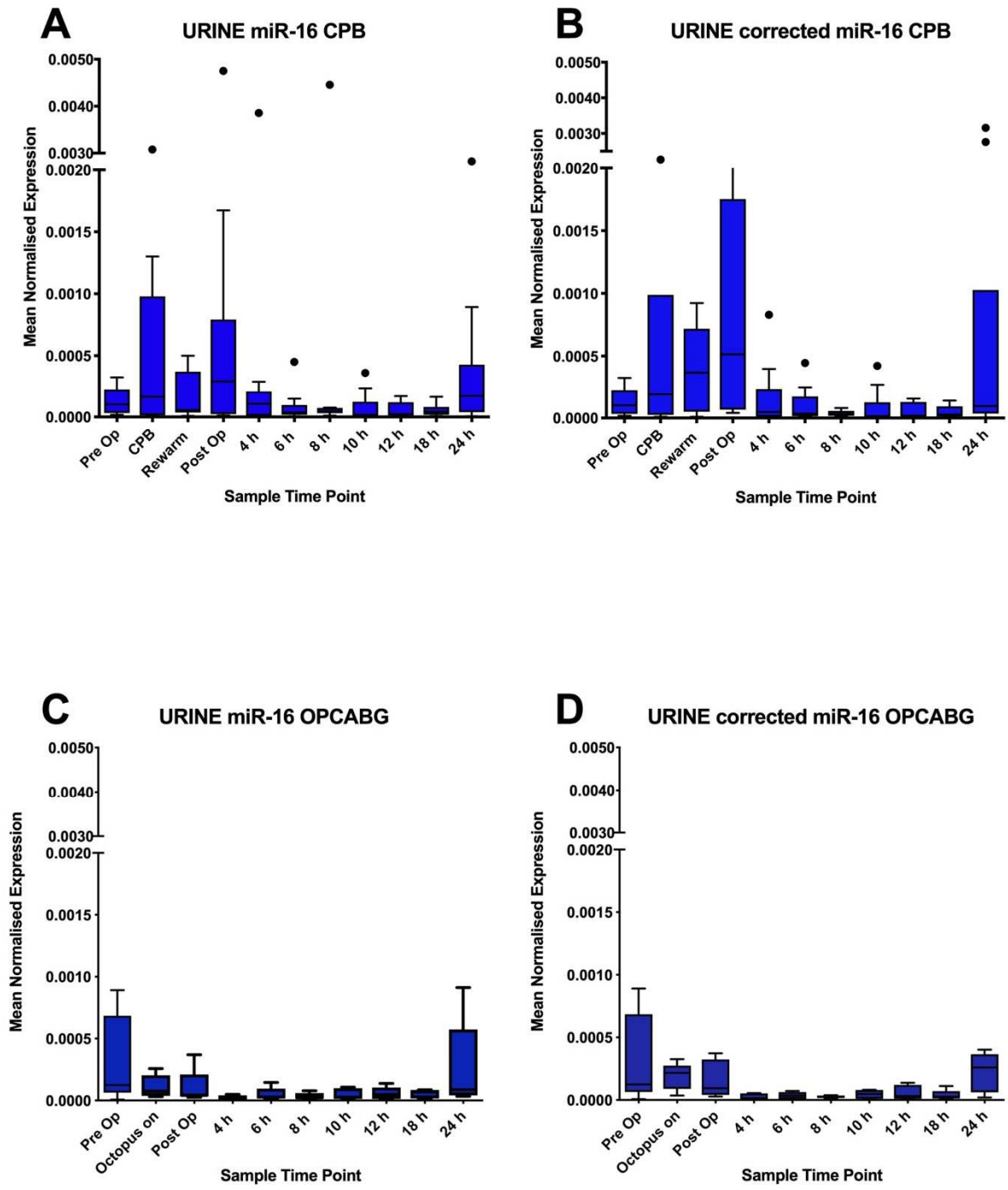


Figure 3.4.3 Expression of miR-16 in urine of patients undergoing cardiac surgery with CPB. **A)** miR-16 levels in the urine of patients before, during and after cardiac surgery with CPB. **B)** miR-16 levels in the urine patients before, during and after cardiac surgery with CPB when corrected for urine concentration using urine creatinine. **C)** miR-16 levels in the urine of patients before, during and after OPCABG. **D)** miR-16 levels in the urine of patients before, during and after OPCABG when corrected urine concentration using urine creatinine**.**

Plasma free haemoglobin levels during cardiac surgery

Mean plasma free Hb levels showed no significant change in patients undergoing cardiac surgery with either CPB or OPCABG (Figure 3.4.4). A small but statistically non significant increase in plasma free Hb was observed postoperatively in patients undergoing CPB (Figure 3.4.4A). Plasma free Hb did however demonstrate a significant positive correlation with levels of miR-16 and miR-210 for patients undergoing cardiac surgery with CPB (Figure 3.4.5A). These correlations were not observed in OPCABG patients (Figure 3.4.5B).

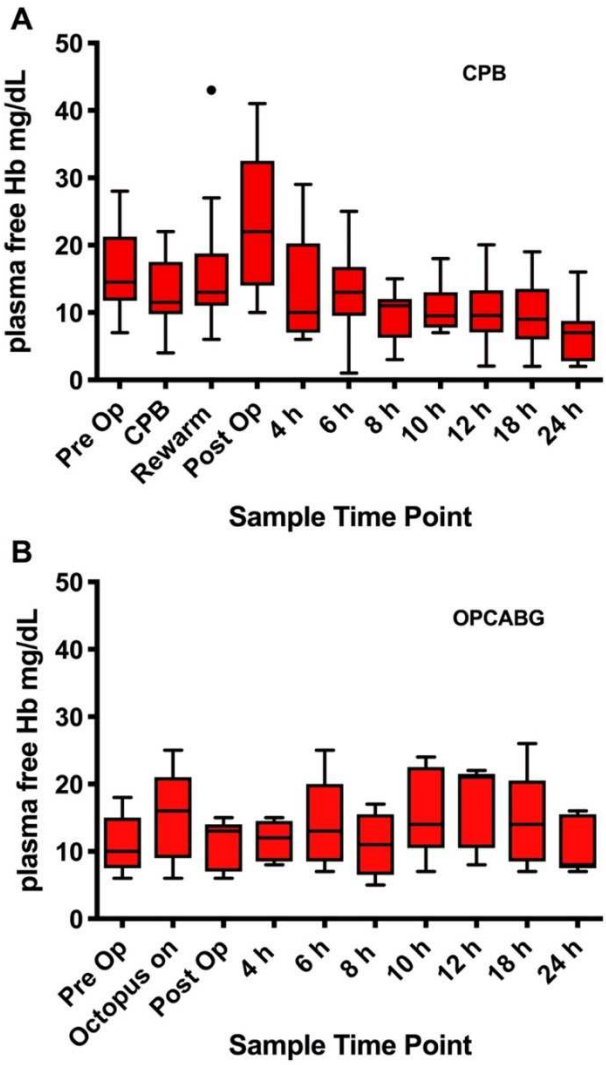


Figure 3.4.4 Mean plasma free haemoglobin (Hb) levels in patient undergoing cardiac surgery.
A) Patients undergoing cardiac surgery with CPB, **B)** Patients undergoing OPCABG**.** There were no significant differences in plasma free Hb levels measured in patients undergoing cardiac surgery with CPB or OPCABG. There were no significant differences observed between each time point within each patient cohort.

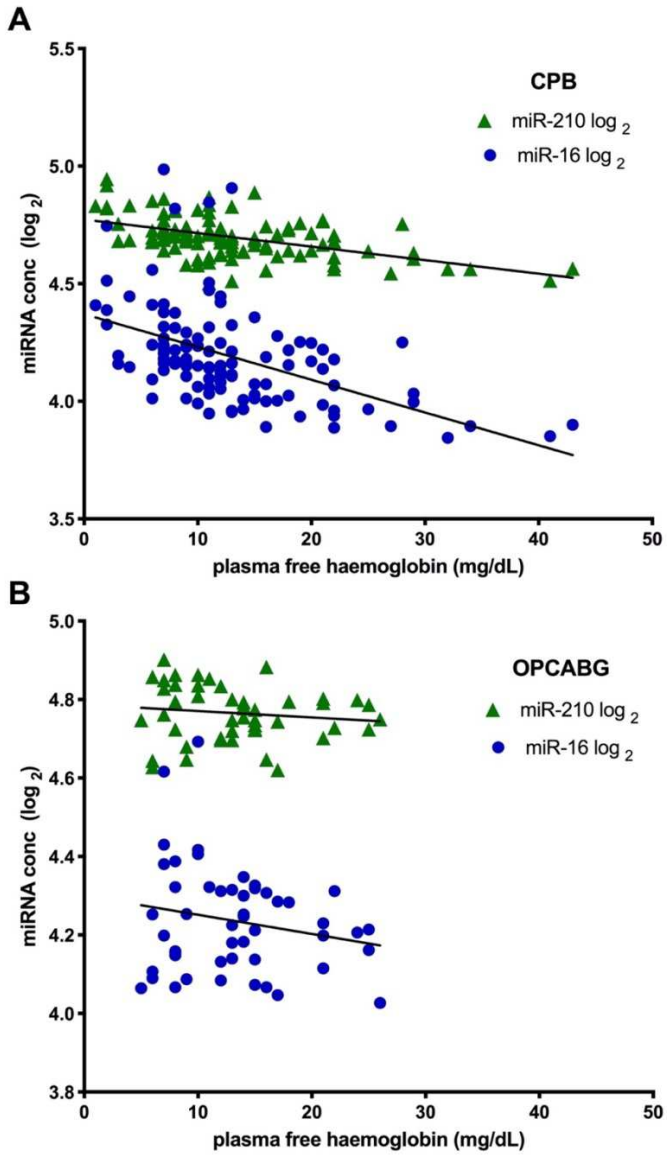


Figure 3.4.5 Correlation between plasma free haemoglobin (Hb), miR-16 (log_2) and miR-210 (log_2) in patients undergoing cardiac surgery.

A) Patients undergoing cardiac surgery with CPB. Quantitation cycle (Cq) values were transformed to log base 2. Cq values represent the number of cycles needed to reach a set threshold fluorescence signal level. A lower Cq value indicates a higher target miRNA expression in a sample. There is an inverse significant correlation between plasma free Hb and miR-16 levels ($r = -0.549$, p (two-tailed) < 0.0001) and plasma free Hb and miR-210 levels ($r = -0.463$, p (two tailed) < 0.0001). Line represents line of best fit. **B)** Patients undergoing OPCABG. No correlation between plasma free Hb and miR-16 levels ($r = -0.122$, p (two-tailed) = 0.398) or plasma free Hb and miR-210 levels ($r = -0.181$, p (two tailed) = 0.207).

Injury markers- troponin T, creatine kinase, serum creatinine

In order to examine for potential links between organ injury and miRNA release, correlations were sought between indicators of myocardial and renal injury and miRNA levels. There were no significant correlations between miR-16 or miR-210 levels with troponin T, CKMB or serum creatinine.

Comparison of circulating small RNA levels: off pump coronary artery bypass grafting vs cardiopulmonary bypass

The total concentration of small RNAs was determined by Bioanalyzer analysis which uses a microfluidics-based platform for sizing and quantification of small RNAs. Total concentration of small RNAs demonstrated a 10-fold mean increase during CPB, with a significant rise seen in the immediate postoperative period, returning to preoperative levels from 4 h postoperatively (Figure 3.4.6A). (See APPENDIX F for example of Bioanalyzer gel electrophoresis result) A substantial increase in total small RNAs was also seen during application of the Octopus®, with levels returning to preoperative levels in the immediate postoperative period, although the rise was not statistically significant (Figure 3.4.6B).

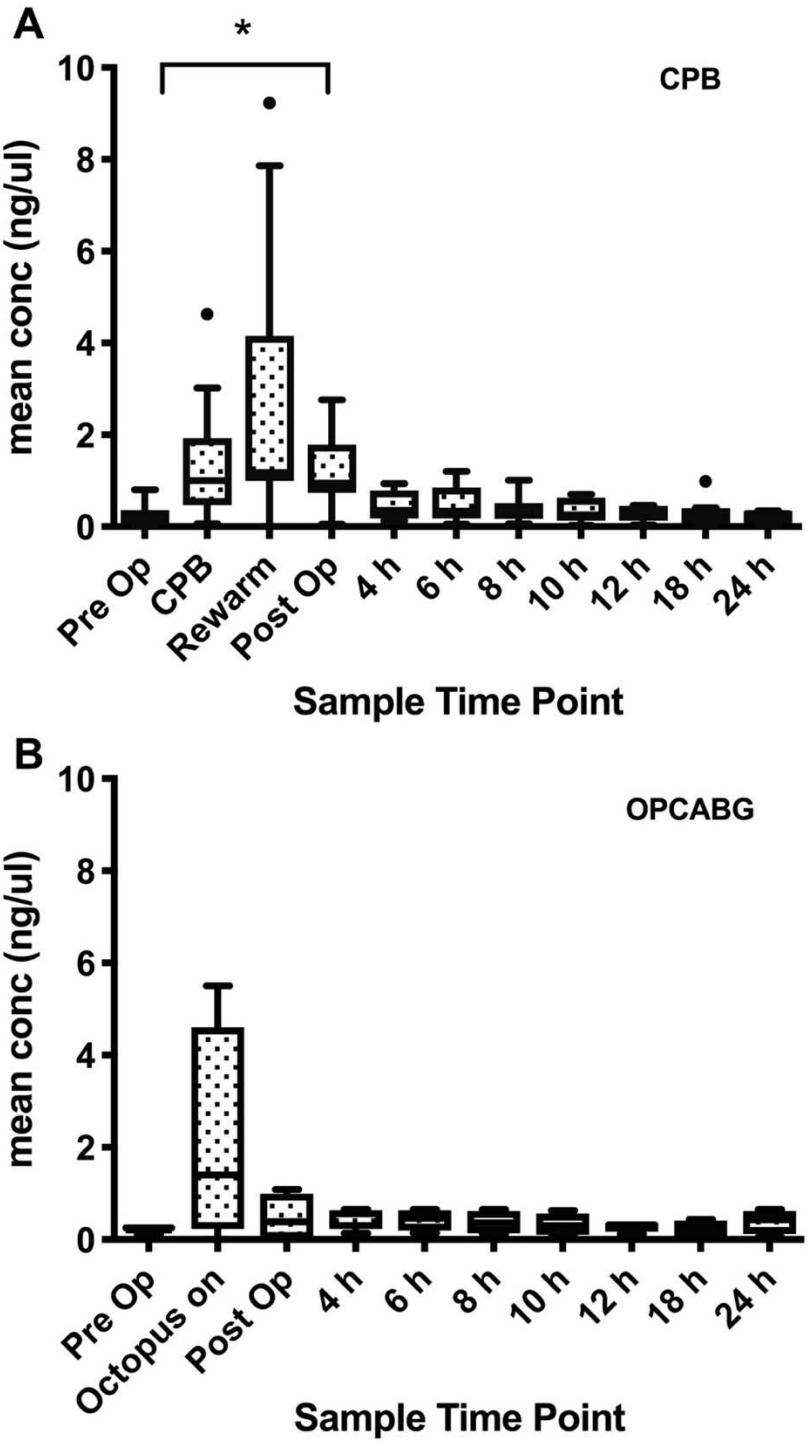


Figure 3.4.6 Quantification of small RNAs in patients undergoing cardiac surgery by Bioanalyzer.

Mean serum concentration of miRNA (gated at nucleotide length 18-25 nucleotides. Average nucleotide length 22 nucleotides) in **A)** Patients undergoing cardiac surgery with CPB (*$p < 0.05$ postoperative vs. preoperative). **B)** Patients undergoing OPCABG.

3.5 Discussion

Circulating miRNAs were successfully isolated and measured in patients undergoing cardiac surgery during and after CPB. Levels of miR-210, miR-16 and total circulating small RNAs markedly increased in the serum of patients upon initiation of CPB and continued to increase during the rewarming phase of CPB. Levels remained elevated into the immediate postoperative period, and returned to preoperative levels after 4 h postoperatively. Levels of miR-210 and total small RNAs increased in the serum of patients during OPCABG but to a lesser degree than patients following cardiac surgery with CPB. No changes in circulating levels of miR-16 were observed during OPCABG.

The CPB period presents a potentially challenging situation for RNA determination because patients are systemically heparinised. Heparin has been shown to influence the results of qRT-PCR analysis interfering with amplification. The only other studies measuring circulating miRNAs during CPB by Yang et al. (2015) and Zhou et al (2013)., did not address the issue of the effect of heparin on miRNA detection by PCR. In this study, treatment of samples with heparinase to degrade heparin, as reported by Wang et al. (2009), allowed successful PCR amplification and determination of miRNA levels (See APPENDIX E1). Furthermore, others have reported that the exposure to heparin does not have an effect on circulating levels of miRNAs (Lorenzen *et al.*, 2011).

Cardiopulmonary bypass also presents a challenging period of biomarker measurement due to the concentration profile of biomarkers being affected by haemodilution associated with CPB. The potential variability in measurement of urinary miRNAs due to differences in urinary dilution was partially overcome by correcting their concentration to urine creatinine. The potential alteration in urinary creatinine kinetics during CPB are unknown. Biomarkers commonly investigated for the early detection of AKI including NGAL, KIM-1, IL-18, cystatin C and cell cycle arrest markers including IGFBP7 and TIMP-2 are typically measured postoperatively. Due to the potential impact of haemodilution and altered urinary kinetics and clearance, there remains paucity of data biomarker measurement during the CPB period.

The lack of a suitable internal reference gene was also addressed in this study. The study by Yang et al. (2015) utilised endogenous small RNA U6 for normalisation of miRNA expression during CPB. Due to the unknown effects of CPB, haemolysis and haemodilution on miRNAs, including miR-16, the variability in expression of an internal reference miRNA would potentially lead to misinterpretation in other miRNAs of interest. In this study, the use of an external spike for normalisation provided a consistent alternative for analysis and interpretation of miRNA expression levels.

MicroRNA-210 expression levels were significantly increased during cardiopulmonary bypass

Levels of circulating miR-210 were significantly increased during the CPB period and immediately into the postoperative period. This is the first study to show, despite the effects of haemodilution, increased miR-210 levels during CPB. While the cellular source responsible for the miR-210 release has not been identified, CPB and cardioplegic arrest is known to cause hypoxic injury to the myocardium and myocardial cells may be contributing to the increased circulating miR-210 observed (Chan *et al.*, 2012; Thum *et al.*, 2007).

Levels of miR-210 were also significantly increased during the period following application of the Octopus® during OPCABG. During OPCABG, it had been thought that the constant source of myocardial blood flow offered greater protection than conventional cardiac surgery with CPB. However, the surgical and mechanical trauma to the heart during OPCABG is sufficient to activate an inflammatory response in the myocardium with increases in lactate, creatine kinase MB (CKMB), troponin I and interleukin 6 during OPCABG and the risks of ischaemia and renal injury are not eliminated (Bappu *et al.*, 2006; Karu *et al.*, 2009; Paparella *et al.*, 2007). Hence the release of miR-210 observed during the grafting period of OPCABG surgery might be due to cardiac ischaemia or an effect of a decrease in cardiac output as the beating heart is manipulated, eliciting a hypoxic stress response from other organ systems. To help determine if the insult of surgery itself contributes to the increase observed in circulating miRNAs, samples were also taken following sternotomy, prior to the administration of heparin and initiation of CPB. Levels of miRNAs remained unchanged from preoperative levels following sternotomy (Results shown in APPENDIX E3).

During the course of this work, Emanueli et al. (2016) reported that the concentration of cardiac-enriched ischaemia-responsive miRNAs, including miR-210, increased in the plasma early after CABG surgery. The results of this study have confirmed this finding and extended it to demonstrate the release of miRNAs during the perioperative CPB period. In addition, the profile of release of miR-210 in relation to OPCABG has not been elucidated previously.

MicroRNA-16 expression levels were increased during cardiopulmonary bypass but not during off pump coronary artery bypass grafting

MiRNA-16 was initially included as an internal housekeeping miRNA for data normalisation for miRNA quantification. MiR-16 is frequently used as an internal control as it is highly expressed and relatively invariant across samples (Muller *et al.*, 2014; H. Schwarzenbach *et al.*, 2015). In contrast, other studies have demonstrated that haemolysis has a substantial impact on the levels of circulating miRNAs in plasma and serum including the release of RBC enriched miR-16 and miR-451, thus questioning the ability of miR-16 as an internal control (Kirschner *et al.*, 2011; Pizzamiglio *et al.*, 2017). The major release of miRNAs including miR-16 from RBC during haemolysis has been reported and our findings are consistent with haemolytic release of small RNAs during bypass (Kirschner *et al.*, 2013). In paediatric patients, haemolysis induced by CPB has been associated with AKI (Mamikonian *et al.*, 2014). Patients with AKI, as defined by the AKIN, displayed significantly higher plasma free Hb levels during surgery compared to non-AKI patients (Vermeulen Windsant *et al.*, 2014). Although this study did not show any significant differences in plasma free Hb during CPB, or in the immediate post CPB period, changes in plasma free Hb correlated significantly with changes in detected miR-16 levels.

The correlation between plasma free Hb and miR-16 levels observed in the patients undergoing cardiac surgery with CPB most plausibly reflects exposure of the blood to the extracorporeal circuit with resultant haemolysis. Levels of plasma free Hb were also associated with changes in miR-210 which may also be attributed to haemolysis (Kirschner *et al.*, 2013). The absence of a significant mean increase in free Hb during CPB may reflect the poor sensitivity of plasma free Hb testing to detect haemolysis at low levels of red cell damage in part due to quenching by haptoglobin and raises the possibility that miR-16 release might be a more sensitive measure of haemolysis or a reflection of sublethal

damage to the red blood cell membrane not reflected in plasma free Hb levels (Olia *et al.*, 2016). In contrast, levels of miR-16 and plasma free Hb remained relatively constant in the OPCABG cohort.

Urinary levels of microRNA-210 are increased following cardiopulmonary bypass

The urinary concentration of miRNAs could potentially be affected by fluid balance, diuretic therapy and urine output. Correcting urine levels of a potential biomarker to urine creatinine is a method used to potentially overcome this (Abassi *et al.*, 2019). Urine concentration of miR-210 and miR-16, when corrected for the effects of urine dilution during CPB, were increased in patients during CPB, and in the immediate postoperative period, returning to preoperative levels after 4h postoperatively. This concern was overcome by correcting urinary levels to urine creatinine. The elevations of miRNA levels during CPB were particularly striking when urine concentration was accounted for by measurement of urinary creatinine (Han *et al.*, 2009). Levels of miR-16 detected in the urine of patients undergoing CPB increased almost 2000%.

Levels of miR-210 showed a similar release in the urine of patients undergoing OPCABG, however when corrected for urinary creatinine concentration the release was less marked. This may reflect inadequate perfusion of the kidney during the perioperative period of CPB leading to either elevated miR-210 production by the kidney in response to hypoxia, or increased circulating miR-210 being eliminated via the kidneys.

Cardiopulmonary bypass induces a global increase in small RNAs

The results of this study demonstrate there is a differential profile of miR-210 and miR-16 release between OPCABG patients and patients undergoing cardiac surgery with CPB. To further gain insight into global miRNA profile changes, Bioanalyzer analysis was carried out. Bioanalyzer analysis uses a microfluidics-based platform for sizing and quantification of small RNAs. These small RNA classes include miRNAs, small nuclear RNAs (snRNA) transfer RNAs (tRNA), ribosomal RNA (rRNA), small nucleolar RNAs (snoRNA) small interfering RNAs (siRNA) and piwi-interacting RNAs (piRNAs) (Zhang, 2009). Small RNAs are defined according to their characteristics related to their origins, structures, associated effector proteins and biological roles. The results of Bioanalyzer analysis confirmed a global release

of miRNAs during and following CPB. While an increase in global miRNAs was also observed during the OPCABG procedure, the release was less marked than during CPB and was not significant. The increased release of small RNAs during CPB suggests a global release of miRNAs in response to CPB. However, Bioanalyzer analysis has limitations in that while the small RNA chip is a good method to measure the amount of small RNA, it is difficult to differentiate miRNAs from all other types of small RNAs and also could mask fragmentation of RNA, with no distinction between miRNAs and RNA fragments of the same size. High throughput RNA sequencing has detected distinct classes of RNA fragments including tRNA fragments ranging from 10 – 45 nt lengths. These tRNA fragments are not products of random RNA cleavage or degradation with studies demonstrating increased levels of circulating tRNA derivatives under conditions of oxidate stress including hypoxia associated with acute renal ischaemia (Anderson & Ivanov, 2014; Mishima *et al.*, 2014). Further investigation of the small RNA populations identified by the Bioanalyzer could include miRNA profiling, either by high throughput qRT-PCR, microarrays or next generation sequencing, to provide further identification and discrimination of small RNA populations observed during and following CPB.

MicroRNA-210 is potentially released due to hypoxia during cardiopulmonary bypass

Cardiac surgery is associated with variable degrees of myocardial damage related to the use of CPB and the ischaemia-reperfusion insult of cardiologic arrest (Emanueli *et al.*, 2016). The results of this chapter focused on miR-210 because of its established induction by hypoxia. In cardiomyocytes, miR-210 exerts cytoprotective effects during hypoxia (Mutharasan *et al.*, 2011). In a murine model of myocardial infarction, miR-210 was shown to improve angiogenesis, inhibit apoptosis and improve cardiac function (Hu *et al.*, 2010). Hence the increase in miR-210 observed during CPB and into the immediate postoperative period could mediate cardioprotective effects and play a role in attenuating the insult of cardioplegic arrest.

These observations raise the question of whether such miRNA release has beneficial or deleterious pathophysiological effects and whether they are predictive of adverse outcomes.

3.6 Study Limitations

In this preliminary study, a detailed release profile of changes in miRNA release of two specific miRNAs during cardiac surgery was determined in a modest number of patients undergoing cardiac surgery with CPB (n = 10) and OPCABG (n = 5). We were unable to determine the cellular source of the observed miRNA release.

3.7 Conclusions

This study verified levels of hypoxically regulated miR-210 and levels of haemolysis susceptible miR-16 are increased in the blood and urine of patients before, during and after cardiac surgery in patients undergoing cardiac surgery with CPB. Levels of miR-210 and miR-16 are increased in the serum and urine of patients undergoing cardiac surgery with CPB also displayed greater changes in miRNA expression profiles to patients undergoing OPCABG. Hypoxic injury to the myocardium and haemolysis may partially account for such release. These results also indicate an independent accumulation of hypoxically induced circulating miR-210 during cardiac surgery with CPB, with a lesser release observed during OPCABG. Levels of miR-210 and miR-16 were also increased in the urine of patients undergoing CPB compared to patients undergoing OPCABG suggesting the release of specific miRNAs during surgery potentially related to the operative, ischaemic and hypoxic insult attributed to CPB. Bioanalyzer investigations also verified levels of small RNAs are increased in patients undergoing cardiac surgery with CPB.

Major findings:

- MiRNAs were successfully isolated and levels measured in the serum and urine of patients undergoing cardiac surgery with CPB.
- Levels of circulating hypoxically regulated miR-210 and haemolysis associated miR-16 were significantly upregulated during and immediately following cardiac surgery with CPB.
- Increased levels of miR-210 and miR-16 were measured in the urine of patients during CPB but there was significant variation between patients and did not achieve statistical significance

- A greater change in small RNAs including miRNAs was observed in patients undergoing cardiac surgery with CPB than patients undergoing OPCABG.

Due to the modest number of patients included in this study, and in the aforementioned studies of miRNA investigations during and following CPB, investigation of miR-210 and miR-16 in a larger cohort is warranted to confirm changes in circulating levels of these miRNAs. Further investigation into the small RNA populations identified by Bioanalyzer analysis to validate the release of miRNAs during CPB is also essential.

CHAPTER 4: DIFFERENTIAL EXPRESSION OF CIRCULATING AND URINARY miRNA-210 and miRNA-16 IN A LARGER COHORT

4.1 Introduction

The results in Chapter 3 found that there was a significant difference in expression of specific circulating and urinary miRNAs in patients undergoing cardiac surgery with CPB when compared to patients undergoing OPCABG. Results also showed a significant release of small RNAs during the CPB period, with miR-210 and miR-16 levels being significantly increased during the CPB period and returning to preoperative levels within 4 hr of surgery. The results from Chapter 3 also highlighted that miRNA expression levels peak during the CPB period with levels returning to preoperative levels within 4 to 6 hr postoperatively.

Investigation of microRNA-210 and microRNA-1 6 in a larger cohort

Previous studies examining miRNA expression levels in patients undergoing cardiac surgery with CPB have been carried out in small patient cohorts, without replication cohorts. Zhou et al. (2013) examined levels of miR-1 in the urine and serum of 20 patients undergoing mitral valve surgery preoperatively, prior to the initiation of CPB, 60 min post termination of CPB and 24 hours postoperatively. Emanueli et al. (2016) measured plasma levels of circulating miR-1, miR-24, miR-133a/b, miR208a/b and miR-210 and plasma exosomes in 15 patients preoperatively, prior to the establishment of CPB, 24 hr and 48 hr postoperatively. Yang et al. (2015) measured changes in levels of myocardial specific miRNAs; miR-1, miR-21, miR208a and miR-499 in 15 patients undergoing combined aortic and mitral valve surgery. The levels of the miRNAs were measured preoperatively, 45 min after aortic cross clamping, 60 min after reperfusion and 24 hr postoperatively. The study by Yang et al. (2015) did not describe methods of treatment of heparinised samples taken during CPB with heparinase nor the use of an appropriate control miRNA for normalisation of expression levels. There remains a paucity of large scale studies examining the expression of miRNA levels in patients undergoing cardiac surgery and specifically during the period of CPB.

In order to confirm the results of Chapter 3 in a larger cohort, miR-210 and miR-16 levels were measured in patients undergoing cardiac surgery with CPB from a second tertiary hospital, establishing whether changes in miRNA levels were also observed in an independent cohort. Patient samples collected for the larger scale study were restricted to samples taken preoperatively, during CPB, immediately following CPB and in the early postoperative period on arrival to the ICU (0 hr) and 3 hr following arrival to the ICU. In collaboration with Professor Roger Evans, Cardiovascular Disease Program, Biomedicine Discovery Institute and Department of Physiology, Monash University, levels of miR-210 and miR-16 were measured in the plasma and urine of 40 patients undergoing cardiac surgery with CPB at Monash Medical Centre (MMC). Plasma and urine samples were collected from patients participating in a study of urinary hypoxia as an intraoperative marker of risk of cardiac surgery associated kidney injury. These studies also facilitated an examination of the relationship between miR-210 levels, AKI and urinary oxygenation which are described in Chapter 5.

MicroRNAs in serum and plasma in patients undergoing cardiac surgery

Both plasma and serum are used for extracellular miRNA detection. Serum was used to detect miRNA expression levels in studies carried out in Chapter 3 while plasma samples were available for examination of miRNAs for this larger cohort study. The essential difference between plasma and serum is the presence of fibrinogen and clotting factors in plasma. Many studies comparing these biological fluids find little or no difference in miRNA quantification (Chen *et al.*, 2008) (D' Alessandra *et al.*, 2010). Wang et al. (2012) reported higher total miRNA concentration in serum for specific miRNAs including miRNAs released upon platelet activation, however for the more abundant miRNAs including miR-16 the concentrations in serum and plasma were the same. In this chapter, a side-by side study was carried out to determine if there were differences in plasma and serum expression levels of the specific miRNAs, miR-210 and miR-16.

A surprising result from Chapter 3 was the elevated circulating miR-16 levels in patients undergoing cardiac surgery with CPB. While miR-16 was initially selected as an internal

control miRNA, the striking increase in miR-16 expression levels reported during CPB and the positive strong correlation with plasma free Hb warranted further investigation of miR-16 levels as a marker of haemolysis in a larger cohort.

4.2 Hypotheses and Aims

Hypothesis: Levels of miR-210 and miR-16 increase during the CPB period and into the early postoperative period.

Aim: To examine whether levels of hypoxically regulated miR-210 and levels of haemolysis susceptible miR-16 increase in the blood and urine of patients before, during and after cardiac surgery in a larger cohort of patients undergoing cardiac surgery with CPB.

Hypothesis: MiR-16 levels correlate with level of haemolysis assessed by plasma free Hb.

Aim: To investigate the role of miR-16 as a marker of haemolysis and examine its correlation with plasma free Hb levels.

Hypothesis: There are no differences in expression levels of specific miRNAs between plasma and serum measured in patients undergoing cardiac surgery with CPB.

Aim: To observe differences in miRNA expression levels in serum versus plasma in patients undergoing cardiac surgery with CPB.

4.3 Methods overview

Patient inclusion

Plasma and urine samples were collected for the study ***Urinary PO_2 and AKI in Cardiac Surgery*** (Human Research Ethics Committee of Monash Health Reference number 12375B). Between January 2015 and August 2017, 65 patients undergoing cardiac surgery requiring CPB were prospectively enrolled. Patients undergoing CABG, valve repair/replacement surgery or combined valve-CABG surgery were included. Exclusion criteria were: i) pre-

existing advanced chronic kidney disease as defined by a baseline serum creatinine of >200 μmol/L, ii) currently on haemodialysis, iii) kidney transplant recipients, iv) preoperative diagnosis of AKI as defined by KDIGO AKI criteria or v) unable to provide informed consent. The patients recruited by MMC into the trial were selected to be at higher risk of AKI with a EuroSCORE of greater than 6 including preoperative risk factors: New York heart association (NYHA) score >3, poor left ventricular (LV) dysfunction, diabetes and older age or the complexity of their surgical procedure. Of the 65 patients enrolled, 25 patients were excluded due to incomplete datasets hence 40 consecutive patients (cohort B) with complete datasets were selected for miRNA analysis. Incomplete data sets were attributed to intraoperative malfunction of equipment measuring urinary oxygen content (UPO_2) and/or missed plasma and urine collection at specific time points in ICU.

Blood and urine samples used for this study were obtained at five time points including a preoperative sample post induction, 45 to 60 min following initiation of CPB, following separation from CPB, on arrival to the ICU (0 hr) and at 3 hr following arrival to the ICU.

Cardiopulmonary bypass procedure

Intravenous heparin (300 IU/kg) was administered immediately before cannulation for CPB and additional doses given to maintain an activated clotting time of 400 s or greater. The circuit included a 40 μm arterial filter, centrifugal pump and a hard-shell membrane oxygenator (Synthesis; Sorin Biomedica, Modena, Italy). The circuit was primed with 1600 mL of Hartmann's solution, 5000 IU of heparin and 1 g of cephazolin. Cardiopulmonary bypass was instituted by cannulation of the ascending aorta and insertion of a single two-stage cannula into the right atrium for CABG and aortic valve replacement (AVR) procedures with bicaval venous cannulation utilised for mitral valve procedures. Centrifugal pumps provided non-pulsatile flow rates of 1.8-2.4 L/min/m^2, patient temperature was allowed to drift to a maintenance temperature of 33-34°C. Cardiotomy suction and cell salvage were used for all cases. Cardiac arrest was induced and maintained by intermittent anterograde and retrograde cardioplegia. Myocardial protection utilised St Thomas solution with blood-cardioplegia administered at ratio of 4:1 (blood:crystalloid) delivered at 34 °C. Target

nasopharyngeal temperature for separation from bypass was 36-36.5 °C with a rewarming rate of 0.5 °C per min. Following weaning from CPB, heparin was reversed with protamine.

Collection of plasma and urine

Arterial blood was collected from the radial arterial catheter into a 4 mL Vacutainer® Plus blood collection K3EDTA tube (Becton, Dickinson and Company) for plasma separation. Samples were centrifuged at 3000 rpm (1010 x *g*) for 10 min at 4°C. Samples were aliquoted into 1.5 mL microcentrifuge tubes and stored at -80°C. Urine (20 mL) was collected from the sample port of the urinary catheter at each time point and stored at -80°C. Samples were transported overnight on dry ice on between Monash University, Melbourne, and Flinders Medical Centre, Adelaide. Samples were received frozen and intact.

RNA extraction from plasma and urine

RNA was extracted from serum or urine and treated with heparinase I according to the method Chapter 2 pg. 50-52. (*RNA extraction from serum and plasma for qRT-PCR; RNA extraction from urine for qRT-PCR).*

RNA extraction from plasma and serum for side by side study

Due to the collection of different types of blood samples, a study was carried out to determine if there were differential expression levels of miR-210 and miR-16 in plasma and serum. Following a power calculation to determine sample size, RNA was extracted from 15 plasma samples (5 time points from 3 patients) and the corresponding time point serum sample collected from cohort A (Chapter 3) patients. RNA was extracted from serum or plasma and treated with heparinase I according to the method outlined in Chapter 2 pg. 50-52. (*RNA extraction from serum and plasma for qRT-PCR).*

MicroRNA qRT-PCR

Reverse transcription and real time PCR were carried out according to the general methods described in Chapter 2 pg.52-53. Relative expression levels were calculated from quantification cycle (Cq) values using Q-gene (Muller *et al.*, 2002).

Statistical Analysis

Statistical analysis was carried out as described in Chapter 2 pg. 58.

4.4 Results

The median age of patients undergoing cardiac surgery was 70, with 20% of the cohort female. Isolated CABG procedures comprised 50% of procedures with a median time on CPB of 105 min and a median X clamp time of 83 min. Following cardiac surgery 21 (52%) of these selected patients developed AKI according to the RIFLE criteria. In hospital mortality was 5% with 2 postoperative deaths reported (Table 4.4.1).

Table 4.4.1 Preoperative, procedural and outcome characteristics of the 40 patients undergoing cardiac surgery with cardiopulmonary bypass- Cohort B

	n = 40
Baseline	
Female	8 (20%)
BMI	28 (21-36)
COPD	5 (12%)
Previous cardiac surgery	5 (12%)
Diabetes	19 (48%)
Hypertension	31 (78%)
Congestive heart failure	9 (23%)
Smoking history	24 (60%)
Ejection fraction < 30%	4 (10%)
Emergency surgery	1 (3%)
Cerebrovascular disease	5 (12%)
Myocardial infarction	14 (35%)
Preoperative Hb, g/L	134 (88-163)
Preoperative creatinine, μmol/L	83 (41-210)
Procedure	
CABG	20 (50%)
Valve repair/replacement	4 (10%)
Valve + CABG	10 (25%)
Other	6 (15%)
CPB duration, min	105 (65-277)
X clamp time, min	83 (13-233)
CPB Naso temperature, min ^{O}C	34.4
CPB MAP, avg mmHg	72
CPB Hb, min g/L	75 (60-108)
CPB Hb, max g/L	89 (65-112)
CPB blood glucose, min mmol/L	7.3 (5.1-10.9)
CPB blood glucose, max mmol/L	10.3 (6.5-13.1)
1st Hb on CPB, g/L	84 (64-114)
Final Hb on CPB, g/L	79 (67-110)
Fluid balance (mL)	
Anaesthetic vol. prior to CPB	500 (100-1000)
CPB prime (static volume)	1600
Volume added during CPB	2250 (200-8900)
Pre CPB urine volume	150 (0-400)
Post CPB urine volume	850 (200-4000)
Outcome	
AKI	21 (53%)
Highest post op creatinine (μmol/L)	114.5 (65-325)
ICU time (hr)	51 (23-1149)
Length of stay (days)	8.5 (5-68)
Mortality	2 (5%)

Data expressed as median and range. All baseline characteristics defined by the Australian and New Zealand Society of Cardiac and Thoracic Surgeons national database. Acute Kidney Injury (AKI) defined by RIFLE. Body

Mass Index (BMI), Chronic Obstructive Pulmonary Disease (COPD), Coronary Artery Bypass Grafting (CABG), Cardiopulmonary Bypass (CPB), Haemoglobin (Hb), Nasopharngeal (Naso), Mean Arterial Pressure (MAP), Intensive Care Unit (ICU). MAP during CPB was recorded manually at 5 min intervals by the research investigator and average calculated over the CPB period. Perfusion related variables including minimum and maximum Hb and blood glucose were collated from the manually completed perfusion record as detailed by the perfusionist.

MicroRNA-210 and microRNA-16 in plasma of patients undergoing cardiac surgery with cardiopulmonary bypass

Both miR-210 and miR-16 were readily detected in plasma by qRT-PCR. The levels of miR-210 and miR-16 were not significantly altered during CPB. However, miR-210 did significantly increase by twofold ($p = 0.0015$) in the immediate postoperative period (Figure 4.4.1A). There were no significant changes in levels of miR-16 measured in the perioperative period. There was a modest increase in miR-16 levels on arrival to the ICU (0 hr) but this did not reach statistical significance (Figure 4.4.1B) ($p = 0.96$).

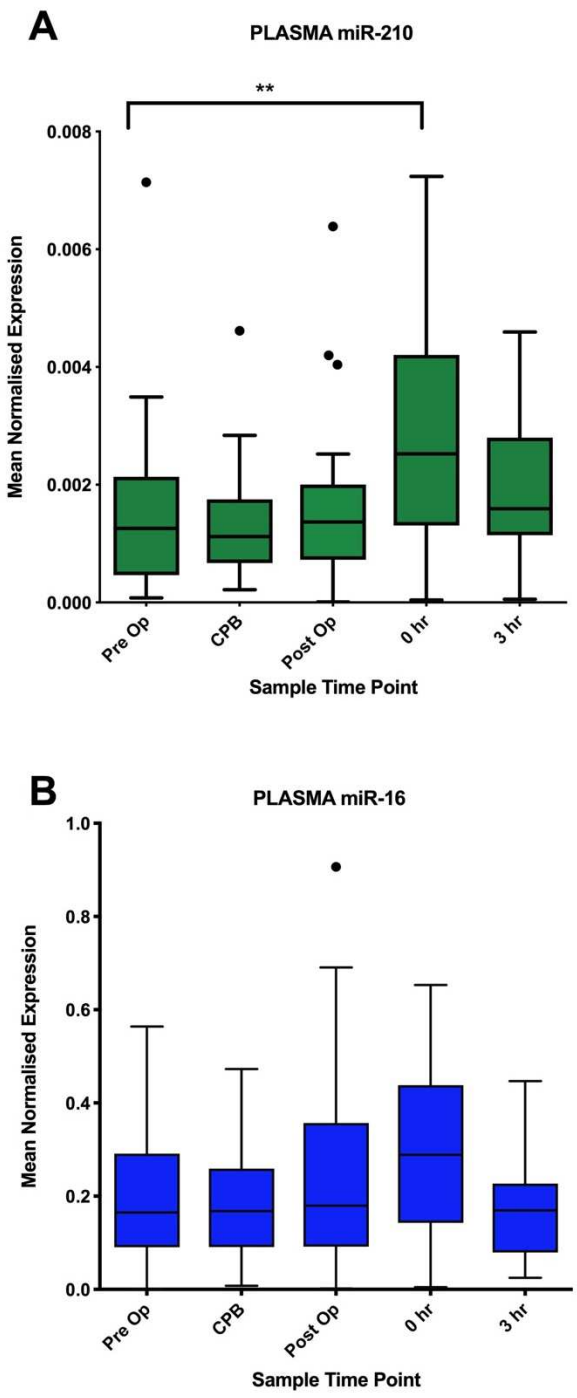


Figure 4.4.1 Expression of miR-210 and miR-16 in plasma of patients undergoing cardiac surgery with CPB.

A) Plasma miR-210 levels from patients before, during and after cardiac surgery with CPB (**p = 0.0015 vs. preoperative). **B)** Plasma miR-16 levels patients before, during and after cardiac surgery with CPB. Data is presented as Box-plots with the whiskers defined using the 25th and 75th percentile ± 1.5 times the inter-quartile range (IQR) (• represent outliers numbers less than the 25th percentile or greater than the 75th percentile by more than 1.5 times the interquartile range).

MicroRNA-210 and microRNA-16 in urine of patients undergoing cardiac surgery with cardiopulmonary bypass

Both miR-210 and miR-16 were detectable in the urine of patients undergoing cardiac surgery, but mean concentrations were substantially less than in plasma (100-fold less for miR-210 and 1500-fold less for miR-16) (Figure 4.4.2A, 4.4.2C). No changes in urine miR-210 or miR-16 levels were statistically significant. After adjusting for urinary concentration by normalising with urinary creatinine there was a 60% increase in normalised miR-210 levels during CPB, however no changes were seen in miR-16 levels during CPB or into the postoperative period (Figure 4.4.2 B, Figure 4.4.2 D).

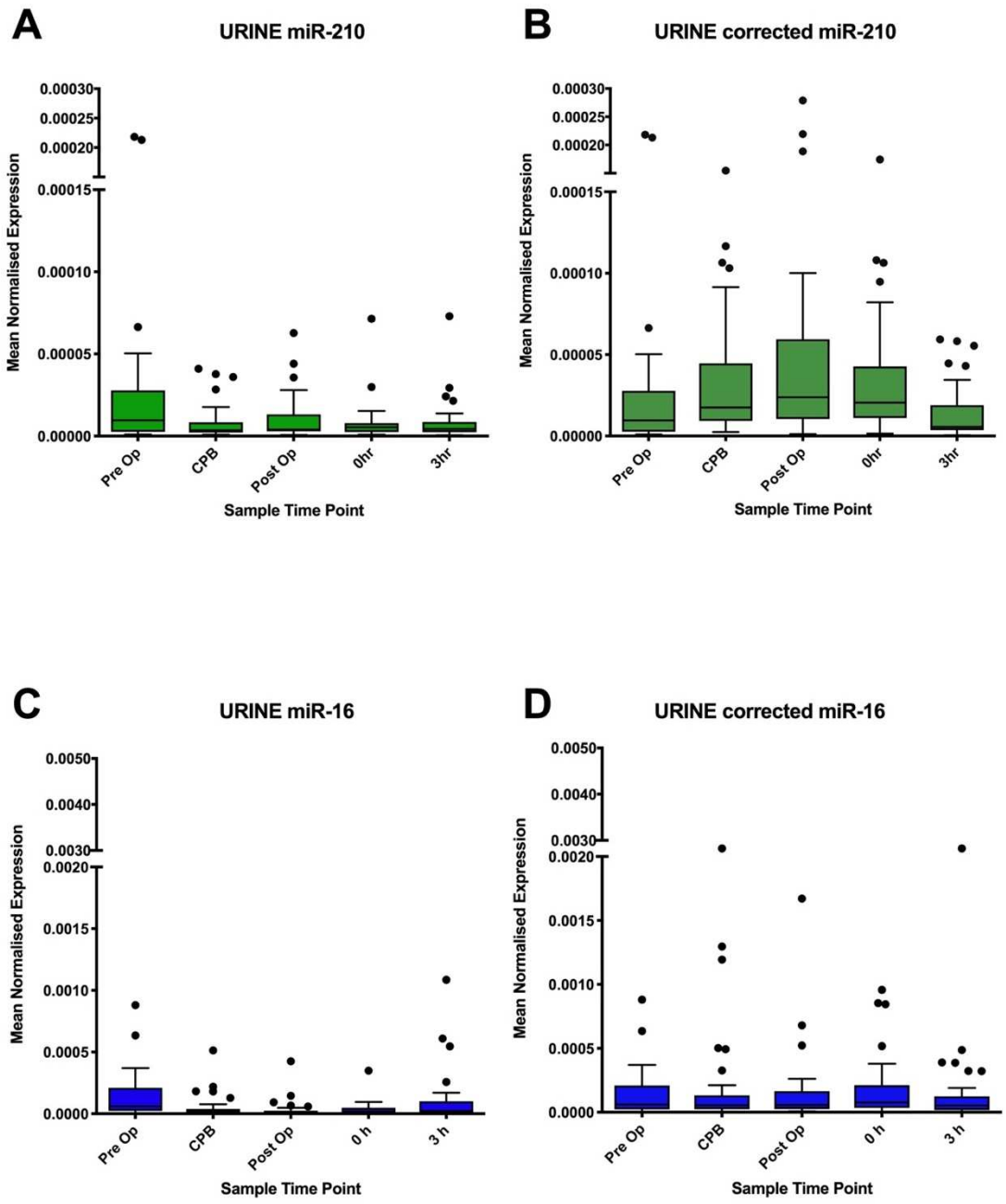


Figure 4.4.2 Expression of miR-210 and miR-16 in urine of patients undergoing cardiac surgery with CPB.

A) miR-210 levels in the urine of patients before, during and after cardiac surgery with CPB. **B)** miR-210 levels in the urine of patients before, during and after cardiac surgery with CPB when corrected for urinary concentration using urine creatinine. **C)** miR-16 levels in the urine of patients before, during and after cardiac surgery with CPB. **D)** miR-16 levels in the urine of patients before, during and after cardiac surgery with CPB when corrected for urine concentration using urine creatinine.

Plasma free haemoglobin levels

Mean plasma free Hb levels were significantly increased in the immediate postoperative period following separation from CPB and this increase continued upon arrival to the ICU, returning to baseline levels within 3hr. (Figure 4.4.3). Plasma free Hb did demonstrate a significant positive correlation with levels of miR-16 and miR-210, with a strong correlation between miR-16 and plasma free Hb observed (r = -0.51, p (two-tailed) < 0.0001) (Figure 4.4.4).

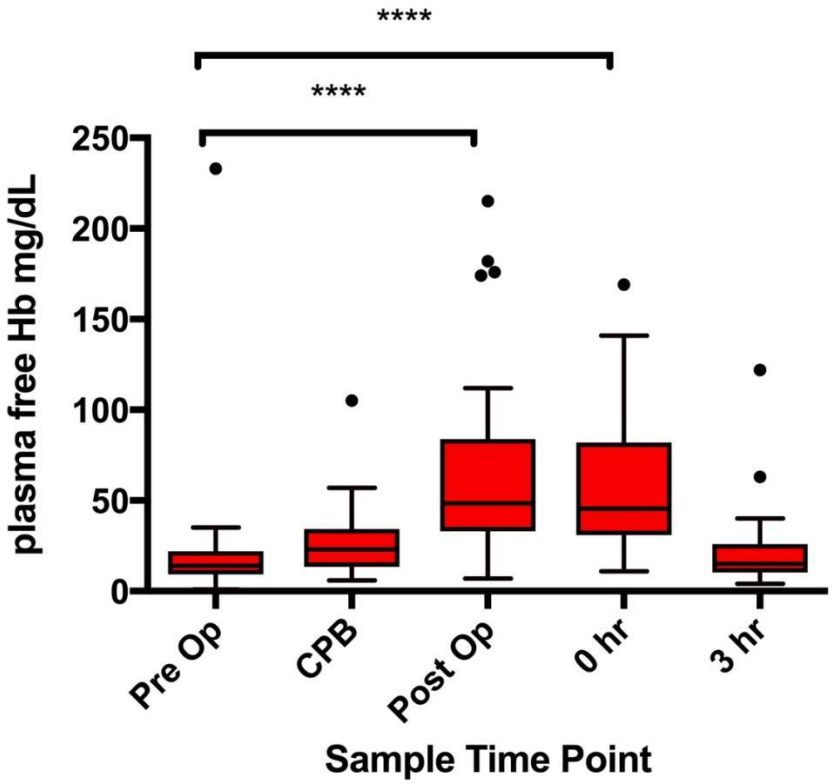


Figure 4.4.3 Mean plasma free haemoglobin (Hb) levels in patients undergoing cardiac surgery with cardiopulmonary bypass.
Significant increases in plasma free Hb were measured postoperatively (****p = 0.0001 postoperative vs. preoperative) and on arrival to the ICU (****p = 0.0001 0 hr ICU vs. preoperative).

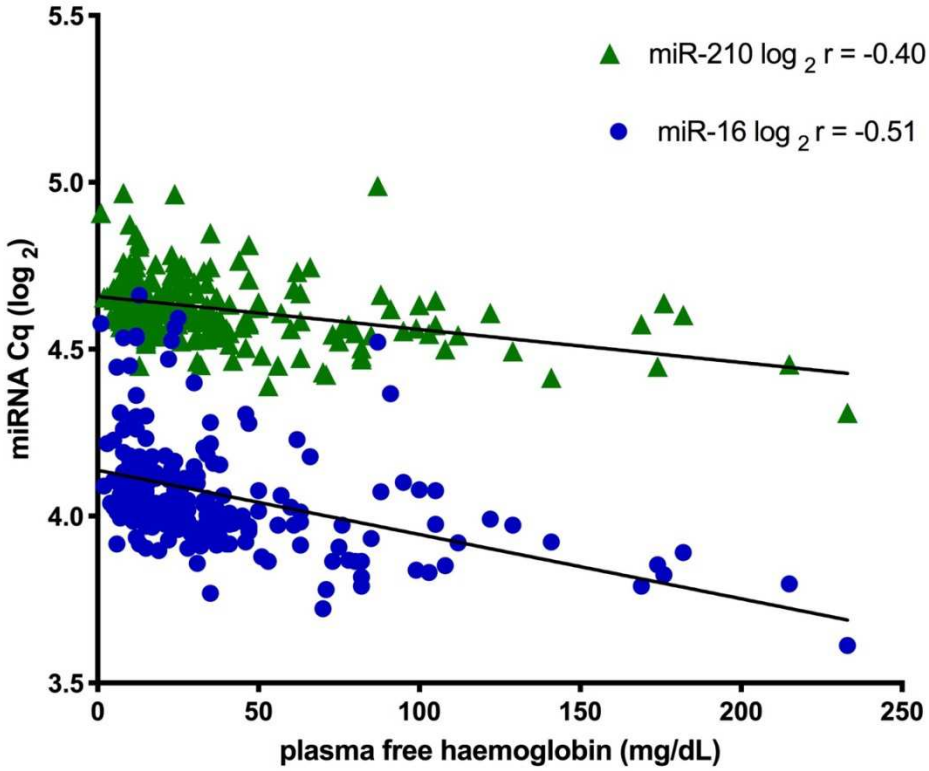


Figure 4.4.4 Correlation between plasma free Hb, miR-210 and miR-16 levels in patients undergoing cardiac surgery with CPB.
Quantitation cycle (Cq) values were transformed to log base 2. Cq values represent the number of cycles needed to reach a set threshold fluorescence signal level. A lower Cq value indicates a higher target miRNA expression in a sample. There is an inverse significant correlation between plasma free Hb and miR-16 levels ($r = -0.51$, p (two-tailed) < 0.0001) and plasma free Hb and miR-210 levels ($r = -0.40$, p (two tailed) < 0.0001). Simple linear regression represents line of best fit.

Differential microRNA-210 and microRNA-16 expression in plasma and serum

In order to determine if there were differences in plasma and serum expression levels of the specific miRNAs, miR-210 and miR-16 a side-by side comparison was carried out in the serum and plasma of cohort A patients. MiRNA-210 expression levels were significantly higher in plasma than serum. There was a strong linear relationship between plasma and serum miR-210 levels, which was confirmed with a Pearson's correlation coefficient of 0.90. Linear regression showed a significant relationship between serum and plasma miR-210 levels (p (two-tailed) < 0.0001) (Figure 4.4.5A). MiRNA-16 levels were significantly higher in plasma than serum. Simple linear regression again showed a significant relationship

between serum and plasma miR-16 levels however, the relationship was less strongly linearly related r = 0.79(p (two-tailed) <0.0004) (Figure 4.4.5B).

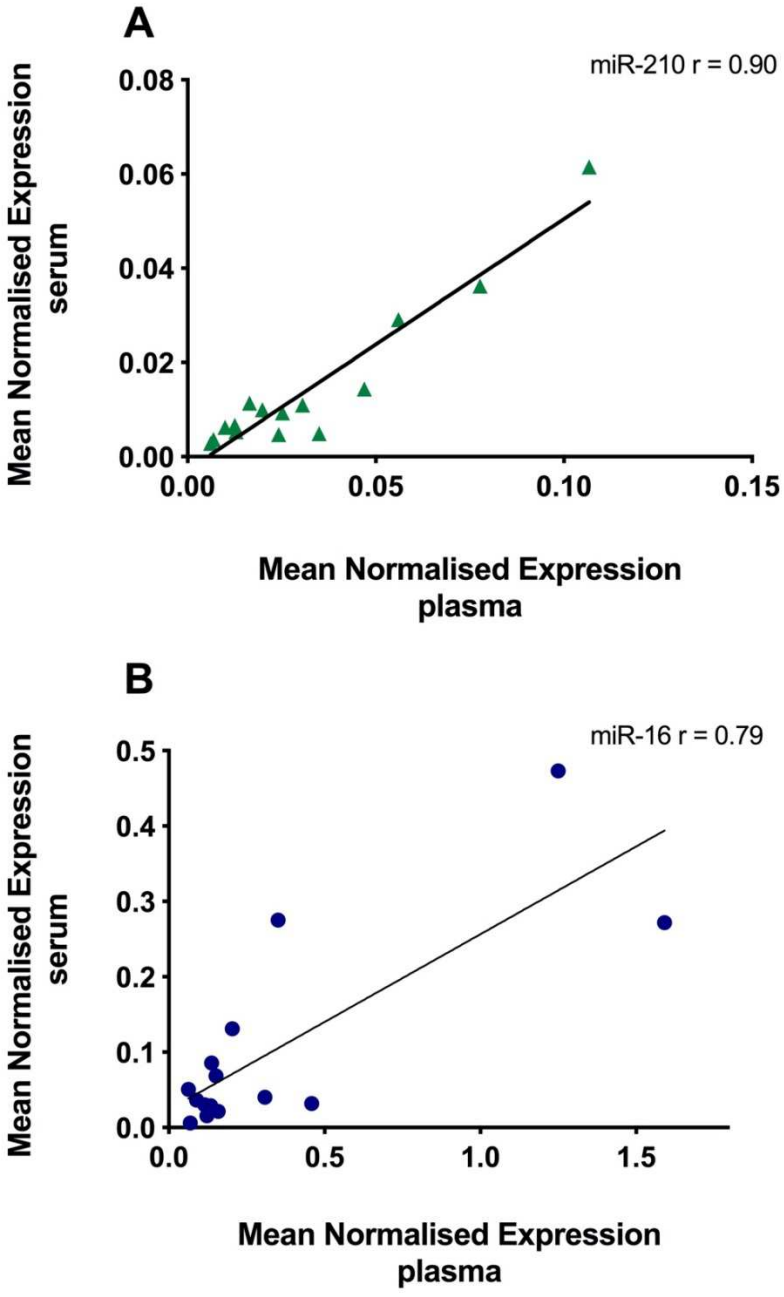


Figure 4.4.5 Correlation between serum and plasma levels of miR-210 and miR-16 in patients undergoing cardiac surgery with CPB.

A) Pearson's correlation between plasma and serum levels of miR-210. There is a significant correlation and significant linear regression exists between plasma and serum miR-210 levels with a r value of 0.90, (p (two-tailed < 0.0001)). **B)** Pearson's correlation between plasma and serum levels of miR-16. A significant regression exists between plasma and serum miR-16 levels with a r value of 0.79, (p (two-tailed) 0.0004).

4.5 Discussion

As in the study described in Chapter 3 (cohort A) circulating and urinary miRNAs were successfully isolated and measured in patients undergoing cardiac surgery with CPB at a second tertiary hospital (cohort B). Examination of the larger cohort of 40 patients found plasma levels of miR-210 to be significantly elevated in the immediate postoperative period following transfer to the ICU and returning to baseline levels within 3 hr. These results support the findings of the pilot study where an increase of hypoxically regulated miR-210 levels were reported in the early postoperative period. Changes in plasma miR-16 levels measured during the perioperative and postoperative period however did not reach significance and did not correlate so clearly with serum levels.

Circulating levels of hypoxically regulated microRNA-210 were increased following cardiopulmonary bypass

The increase in hypoxically released miR-210 levels measured in the early postoperative period and the known association of miR-210 release in to the circulation in response to hypoxic injury, may reflect a hypoxia insult induced during CPB. While the cellular source responsible for the miR-210 release has not been identified, CPB and cardioplegic arrest is known to cause hypoxic injury to the myocardium and myocardial cells may be contributing to the increased circulating miR-210 (Chan *et al.*, 2012; Thum *et al.*, 2007). Studies have shown in patients with MI, miR-210 levels are upregulated potentially in response to hypoxic injury to the myocardium (D' Alessandra *et al.*, 2010; Wang *et al.*, 2015).

A study by Lorenzen et al. (2011) on circulating miR-210 levels in patients with AKI included patients with acute MI as disease controls. The study showed a borderline upregulation of miR-210 in the group of patients with acute MI. The most highly significant levels of miR-210 were observed in patients with AKI when compared to the disease control group and age-matched health controls. The patients enrolled in the current study were deliberately approached for enrolment as they were likely at a higher risk of developing CSA-AKI. This allowed an examination of the relationship between miR-210 expression levels and AKI to be investigated. Correlation between miR-210 levels, AKI and urinary oxygenation, a known predictor of AKI, will also be explored in Chapter 5.

While a significant increase in miR-210 was observed during the CPB period in Chapter 3, these results were not observed in the replication study. MiR-16 levels were also not significantly increased during or following CPB in the larger cohort study. Multiple differences exist between the patient cohorts including management of CPB, surgical and anaesthetic techniques. The blood sample used to measure circulating miRNA levels was also different between the two cohorts. While serum was used for miRNA analysis of cohort A patients, plasma was collected for an independent study in the larger replication cohort (cohort B).

One of the most plausible explanations for the absence of a significant increase in miRNA expression levels during CPB may be due to the volume of administration of crystalloid and consequent level of haemodilution between the two centres. On average patients at cohort B hospital received 6 times the crystalloid volume patients received during the perioperative period at patients undergoing cardiac surgery at cohort A hospital. The increased volume administered by the anaesthetist and perfusionist at cohort B hospital is reflected in the significantly lower Hb recorded in the first arterial blood gas measured within 10 min of the institution of CPB. While preoperative Hb did not vary significantly between the two cohorts, there was a significant difference in the first Hb measured on CPB, with a 35% reduction in Hb from preoperative levels in cohort B patients from preoperative levels.

There remains a paucity of data of biomarker measurement during the unique CPB period. In a meta-analysis carried out by Ho et al. (2015) reporting urinary, plasma and serum biomarkers' utility for predicting CSA-AKI in adults, they found of the 28 studies reporting intraoperative and/or early postoperative measurement of urine, plasma or serum biomarkers that only 4 of these studies measured biomarkers intraoperatively with the samples taken immediately after CPB. The authors concluded there are comparatively few data for the discrimination of biomarkers in the intraoperative period of cardiac surgery, a time of potential active management to mitigate kidney injury. The result of this study have shown the administration of crystalloid volume and differences in perioperative perfusion and anaesthestic volume management may have a potential dilution effect on miRNA levels and needs to be considered when looking at potential biomarker measurement during CPB.

Despite the effects of haemodilution, the results of this study have shown an increase in circulating and urinary miR-210 levels was observed in patients following CPB.

Urinary levels of microRNA-210 are increased following cardiopulmonary bypass

In keeping with the results of chapter 3, urine levels of miR-210, when corrected for the effects of urine dilution with urine creatinine, were increased during CPB and into the early postoperative period. While urinary miR-16 levels increased almost 2000% during CPB in cohort A patients, this considerable increase was not observed in the replication cohort with a modest increase of 20% in urinary miR-16 levels during CPB. The differences in urinary miRNA levels observed between the two cohorts may be attributed to the variations in volume management, use of diuretics and urine output. Patients in cohort B had higher urine output and increased use of the loop diuretic furosemide. Measurement of miRNA and biomarkers in urine remains difficult due to the unknown effect of volume addition, use of diuretics and the unknown effects of CPB on kidney function.

Plasma free haemoglobin levels correlate with microRNA-16 expression

The correlation between plasma free Hb and miRNAs levels reported in the pilot study was confirmed in the larger cohort study. Historically the use of centrifugal pumps has been attributed to less haemolysis and reduced plasma free Hb release (Morgan *et al.*, 1998). Due to the use of centrifugal pumps by the cohort B hospital it was suggested there would be less haemolysis. A meta-analysis of randomised controlled trials comparing centrifugal pumps and roller pumps in adult cardiac surgery showed no significant differences in haematological variables including plasma free Hb levels (Saczkowski *et al.*, 2012). Despite the use of centrifugal pumps, significantly increased levels of free Hb were detected in the plasma of cohort B patients in the immediate postoperative period following CPB with levels remaining elevated upon arrive al to the ICU.

Potential explanation for the increased plasma free Hb levels could be due to the use of cardiotomy suction in all cases. Cardiotomy suction is not used for isolated CABG procedures at the cohort A hospital and thus was only used for 6 of the 10 patients included in the cohort A study. Cardiotomy suction allows heparinised blood shed into the operative

field to be returned to the CPB circuit. Evidence suggests that the return of shed blood increases the burden of microembolisation and potentiates the systemic inflammatory response. The concurrent suction of air results in highly turbulent flow with high stresses at the air-fluid interface causing haemolysis, and elevated levels of plasma free Hb (Jewell *et al.*, 2003). The return of cardiotomy suction blood has been attributed to being the principle source of plasma free Hb during CPB (Hansbro *et al.*, 1999). At the cohort B hospital cardiotomy suction is used for all cardiac surgery procedures. The extent of use of cardiotomy suction during the CPB period at the cohort B hospital is not known. High suction flow rates and excessive return of blood via the cardiotomy suction may contribute to the higher levels of plasma free Hb observed.

The correlation between plasma free Hb and miR-16 levels observed in the cohort B patients most plausibly reflects exposure of the blood to the extracorporeal circuit and use of cardiotomy suction with resultant haemolysis. The correlation of miR-16 to levels of plasma free Hb reinforce miR-16 as a potential marker of haemolysis. MiR-16 levels may also be affected by RBC transfusion. Future studies could investigate the effect of RBC transfusion and the associated haemolysis on miR-16 levels during cardiac surgery.

Differential microRNA-210 and microRNA-16 expression in plasma and serum

Both plasma and serum are used for extracellular miRNA detection. Serum was used to detect miRNA expression levels in the pilot study while plasma samples were available for examination of miRNAs in the larger cohort study. Many studies comparing these biological fluids find little or no difference in miRNA quantification dependent on the miRNA. There is as lack of consensus and no definitive choice for biomarker fluid for miRNA analysis. The results of this study demonstrated miRNA levels of miR-210 were 2X times greater in plasma than serum and miR-16 levels were 3 times greater in plasma than serum. While there was a strong correlation between miR-210 levels measured in serum and plasma, the correlation was poorer for miR-16 levels. The poorer correlation of miR-16 levels between plasma and serum may be attributable to the differences in the specific miRNA expression between the two fluids due to the composition of the fluid with plasma containing platelets and other clotting factors. Serum is generally a cleaner sample typically free of cells and platelets

because they are trapped in the fibrin meshwork of clot in the collection tube. However, serum does contain the clotting metabolites that result from the clotting process.

4.6 Limitations

Differences in methods of data collection between the two sites limit data analysis and interpretation of results. Data is collected manually at cohort B hospital during CPB and involves the perfusionist manually entering data, including anaesthetic machine parameters such as MAP and heart lung machine parameters such as arterial pump flow, every 5-10 min. At cohort A hospital data collection is automated utilising an integrated data management system, with data collected from the anaesthetic machine and heart lung machine every 20 sec, hence providing a comprehensive unbiased representation of events during CPB. Results of a study by Ottens et al. (2005) observing manual recorded versus computer generated perfusion records generated during CPB showed significant variations in the information recorded. Areas that showed the most inconsistency included measurement of the MAP, arterial pump flow, blood temperatures, cardioplegia delivery details, and the recording of events, with the electronic record superior in the integrity of the data. The study showed the use of an automated system provided the opportunity to minimise transcription error and bias. The study highlighting the limitation of spot recording of perfusion events in the overall record keeping for perfusion management (Ottens *et al.*, 2005).

For miRNA measurement, data normalisation is still a challenge, especially for circulating miRNAs. While the use of the external control in this study, *C. elegans* allowed for normalisation and to measure technical variability, a set of internal controls to control for biological variability is not well- developed, thus there are no specific miRNAs suitable as an internal control (Kroh *et al.*, 2010). The effect of haemodilution may also potentially impact on the use of internal controls as shown in this study due to the variability observed between cohort A and cohort B miRNA measurement during CPB. The effect of CPB on circulating miRNAs and the unknown factors that modulate circulating miRNA levels during CPB also limits the use of an internal control.

4.7 Conclusions

Results of the larger replication cohort support the results of Chapter 3 in showing that levels of miR-210 were increased in plasma and urine of patients undergoing cardiac surgery with CPB. A significant accumulation of hypoxically induced circulating miR-210 was also seen during the early postoperative period. While there were no significant changes in levels of miR-16 measured in the perioperative period, there was a modest increase in miR-16 levels on arrival to the ICU. The absence of a significant increase in miRNA levels during CPB may be attributed to the effects of volume administration and haemodilution. A positive correlation of miR-16 levels with level of haemolysis as assessed by plasma free Hb levels support the role of miR-16 as a marker of haemolysis. A side-by side study found levels of plasma and serum miR-210 and miR-16 were highly correlated, however levels were higher in plasma than serum. This result reinforces the need for a standardised approach to sample selection for miRNA measurement due to the variability that potentially exists in different biological samples.

Major findings:

- Levels of miR-210 were increased in the early postoperative period in patients undergoing cardiac surgery with CPB
- Differences in perfusion and anaesthetic practices between different institutions may impact miRNA levels measured during CPB due to differences in fluid levels and consequent haemodilution
- miR-16 levels correlate with level of haemolysis assessed by plasma free Hb
- Levels of miR-210 and miR-16 measured were higher in plasma than in serum

The increases in miR-210 levels observed during and following CPB in this study raise the interesting question of whether the increased levels are associated with an increased risk of AKI and whether they are elevated due to more severe renal hypoxia during AKI. This will specifically be examined in Chapter 5.

CHAPTER 5: CIRCULATING AND URINARY microRNA EXPRESSION AND URINARY OXYGEN CONTENT IN ACUTE KIDNEY INJURY

5.1 Introduction

The results of Chapter 3 described that in patients undergoing cardiac surgery with CPB there was an increase in hypoxically regulated miR-210 during the CPB period that continued into the immediate postoperative period. The increase in miR-210 in the early postoperative period following CPB was confirmed in a replication of the study in a larger cohort of patients from a second tertiary hospital described in Chapter 4. In this chapter, the findings were extended to examine whether there were differences in miR-210 expression between patients with and without AKI and whether there was correlation between miR-210 levels and a direct indicator of renal oxygenation, urinary oxygen content (uPO_2).

Urinary oxygen content and acute kidney injury

Studies have shown that measurement of urine oxygen tension in the renal pelvis is a sensitive surrogate measure of the oxygenation of the immediately adjacent renal medulla. As urine flows through the tubules of medullary thick ascending limb into the renal pelvis, the urine PO_2 equilibrates with the medullary PO_2, which in turn reflects medullary perfusion and oxygen consumption (Leonhardt & Landes, 1963; Stafford-Smith & Grocott, 2005). In a study carried out by Wang et al. (2008) measuring urine oxygen tension using *in vivo* MRI measurements, they reported normal mean bladder urine PO_2 ranged from 23 mmHg to 45 mmHg in healthy subjects. When using oxygen probes to measure uPO_2, normal urine PO_2 has been reported to range from 50 mmHg to 80 mmHg (Giannakopoulos *et al.*, 1997; Pickwell, 1964). Urine PO_2 can change in response to a variety of physiological and pathological stimuli. In patients with renal failure the uPO_2 increases and this change is through to reflect the reduced medullary metabolic function in such patients (Giannakopoulos *et al.*, 1997).

As previously discussed, the aetiology of AKI after cardiac surgery is complex. Hypoxia in the renal medulla appears to be a hallmark of AKI being present during and after experimental

CPB and in other pathophysiologies of AKI including sepsis, ischaemia reperfusion injury, administration of radiocontrast agents and rhabdomyolysis. In 1996, Kainuma et al. reported continuous urinary oxygen tension monitoring in patients undergoing cardiac surgery. Ninety-eight consecutive patients undergoing elective cardiac surgery with CPB were studied. A Foley catheter joined to a urinary tube was placed in the urinary bladder of each patient. A polarographic oxygen electrode connected to an oxygen tension monitor was inserted into the urinary tube to continuously measure uPO_2. The authors reported uPO_2 was constant in the perioperative period prior to initiation of CPB and then progressively decreased following initiation of CPB. UPO_2 partially recovered at weaning from CPB but did not return to preoperative levels until the early postoperative period. Postoperative serum creatinine concentrations were significantly higher in patients whose uPO_2 decreased following CPB. The rate of recovery in uPO_2 following CPB was also associated with peak values of postoperative serum creatinine concentrations. The authors suggested measurement of uPO_2 during CPB might be a predictor of postoperative renal dysfunction.

In animal studies carried out by Lankadeva et al. (2016), bladder uPO_2 correlates strongly with renal medullary PO_2 in sheep during development of hyperdynamic septic AKI and in rabbits during pharmacologically induced changes in renal medullary PO_2. The study found sepsis induced AKI was associated with medullary tissue hypoperfusion and hypoxia. Resuscitation with the vasoconstrictor noradrenaline caused a transient improvement in renal function, but this was associated with a reduction in medullary perfusion and tissue PO_2. Perfusion and tissue PO_2 in the renal cortex remained unchanged during the resuscitation period. The reduction in medullary PO_2 reported during sepsis and noradrenaline resuscitation could be detected by measurement of uPO_2. The authors concluded there is a strong relationship between simultaneously measured medullary tissue PO_2 and uPO_2.

A study carried out at cohort B hospital examined the association between postoperative AKI and intraoperative uPO_2, measured via a fibre optic probe in the tip of the urinary catheter. The results of the study found uPO_2 fell during the operative period, often

reaching its nadir during rewarming or after weaning from CPB, with nadir uPO_2 lower in patients who developed AKI than in the patients who did not. Patients who developed AKI after their cardiac surgery experienced longer and more severe periods of low intraoperative uPO_2 than patients who did not develop AKI. The authors concluded low intraoperative uPO_2 was an independent predictor of postoperative AKI, potentially providing prognostic information during the surgical procedure, which may provide a unique opportunity to intervene during the intraoperative period to improve renal oxygenation and hence potentially reduce the risk of AKI (Zhu *et al.*, 2018).

Urinary oxygen content and cardiac surgery with cardiopulmonary bypass

The 40 patient samples used for analysis in the larger cohort study were collected as part of the study being undertaken at MMC examining the role of continuous bladder uPO_2 measurement during cardiac surgery. The study enrolled 65 patients undergoing cardiac surgery requiring CPB. The patients enrolled in the study were recruited to be at an increased risk of AKI with a EuroSCORE greater than 6 with known pre-operative risk factors for increased risk of CSA-AKI including a higher NYHA score, LV dysfunction, diabetes and older age.

The study compared the efficacy of continuous intraoperative measurement of bladder urine uPO_2 with traditional and emerging biomarkers of AKI. Serum samples were collected for measurement of the biomarker serum creatinine. Plasma and urine samples were collected for urinary and plasma NGAL and cystatin C measurement. The study was carried out to test the hypothesis that low intraoperative uPO_2 predicts postoperative AKI more reliably, and earlier, than standard biomarkers including serum creatinine or biomarkers such as NGAL or cystatin C.

The results of the study showed that urinary hypoxia during surgery, predicts the subsequent development of CSA-AKI. Nadir uPO_2 was lower in patients who developed AKI (8.9 ± 5.6 mmHg) than in patients who did not (14.9 ± 10.2 mmHg, $p = 0.008$). Patients who developed AKI had longer periods of $uPO_2 \leq 15$ mmHg and 10 mmHg than patients who did not. Odds of AKI increased when uPO_2 fell to ≤ 10 mmHg (3.60 [1.27-10.21], $p = 0.02$), or ≤ 5

mmHg (3.60 [1.04-12.42], p = 0.04) during the operation (Zhu *et al.*, 2018). Continuous intraoperative measurement of uPO_2 during CPB provided an early warning of the risk of AKI, at a time when it was potentially possible to intervene to prevent AKI. In the collaborative study with Monash University, 40 patients were selected from the 65 patients enrolled in the original study. The 40 patients selected were consecutive patients within the cohort with complete datasets and no sample time points missed. Excess plasma and urine samples collected from the 40 patients were used for miRNA analysis, enabling the correlation of hypoxically regulated miRNAs, including miR-210 with actual measurements of renal oxygenation. This patient group allowed exploration of a relationship between hypoxically regulated miR-210 and uPO_2 and whether differential patterns of miRNA profiles existed in patients that developed CSA-AKI.

Hypoxically regulated microRNA-210 and acute kidney injury

MiRNA-210 is known as a master hypoxamir and plays a unique and complex role in the cellular responses to hypoxia. The results from Chapter 3 and Chapter 4 have shown the expression of circulating miR-210 is upregulated during CPB and into the immediate postoperative period. This chapter investigates whether miRNAs show differential expression in patients that develop CSA-AKI when compared to patients that do not develop AKI postoperatively and observes the relationship between selected miRNA expression and the level of urinary oxygenation. Levels of hypoxically regulated miR-210 are examined as an indicator of renal hypoxia and thereby a potential biomarker for the development of AKI.

MicroRNA-16, plasma free haemoglobin and acute kidney injury

A surprising result from Chapter 3 was the elevated circulating miR-16 levels in patients undergoing cardiac surgery with CPB. The positive strong correlation of haemolysis associated miR-16 with plasma free Hb observed in Chapter 3 was confirmed in Chapter 4. Changes in plasma free Hb have also been significantly associated with the development of AKI postoperatively (Mamikonian *et al.*, 2014). Further investigation of miR-16 levels as a marker of haemolysis and levels of plasma free Hb in patients who develop AKI is warranted.

5.2 Hypotheses and Aims

Hypothesis: Levels of circulating and urinary miR-210 and miR-16 are differentially expressed in patients who develop CSA-AKI.

Aim: To determine if circulating and urinary miR-210 and miR-16 are differentially expressed in patients who developed CSA-AKI.

Hypothesis: Levels of circulating miR-210 are significantly correlated with uPO_2 measured during CPB.

Aim: To examine if miR-210 expression varies with uPO_2, a predictive marker of AKI following CPB.

Hypothesis: MiR-16 and plasma free Hb levels will be higher in patients who develop AKI following cardiac surgery.

Aim: To investigate the role of miR-16 as a marker of haemolysis, plasma free Hb and their association with AKI.

5.3 Methods overview

Patient inclusion

Plasma and urine samples were collected from 40 patients enrolled in the Urinary PO_2 and AKI in Cardiac Surgery study carried out at MMC and Monash University, as previously described in Chapter 4.

Continuous measurement of urinary oxygen tension

The urinary bladder was catheterised with a standard Foley urinary catheter (Medtronic, Minneapolis, USA). A sterilised fibre optic luminescence optode (NX-LAS-1/O/E-5m, Oxford Optronix, Abington, UK) was advanced through the lumen of the catheter, to the catheter tip. The sensing tip of the probe was in direct contact with bladder urine. The bladder

catheter was then connected to a standard urine collection system (Precision 400, Covidien, Minneapolis, MN). The fiber optic probe was connected to a luminescence oximeter (Oxylite Pro, Oxford Optronix, Abington, UK) interfaced with a laptop computer running LabChart software (Version 8, ADInstruments, Bella Vista, NSW, Australia).

Urinary oxygen content was measured at 6 s intervals across 5 time points of surgery; after induction of anaesthesia prior to initial incision, before commencement of CPB, during CPB prior to rewarming, during rewarming on CPB and after separation from CPB. Additional data from the patient monitoring systems were recorded every 5 min. Before and after CPB, these included nasopharyngeal temperature, heart rate, systolic, diastolic and mean arterial pressure, systolic, diastolic and mean pulmonary artery pressure, central venous pressure, arterial oxygen saturation (pulse oximetry) and end expired PCO_2. During CPB, temperature of patient's blood, arterial pump flow, MAP, arterial PCO_2 and PO_2, haematocrit and blood haemoglobin, arterial and venous saturations, systemic DO_2 and consumption and fractional oxygen extraction were recorded.

Collection of plasma and urine

Arterial blood was collected from the radial arterial catheter into a 4 mL Vacutainer® Plus blood collection K3 EDTA tube (Becton, Dickinson and Company) for plasma separation. Samples were centrifuged at 3000 rpm (1010 x *g*) for 10 min at 4°C. Samples were aliquoted into 1.5 mL microcentrifuge tubes and stored at -80°C. Urine (20 mL) was collected from the sample port of the urinary catheter at each time point and stored at -80°C. Samples were transported overnight on dry ice between Monash University, Melbourne, and FMC, Adelaide. Samples were received frozen and intact.

RNA extraction from plasma and urine

RNA was extracted from plasma or urine and treated with heparinase I according to the method outline in Chapter 2 pg. 50-52. (*RNA extraction from serum and plasma for qRT-PCR; RNA extraction from urine for qRT-PCR).*

MicroRNA qRT- PCR

Reverse transcription and real time PCR were carried out according to the general methods described in Chapter 2 pg. 52-53. Relative expression levels were calculated from quantification cycle (Cq) values using Q-gene (Muller *et al.*, 2002).

Statistical Analysis

To determine associations between levels of circulating miRNAs and AKI, and perioperative risk factors and AKI, univariate logistic regression was performed Statistical analysis was carried out as described in Chapter 2 pg. 58.

5.4 Results

To determine any clinical or investigational differences, the characteristics of the patients who did and did not develop AKI following cardiac surgery were reported and are summarised in table 5.4.1. Twenty-one (52%) of the 40 patients undergoing cardiac surgery with CPB developed AKI postoperatively, according to the RIFLE criteria. Of the patients that developed AKI, 11 (52%) developed RIFLE class R, 6 (29%) developed RIFLE class I and 4 (19%) developed RIFLE class F. Significant differences in Hb levels were observed between the two groups. Preoperative Hb levels were significantly lower in patients who developed AKI when compared to patients who did not develop AKI ($p = 0.014$). This trend continued during the CPB period with the first Hb recorded on initiation of CPB ($p = 0.004$) and nadir Hb recorded during CPB ($p = 0.034$) significantly lower in the AKI patient cohort. Nadir UPO_2 levels were significantly lower in the AKI patient cohort when compared to patients who did not develop AKI postoperatively ($p = 0.042$).

Table 5.4.1 Preoperative, procedural and outcome characteristics of the 40 patients undergoing cardiac surgery with CPB who did and did not develop AKI

	No AKI (n=19)	**AKI (n=21)**	P value
Baseline			
Age, years	71 (49-83)	70 (47-81)	0.810
Female	3	5	0.526
BMI	28 (21-36)	28 (22-36)	0.992
COPD	2	3	0.719
Previous cardiac surgery	3	2	0.549
Diabetes	6	13	0.055
Hypertension	15	16	0.834
Congestive heart failure	2	7	0.084
Smoking history	13	11	0.301
Ejection fraction < 30%	1	3	0.342
Emergency surgery	0	1	
Cerebrovascular disease	4	1	0.119
Myocardial infarction	6	8	0.666
Preoperative Hb, g/L*	139 (115-163)	130 (88-152)	*0.014*
Preoperative creatinine, μmol/L	83 (41-116)	87 (49-210)	0.406
Procedure			
CABG	9	11	0.751
Valve repair/replacement	2	2	0.915
Valve + CABG	6	4	0.360
Other	2	4	0.451
CPB duration, min	101 (65-261)	114 (49-277)	0.726
X clamp time	83 (13-233)	98 (45-200)	0.429
CPB Naso temperature, min ^{O}C	34.4	34.4	0.298
CPB MAP, avg mmHg	73	71	0.180
CPB Hb, min g/L*	82 (67-108)	74 (60-100)	*0.034**
CPB Hb, max g/L	92 (73-112)	85 (65-103)	0.103
CPB blood glucose, min mmol/L	7.3 (5.1-10.1)	7.4 (5.8-10.9)	0.342
CPB blood glucose, max mmol/L	10.3 (7.1-13.1)	10.2 (6.5-11.4)	0.992
1st Hb on CPB*, g/L	91 (71-114)	80 (60-103)	*0.004**
Final Hb on CPB, g/L	86 (67-110)	76 (64-96)	0.105
Red blood cell transfusion > 1 unit	1	6	0.052
Nadir uPO_2*, mmHg	13.1 (4.7-19)	8.1 (3.5-21)	*0.042**
Fluid Balance (mL)			
Anaesthetic vol. prior to CPB	500 (100-1000)	300 (100-1000)	0.226
Volume added during CPB	2400 (400-3400)	2100 (200-8900)	0.992
Pre CPB urine volume	200 (0-400)	150 (50-400)	0.779
Post CPB urine volume	700 (200-2800)	1000 (300-4000)	0.624
Outcome			
Highest post op creatinine* (μmol/L)	98 (65-121)	170 (86-325)	*<0.00001*
ICU time (hr)	47 (23-554)	70 (20-1149)	0.107
Length of stay (days)	7 (5-15)	11 (6-68)	0.073
Mortality	0	2	

Data expressed as median and range. Significant differences include 1st Hb on CPB*and final Hb on CPB* from baseline Hb levels and highest postoperative creatinine* ($p < 0.05$, unpaired t-test). All baseline characteristics defined by the Australian and New Zealand Society of Cardiac and Thoracic Surgeons national database. Acute Kidney Injury (AKI) defined by RIFLE. Body Mass Index (BMI), Chronic Obstructive Pulmonary Disease (COPD), Haemoglobin (Hb), Coronary Artery Bypass Grafting (CABG), Cardiopulmonary Bypass (CPB), Urinary Oxygen Content (uPO_2), Nasopharngeal (Naso), Mean Arterial Pressure (MAP), Intensive Care Unit (ICU). MAP during CPB was recorded manually at 5 min intervals by the research investigator and average calculated over the CPB period. Perfusion related variables including minimum and maximum Hb and blood glucose were collated from the manually completed perfusion record as detailed by the perfusionist.

Circulating microRNA-210 and microRNA-16 in patients with and without Acute Kidney Injury following cardiac surgery

MiRNA-210 and miR-16 were readily detected in plasma by RT-qPCR. On arrival to the ICU (0 hr), levels of miR-210 were significantly increased in patients who did develop AKI with a 100% increase from preoperative levels (Figure 5.4.1C) ($p < 0.05$). Levels of miR-210 were also significantly increased in patients who did not develop AKI on arrival to the ICU however the release was less striking with a 50% increase from preoperative levels (Figure 5.4.1A) ($p < 0.05$). Interestingly, in patients who did not develop AKI there was a significant reduction in miR-210 levels measured during CPB (Figure 5.4.1.A).

In patients who did develop AKI, miR-16 levels did increase by 125% from preoperative levels when measured on arrival to the ICU (0 hr), however the result did not reach statistical significance. There were no other significant differences in miR-16 levels observed in patients who did (Figure 5.4.1.D p=n.s) or did not develop AKI (Figure 5.4.1B, p= n.s)

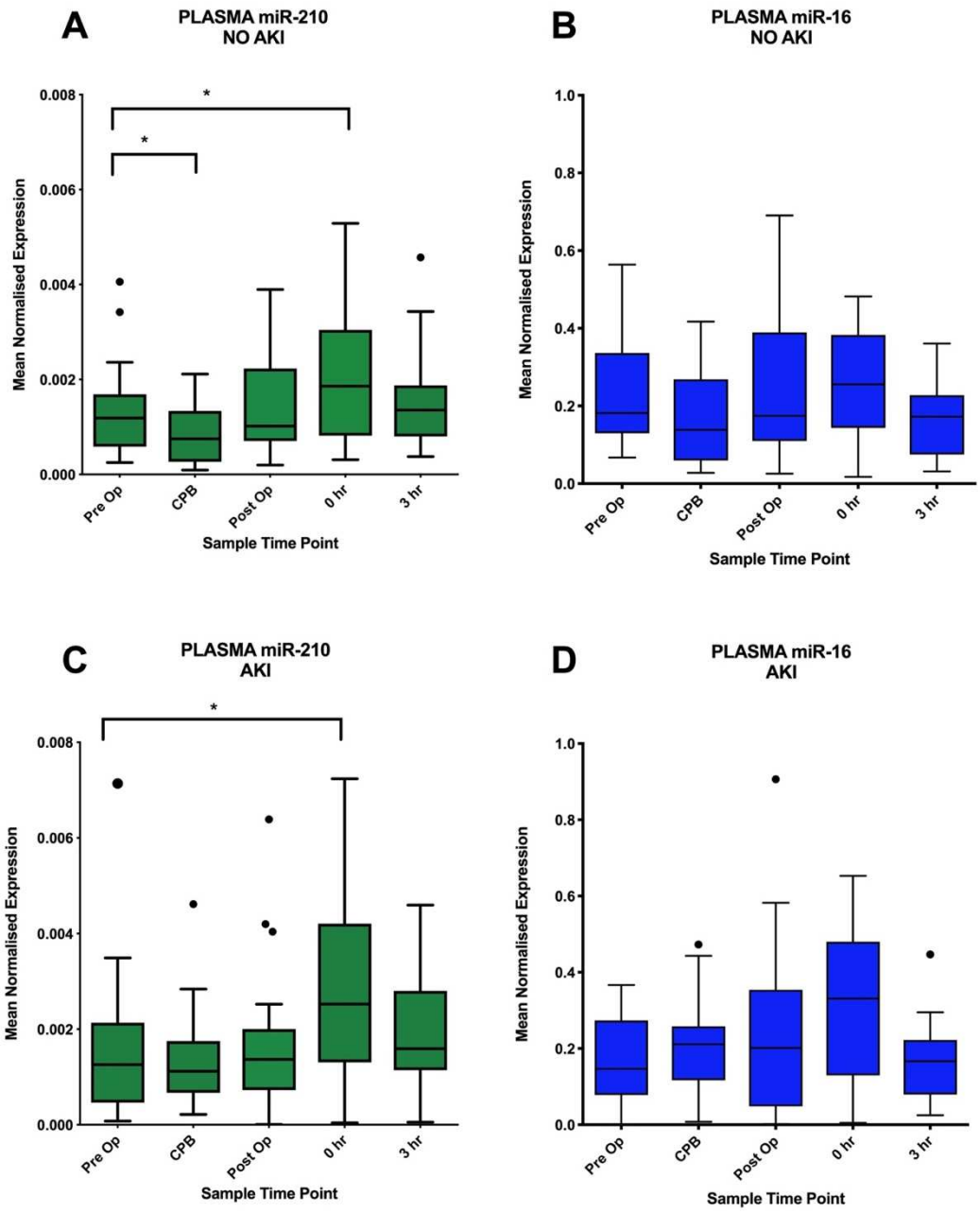


Figure 5.4.1 Expression of miR-210 and miRNA in plasma of patients undergoing cardiac surgery with CPB who did and did not develop AKI.

A) Plasma miR-210 levels from patients before, during and after cardiac surgery who did not develop AKI post-operatively (*p < 0.05 vs. preoperative). **B)** Plasma miR-16 levels from patients before, during and after cardiac surgery who did not develop AKI postoperatively. **C)** Plasma miR-210 levels from patients before, during and after cardiac surgery who developed AKI postoperatively **(***p < 0.05 vs. preoperative. **D)** Plasma miR-16 levels from patients before, during and after cardiac surgery who developed AKI postoperatively. Data is presented as Box-plots with the whiskers defined using the 25th and 75th percentile ± 1.5 times the inter-quartile range (IQR) (• represent outliers numbers less than the 25th percentile or greater than the 75th percentile by more than 1.5 times the interquartile range).

MicroRNA-210 and microRNA-16 in urine of patients with and without acute kidney injury following cardiac surgery

Both miR-210 and miR-16 were detectable in the urine of patients who did and did not develop AKI postoperatively (Figure 5.4.2, Figure 5.4.3). When correcting for urine concentration using urinary creatinine, levels of miR-210 observed during CPB increased by 180% from preoperative levels in patients who did develop AKI, compared to a modest 40% measured at the same time point in patients who did not develop AKI (Figure 5.4.2D, Figure 5.4.2B).

Levels of urinary miR-16, when corrected for urine concentration using urine creatinine, were not significantly different in patients who did and did not develop AKI postoperatively (Figure 5.4.3B, Figure 5.4.D). While there were no significant differences in urinary miR-16 levels, there was a 75% increase of miR-16 levels observed on arrival to the ICU (0 hr) in patients who did develop AKI potentially reflecting the increased plasma miR-16 levels measured at the same time point.

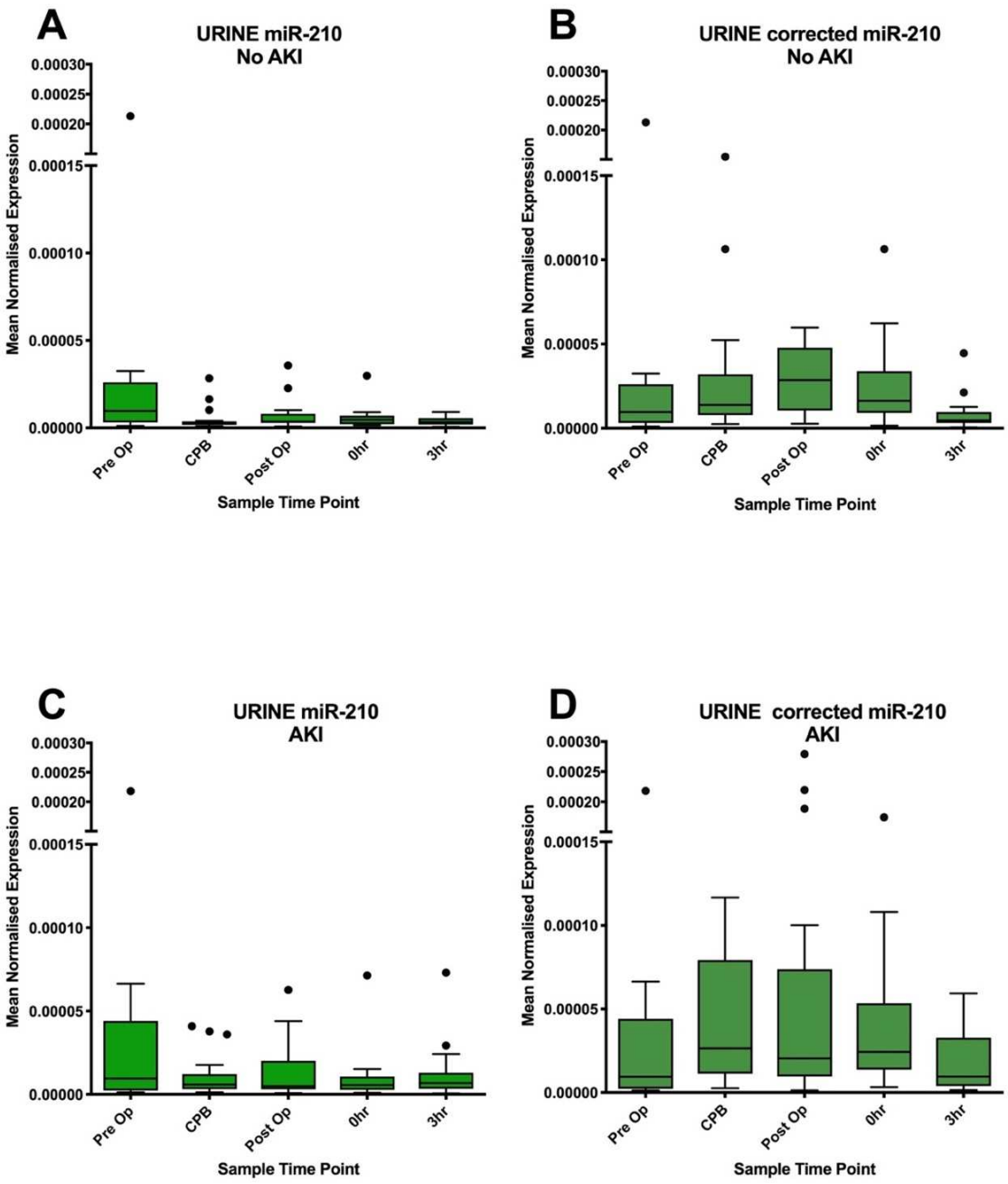


Figure 5.4.2 Expression of miR-210 in urine of patients undergoing cardiac surgery with CPB who did and did not develop AKI.

A) miR-210 levels in the urine of non-AKI patients before, during and after cardiac surgery. **B)** miR-210 levels in the urine of non-AKI patients before, during and after cardiac surgery when corrected for urine concentration using urine creatinine. **C)** miR-210 levels in the urine of AKI patients before, during and after cardiac surgery. **D)** miR-210 levels in the urine of AKI patients before, during and after cardiac surgery when corrected for urine concentration using urine creatinine.

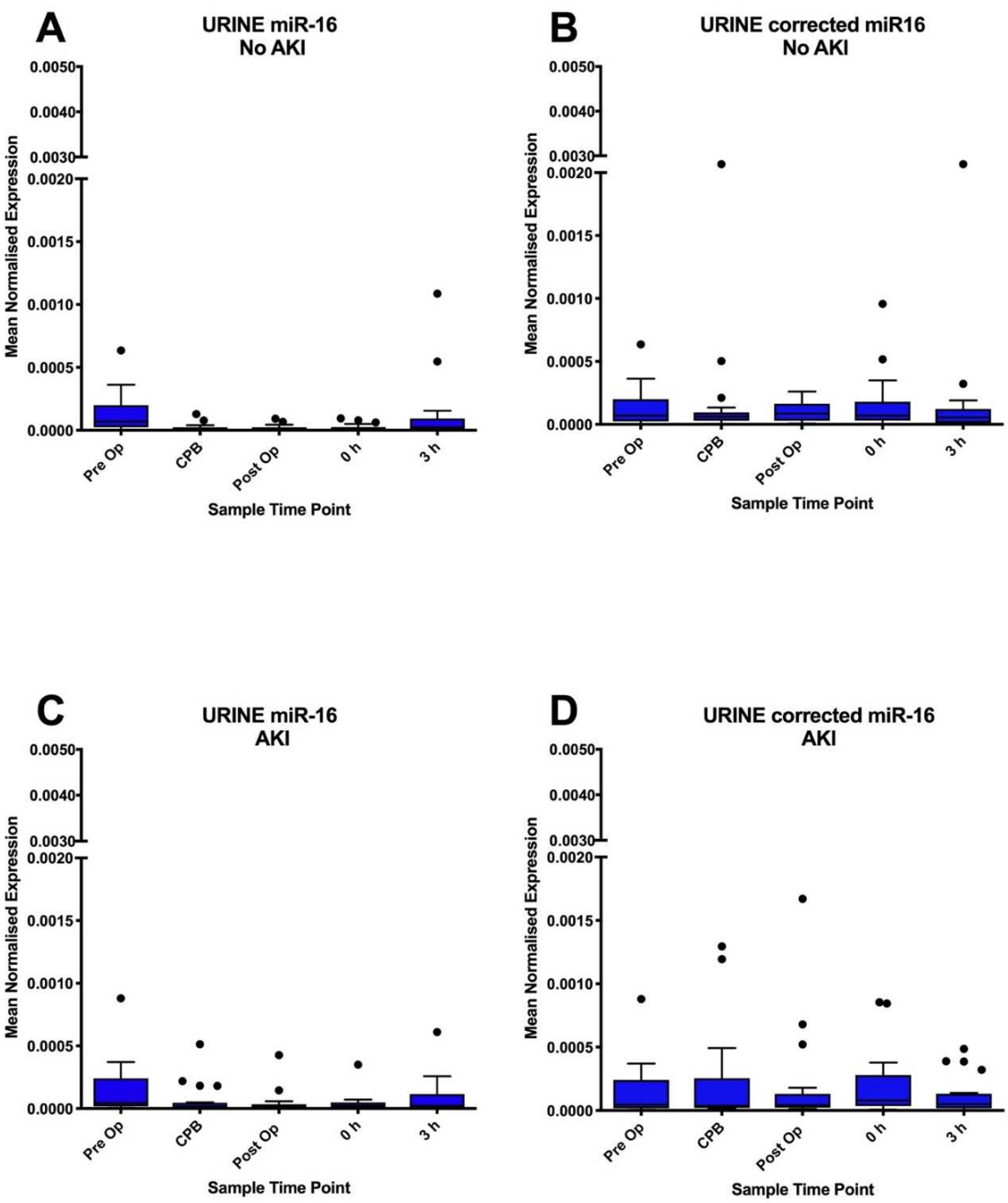


Figure 5.4.3 Expression of miR-16 in urine of patients undergoing cardiac surgery with CPB who did and did not develop AKI.

A) miR-16 levels in the urine of non-AKI patients before, during and after cardiac surgery. **B)** miR-16 levels in the urine of non-AKI patients before, during and after cardiac surgery when corrected for urine concentration using urine creatinine. **C)** miR-16 levels in the urine of AKI patients before, during and after cardiac surgery. **D)**. miR-16 levels in the urine of AKI patients before, during and after cardiac surgery when corrected for urine concentration using urine creatinine.

Plasma free haemoglobin levels

Mean plasma free Hb levels were significantly increased in both groups. A larger proportion of patients with AKI had a significant release of plasma free Hb during the perioperative and postoperative period. In patients who did develop AKI, there was a significant increase in plasma free Hb levels measured during CPB, in the immediate postoperative period with levels remaining significantly elevated upon arrival to the ICU ($p = 0.133$ CPB vs preoperative: $p = 0.0021$ post op vs preoperative; $p = 0.0005$ 0 hr vs preoperative) (Figure 5.4.4B). In patients who did not develop AKI postoperatively, there was also a significant increase in plasma free Hb levels detected in the immediate postoperative period but this was not maintained upon arrival to the ICU ($p = 0.0001$) (Figure 5.4.4A).

In order to examine for correlations between miRNA levels and plasma free Hb that might be associated with miRNA release from red blood cells, levels of miR-16 and plasma free Hb were examined. In patients who developed AKI postoperatively, plasma free Hb levels demonstrated a strongly significant positive correlation with levels of miR-16 ($r = -0.59$ p (two-tailed) < 0.0001) and miR-210 ($r = -0.48$, p (two tailed) < 0.0001) (Figure 5.4.5B). These correlations were not observed in patients who did not develop AKI postoperatively (Figure 5.4.5A)

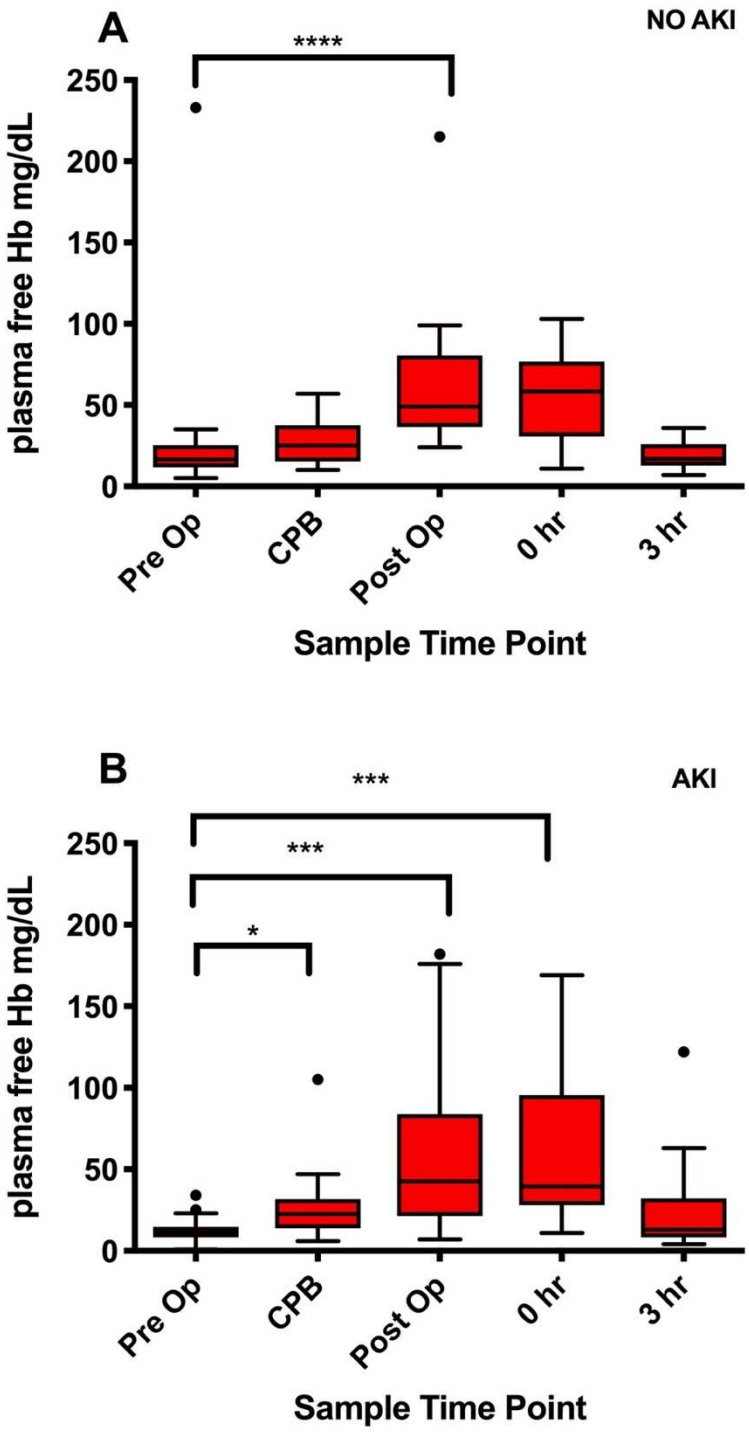


Figure 5.4.4 Mean plasma free haemoglobin (Hb) levels in patients undergoing cardiac surgery with CPB who did and did not develop AKI

A) non-AKI patients before, during and after cardiac surgery (****p = 0.0001 postoperative vs. preoperative) and **B)** AKI patients before, during and after cardiac surgery (*p = 0.0133 CPB vs preoperative, ***p = 0.00021 postoperative vs preoperative, ***p = 0.0005 0 hr vs preoperative)

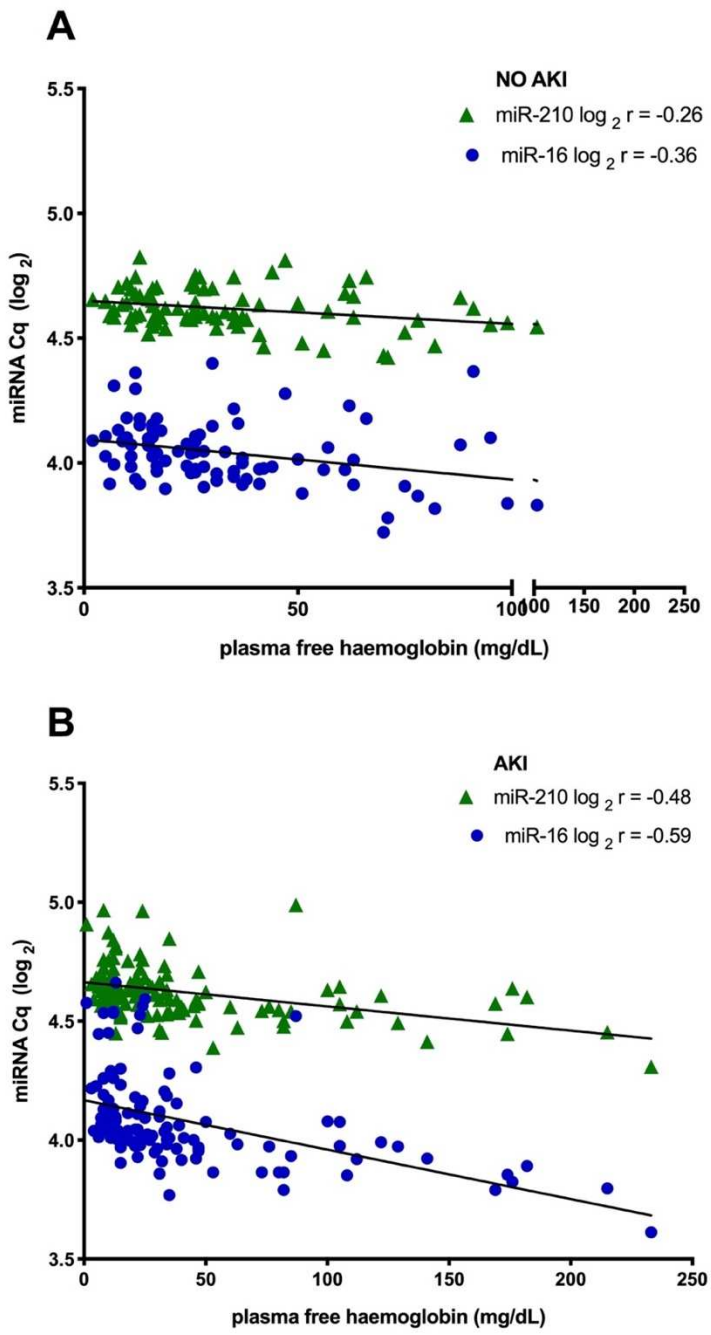


Figure 5.4.5 Correlation between plasma free Hb and miR-210 and miR16 levels in patients undergoing cardiac surgery with CPB who did and did not develop AKI.

A) Patients who did not develop AKI following cardiac surgery. Quantitation cycle (Cq) values were transformed to log base 2. Cq values represent the number of cycles needed to reach a set threshold fluorescence signal level. A lower Cq value indicates a higher target miRNA expression in a sample. There is an inverse significant correlation between plasma free Hb and miR-16 levels (r = -0.36, p (two-tailed) 0.0006) and plasma free Hb and miR-210 levels (r = -0.26, p (two tailed) 0.0126). **B)** Patients who did develop AKI following cardiac surgery with CPB. There is a strong inverse significant correlation between plasma free Hb and miR-16 levels (r = -0.59 p (two-tailed) < 0.0001) and plasma free Hb and miR-210 levels (r = -0.48, p (two tailed) <0.0001). Simple linear regression represents line of best fit.

Urinary oxygen content and circulating microRNA-210 and microRNA-16 in patients with acute kidney injury

In patients who developed AKI postoperatively, the lowest uPO_2 recorded during CPB demonstrated a stronger significant positive correlation with levels of plasma miR-210 measured during CPB. The correlation between UPO_2 and miR-16 levels was not as marked during CPB (Figure 5.4.7B**.** miR-210 r = 0.53, p (two-tailed t-test= 0.013); miR-16 r= 0.29, p (two-tailed t-test) = 0.187). The significant positive relationship between uPO_2 and miR-210 levels extended into the immediate postoperative period for miR-210, with a weaker but significant correlation between lowest urinary PO_2 and miR-16 levels (Figure 5.4.7C miR-210 r= 0.54, p (two-tailed t-test = 0.010); miR-16 r=0.44, p (two-tailed t-test = 0.044). No significant correlations were observed between uPO_2 and preoperative miR-210 or miR-16 plasma levels (Figure 5.4.7)

In patients who did not develop AKI postoperatively, there was no significant correlation between lowest uPO_2 measured during CPB and plasma levels of miR-210 and miR-16 levels measured preoperatively, during CPB or into the early postoperatively period (Figure 5.4.6).

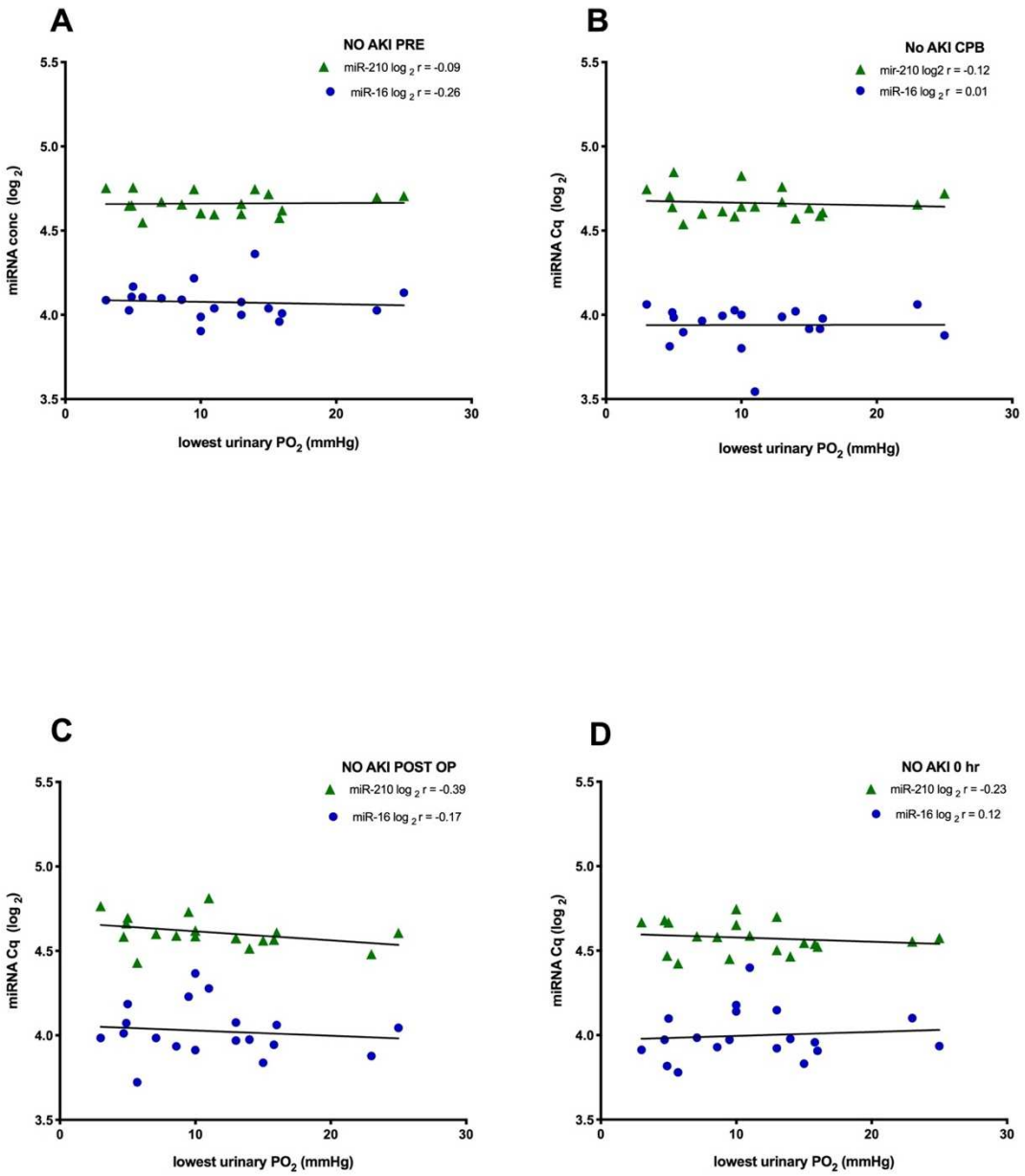


Figure 5.4.6 Correlation between nadir uPO_2 measurement during CPB and plasma levels of miR-210 and miR-16 in patients who did not develop AKI.

Quantitation cycle (Cq) values were transformed to log base 2. Cq values represent the number of cycles needed to reach a set threshold fluorescence signal level. A lower Cq value indicates a higher target miRNA expression in a sample. P values reported from two tailed t-tests. **A)** preoperatively (miR-210 r = -0.09, p = 0.709; miR-16 r =-0.26, p = 0.278), **B)** During CPB (miR-210 r = -0.12, p = 0.622; miR-16 r = 0.01, p = 0.974, **C)** Postoperatively (miR-210 r = -0.39, P = 0.097; miR-16 r = -0.17, p = 0.485) and **D)** Arrival in ICU (0 hr) (miR-210 r = -0.23, p = 0.324; miR-16 r = 0.12, p = 0.613).

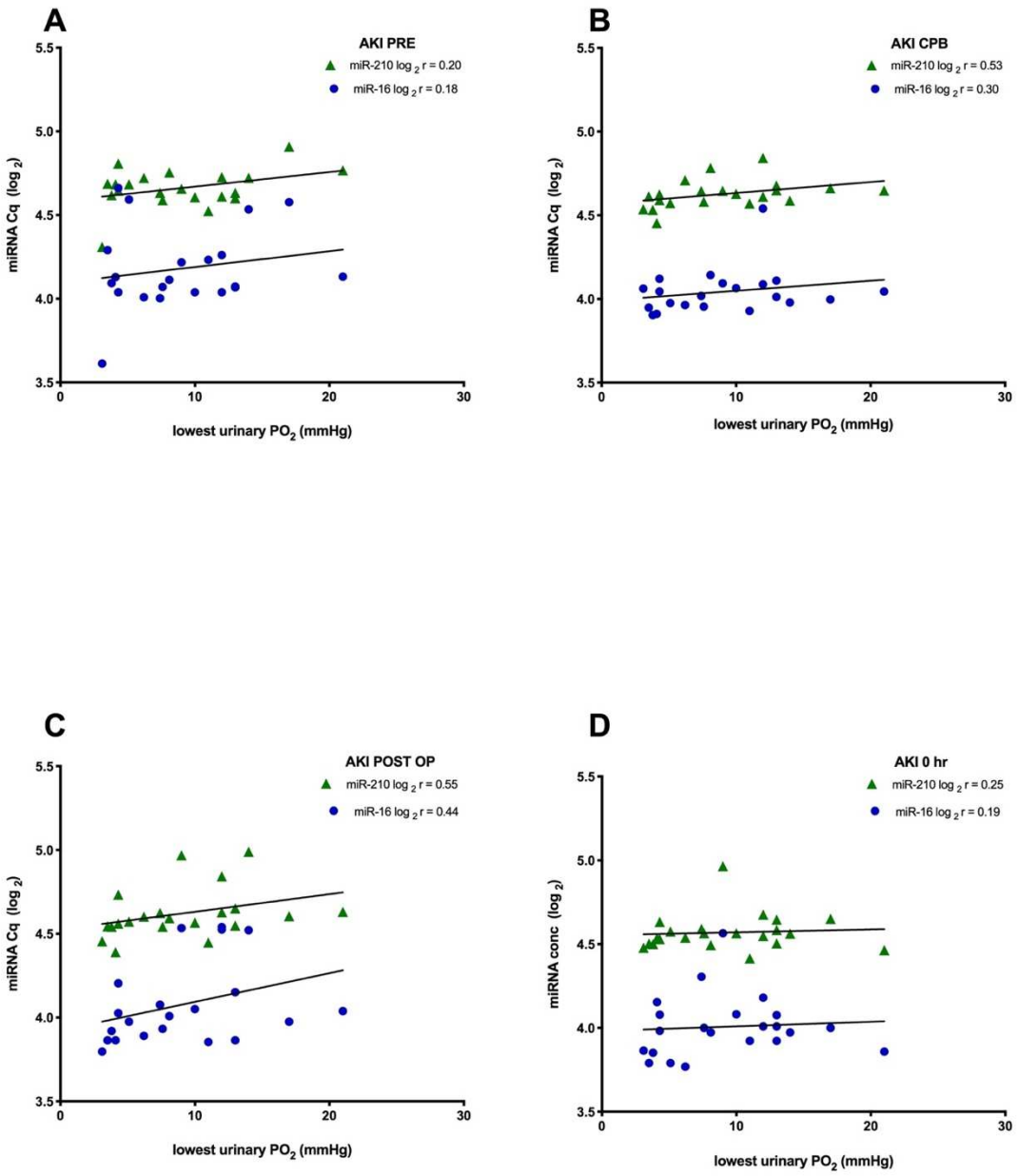


Figure 5.4.7 Correlation between nadir urinary PO_2 measurement during CPB and plasma levels of miR-210 and miR-16 in patients who did develop AKI.

Quantitation cycle (Cq) values were transformed to log base 2. Cq values represent the number of cycles needed to reach a set threshold fluorescence signal level. A lower Cq value indicates a higher target miRNA expression in a sample. P values reported from two tailed t-tests. **A)** preoperatively (miR-210 r = 0.20, p = 0.424; miR-16 r = 0.18, p = 0.384). **D)** Arrival in ICU (miR-210 r = 0.25, p = 0.250; miR-16 r =0.19, p = 0.380). **B)** During CPB there is a significant correlation between lowest uPO_2 and miR-210 levels but not miR-16 levels (miR-210 r = 0.53, p = 0.013); miR-16 r = 0.30, p = 0.187). **C)** Postoperatively there is a significant correlation between lowest uPO_2 and miR-210 and miR-16 (miR-210 r = 0.55, p = 0.010); miR-16 r = 0.44, p = 0.044).

Association of microRNA-210 levels with clinical variables and the incidence of acute kidney injury

Preoperative Hb and minimum Hb on CPB were found to be predictors of AKI (OR = 0.52, [95% CI 0.31 - 0.88], p = 0.015, OR=0.50, [95% CI 0.26-0.94], p=0.034, respectively (Table 5.4.2, Table 5.4.3). The likelihood of developing AKI in patients with increasing plasma miR-210 measured during CPB did not achieve conventional statistical significance (OR = 2.0, [95% CI 0.97 - 4.13], p = 0.06) (Table 5.4.4).

Table 5.4.2 Univariate regression analysis risk factors for AKI – preoperative factors

Preoperative Variable	**Odds Ratio**	**95% CI**	**P value**
Age (years)	0.99	0.92-1.05	0.783
Gender (male/female)	1.66	0.33-8.17	0.529
BMI (kg/m^2)	1.00	0.88-1.15	0.908
Diabetes (yes/no)	3.52	0.95-13.02	0.059
Hypertension (yes/no)	1.6	0.30-8.30	0.576
Congestive heart failure (yes/no)	4.25	0.75-23.81	0.100
Ejection Fraction (%)	0.97	0.92-1.01	0.237
Redo (yes/no)	1.41	0.21-9.54	0.721
Preoperative Hb (g/dL)*	0.52	0.31-0.88	*0.015**

Table 5.4.3 Univariate regression analysis for AKI – perioperative factors

Perioperative Variable	Odds Ratio	95% CI	P value
CPB duration (minutes)	1.00	0.99-1.01	0.387
Minimum Hb on CPB (g/dL)*	0.50	0.26-0.94	*0.034**
Highest lactate on CPB (mmol/L)	1.67	0.84-3.33	0.139
Lowest urinary PO_2 on CPB (mmHg)	0.64	0.33-1.26	0.201
Intraoperative crystalloid volume admin > 3L (yes/no)	1.11	0.14-8.82	0.916
Intraoperative crystalloid volume admin > 4L (yes/no)	0.53	0.12-2.22	0.388
Intraoperative crystalloid volume admin > 5L (yes/no)	0.88	0.18-4.15	0.874
>1 Red blood cell units administered during CPB (yes/no)	7.2	0.77-66.6	0.082
Preop plasma free Hb	0.81	0.43-1.54	0.535
CPB plasma free Hb	0.63	0.32-1.23	0.180
Postop plasma free Hb	0.95	0.51-1.79	0.894
Arrival to ICU plasma free Hb	0.88	0.46-1.67	0.705
3hr ICU plasma free Hb	0.67	0.34-1.31	0.247
Highest plasma free Hb	0.83	0.44-1.58	0.582

Table 5.4.4 Univariate regression analysis for AKI- miRNA levels

miRNA Variable	Odds Ratio	95% CI	P value
Preoperative plasma miR-210	1.06	0.56-1.99	0.846
CPB plasma miR-210	2.00	0.97-4.13	0.060
Postoperative plasma miR-210	1.12	0.59-2.11	0.721
Arrival to ICU plasma miR-210 (0 hr)	1.21	0.64-2.29	0.549
ICU plasma miR-210 (3 hr)	1.34	0.70-2.56	0.375
Preoperative plasma miR-16	0.66	0.34-1.29	0.232
CPB plasma miR-16	1.44	0.74-2.77	0.276
Postoperative plasma miR-16	0.83	0.43-0.59	0.567
Arrival to ICU plasma miR-16 (0 hr)	1.15	0.61-2.18	0.648
ICU plasma miR-16 (3 hr)	0.89	0.47-1.67	0.721

5.5 Discussion

The results of this chapter found that levels of circulating miR-210 are differentially expressed in patients who develop AKI following cardiac surgery with CPB. Levels of miR-210 were significantly increased in the early postoperative period in patients who developed AKI with a 100% increase from preoperative levels. Levels of miR-210 were also significantly increased in patients who did not develop AKI on arrival to the ICU however the release was less marked. Levels of urinary miR-210 measured during CPB increased by 180% in patients who developed AKI compared with a 40% increase in urinary miR-210 levels patients who did not develop AKI. Interestingly during CPB, levels of circulating miR-210 were found to be significantly correlated with an independent predictor of postoperative AKI, nadir uPO_2.

Acute kidney injury following cardiac surgery with cardiopulmonary bypass

Several studies have examined the risk factors associated with the development of AKI after cardiac surgery with CPB. In almost all studies, certain risk factors have been repeatedly associated with an increased risk of AKI including female gender, reduced LV function or the presence of congestive heart failure, diabetes, peripheral vascular disease, preoperative use of an intra-aortic balloon pump, chronic obstructive pulmonary disease, the need for emergent surgery, low preoperative Hb levels and an elevated preoperative serum creatinine (Rosner & Okusa, 2006). Investigation of the patient cohort who developed AKI found preoperative factors including diabetes and congestive heart failure were more frequent in patients who developed AKI, these preoperative risk factors however failed to reach statistical significance in this small patient cohort (Table 5.4.1).

Circulating microRNA-210 levels were increased in acute kidney injury

The expression level of circulating plasma miR-210 was significantly increased by 100% in the immediate postoperative period in patients who developed AKI following cardiac surgery with CPB. In patients who did not develop AKI a significant increase was also observed in the immediate postoperative period however the increase was less marked. Surprisingly a significant decrease in miR-210 levels was observed on initiation of CPB in patients who did not develop AKI. This may be a reflection of the increased volume of administration of crystalloid fluid and consequent level of haemodilution in the patient

cohort. Patients who did not develop postoperative AKI received on average 2900 mL of fluid intraoperatively compared to 2400 mL administered to patients who developed AKI. Other studies have reported increased postoperative levels of circulating miR-210 in patients with AKI following cardiac surgery, suggesting the role of miR-210 as a novel biomarker for the development of AKI demonstrating earlier detection than by serum creatinine levels (Aguado-Fraile *et al.*, 2015; Lorenzen *et al.*, 2011).

In patients who did develop AKI, miR-16 levels did increase by 125% from preoperative levels when measured on arrival to the ICU (0 hr), however the result did not reach significance. There were no other significant differences in miR-16 levels observed in patients who did or did not develop AKI.

Low preoperative haemoglobin and haemodilution increases the risk of cardiac surgery associated acute kidney injury

Patients who did develop AKI postoperatively received on average 500 mL less crystalloid fluid during the perioperative period when compared to patients who did not develop AKI postoperatively. Despite the reduced volume administered to patients who developed AKI, the patient cohort had significantly lower preoperative Hb, lower 1st Hb measured on CPB and lower nadir Hb measured on CPB than patients who did not develop AKI. Univariate analysis revealed preoperative Hb and nadir Hb measured on CPB were predictive of AKI. Multiple large studies have found a relationship between haemodilution, lower haematocrit and increased incidence of AKI (Table 1.2.2) potentially due to impairment of oxygen delivery to an already hypoxic renal medulla.

Anaemia induced tissue hypoxia may be a central mechanism of organ injury and morbidity during the acute haemodilution observed during CPB. Upregulation of hypoxia-inducible factor (HIF) dependent cellular responses have been shown to maintain oxygen haemostasis in anaemic animals undergoing acute haemodilution (Hare *et al.*, 2018). Most studies suggest a direct connection between miR-210 expression and HIF (Bavelloni *et al.*, 2017). The extent to which haemodilution can compromise renal oxygenation and contribute to

AKI is unclear. There is increasing evidence that excessive fluid administration in ICU patients is associated with increased morbidity including AKI (Ostermann *et al.*, 2015).

Plasma free haemoglobin levels correlate with microRNA-16 expression in patients with acute kidney injury

Plasma free Hb is a well-known renal toxin associated with AKI in patients undergoing CPB and has been found to be a predictor of AKI in other forms of extracorporeal support including extracorporeal membrane oxygenation (Lyu *et al.*, 2016). Adverse outcomes related to high levels of plasma free Hb can be explained by its propensity to cause direct kidney injury due to renal tubular obstruction and the enhanced nitric oxide binding association of plasma free Hb in the renal microvasculature leading to arteriolar vasoconstriction and subsequent renal tissue hypoxia (Deuel *et al.*, 2016).

Significant increases in plasma free Hb were observed at all perioperative time points in patients who developed AKI postoperatively, while increases were only observed following separation from CPB in patients who did not develop AKI. In a case control study of 10 AKI patients and 10 risk matched controls, patients who developed AKI had twice the plasma free Hb at the end of CPB than those who did not develop AKI (289.0 $\pm$ 37.8 mg/dL vs 104.4 mg/dL, $p = 0.01$) (Billings *et al.*, 2011). Cardiopulmonary bypass related haemolysis is caused by a variety of factors including mechanical shear stress within the extracorporeal circuit including roller and centrifugal pumps, turbulent passage through the oxygenator, reservoir, filters, arterial and venous cannulae, cardiotomy suction, the air to blood interface, cell salvage and transfusion (Ricci *et al.*, 2014). Due to the numerous factors contributing to its release, a degree of plasma free Hb release is expected on CPB. The significant increase observed in the AKI cohort during CPB, immediately postoperatively and on arrival to the ICU, shows a greater degree of plasma free Hb release despite no significant differences in conduct of perfusion or length of time on CPB between the two groups.

There was a strong correlation between plasma free Hb and miR-16 levels observed in the AKI cohort which was not observed in patients who did not develop AKI. This most likely

reflects the greater degree of plasma free Hb in patients who developed AKI. Following univariate analysis plasma free Hb levels was not predictive of the development of AKI.

Circulating miR-210 levels correlate with urinary oxygen content

In patients who developed AKI postoperatively, the lowest uPO_2 recorded during CPB demonstrated a significant correlation with plasma levels of miR-210 measured during CPB. Animal studies and computational models have shown medullary hypoxia occurs during and after CPB with urinary hypoxia reflecting hypoxia in the renal medulla (Sgouralis *et al.*, 2015). There is also clinical evidence that renal oxygen delivery falls during CPB and that postoperative AKI is associated with intraoperative desaturation of renal tissue (Choi *et al.*, 2014). Urinary hypoxia has been identified as an intraoperative marker of risk of AKI following cardiac surgery (Zhu *et al.*, 2018). The strong positive correlation of plasma miR-210 and uPO_2 potentially reflects inadequate perfusion of the kidney and hypoxia during the perioperative period of CPB leading to elevated miR-210 production by the kidney.

In light of the strong positive correlation of plasma miR-210 measured on CPB and uPO_2, univariate analysis was carried out to evaluate the predictive capacity of plasma miR-210 for the development of AKI. Several established risk factors predictive for AKI were identified by univariate logistic regression including preoperative Hb and minimum Hb on CPB. Whilst there was a two-fold increase in miR-210 levels during CPB in patients who developed AKI, the relationship between plasma miR-210 measured during CPB did not reach statistical significance for predicting postoperative AKI. Possibly this finding could be attributed to the small sample size in the analysis, since other known predictors of AKI, including diabetes and transfusion, also did not reach statistical significance in this patient cohort.

The observations of the study highlight the importance of intraoperative strategies to potentially optimise perfusion to patients that are anaemic and vulnerable to the effects of excessive haemodilution and transfusion during CPB. The differential expression of miRNAs observed in patients either developing or not developing AKI may indicate selective miRNAs are released during CPB and warrants further investigation of specific miRNA release during

CPB. The strong correlation of uPO_2 and plasma miR-210 during CPB is further supportive evidence of the potential role of hypoxia in the development of AKI.

5.6 Limitations

This study is limited by the modest patient numbers in each cohort. The study was observational and carried out at a single centre. The miRNA studies reported in this chapter were measured in samples taken for a separate study being undertaken at MMC. MiRNA studies were carried out on plasma and urine samples previously collected and stored at -80°C.

5.7 Conclusions

The results of this study show that levels of circulating and urinary miR-210 are differentially expressed in patients who develop AKI following cardiac surgery with CPB. MiR-16 levels and their correlation with haemolysis assessed by plasma free Hb, showed a differential profile between patients who developed AKI postoperatively and patients who did not. High circulating miR-210 levels measured during CPB in patients who develop AKI postoperatively are also significantly correlated to low urinary oxygen tension levels.

Major findings:

- Higher levels of circulating and urinary miR-210 and miR-16 expressed in patients who develop CSA-AKI
- In patients who develop CSA-AKI, miR-16 levels correlate with level of haemolysis assessed by plasma free Hb
- During CPB, levels of circulating miR-210 are significantly correlated with uPO_2 in patients who develop CSA-AKI suggesting a specific hypoxic release of miR-210 during CPB.

The results of this chapter show preoperative Hb and minimum Hb on CPB were the most powerful independent predictors of AKI. The reduction in oxygen delivery attributed to lower Hb levels in combination with the increased levels of hypoxically induced miR-210 levels measured in the serum and urine of patients who develop AKI and the strong

correlation of miR-210 with uPO2 all provide evidence supporting the critical role of renal oxygenation in CSA-AKI.

The differential release profiles of miR-210 and miR-16 in patients who developed AKI support the suggestion that specific miRNAs are released due to the hypoxic insult attributed to CPB in a subset of vulnerable patients with an increased risk of AKI. Further investigation into the release profiles of other miRNAs is warranted to determine if there is a global release of miRNAs during CPB or if there are specific miRNAs increased during CPB predicting renal hypoxia and ultimately predicting AKI. Investigation into the tissue of origin of miRNAs specifically released during CPB may help to gain further understanding of the molecular effect of hypoxia and CPB.

CHAPTER 6: PROFILING OF CIRCULATING microRNAs IN PATIENTS UNDERGOING CARDIOPULMONARY BYPASS

6.1 Introduction

The work thus far has addressed whether miRNAs can be detected in blood and urine during and following CPB and whether hypoxically regulated miRNAs are released and correlate with markers of renal hypoxia and the development of AKI. In addition to the upregulation of specific miRNAs observed during and following CPB, a global release of small RNAs was suggested by the Bioanalyzer analysis that utilises microfluidic electrophoretic separation and fluorescent detection. The work described in this chapter utilises an independent method of miRNA profiling (high throughput qRT-PCR) capable of determining the expression of all well characterised human miRNAs. This method was selected to enable the following specific questions to be addressed:

1. Is there a generalised increase in circulating small RNAs during CPB?
2. Is there preferential release of miRNAs associated with hypoxia?
3. Are the miRNAs released during and following CPB tissue specific?

Is there generalised increase in circulating small RNA during cardiopulmonary bypass?

The results of chapter 3 and 4 have shown that expression of miR-210 and miR-16 are upregulated during CPB and into the postoperative period with levels returning to preoperative levels after 4 h. To determine if the upregulation of miR-210 and miR-16 was due to a non-specific global release of miRNAs and other classes of RNAs, analysis with the Bioanalyzer was carried out. The Bioanalyzer uses a microfluidics-based platform for sizing and quantification of small RNAs. As reported in Chapter 3, Bioanalyzer analysis of RNA extracted from serum found total concentration of small RNAs demonstrated a 10-fold mean increase during CPB, which continued into the immediate postoperative period. Small RNAs are defined according to their characteristics related to their origins, structures, associated effector proteins and biological roles. These small RNA classes include miRNAs, snRNA, tRNA, rRNA, snoRNA, siRNA and piRNAs (Zhang, 2009). An independent method of

expression profiling and analysis was carried out to determine if the release of small RNAs suggested by the Bioanalyzer measurements is indicative of a global release of miRNAs or of specific subclasses of small RNAs.

Are levels of microRNAs known to show hypoxic regulation increased during cardiopulmonary bypass?

The focused examination of the levels of a specific hypoxically regulated miRNA in Chapter 3 and 4 have shown that miR-210 levels were upregulated during CPB and into the postoperative period. The results of Chapter 5 revealed that the lowest uPO_2 recorded during CPB demonstrated a significant positive correlation with levels of plasma miR-210 measured during CPB in patients who developed CSA-AKI. Low uPO_2 has been found to be a renal marker of hypoxia and thus an independent predictor of postoperative AKI (Zhu *et al.*, 2018). Microarray-based expression profiling by Kulshreshtha et al. (2007) revealed that a specific spectrum of microRNAs, including miR-23, miR-24, miR-26, miR-27, miR-103, miR-107, miR-181, miR-210, and miR-213 are induced in response to hypoxia. Lorenzen et al. (2011) reported that circulating levels of hypoxically regulated miR-210 was upregulated in patients with AKI, including CSA-AKI. Furthermore, the authors concluded that miR-210 was a strong and independent predictor of survival in critically ill patients with AKI. Further investigation of other miRNAs utilising miRNA expression profiling was undertaken to identify if other hypoxically induced miRNAs are upregulated during CPB.

Is there release of tissue specific microRNAs during and following cardiopulmonary bypass?

Peripheral blood is the main source for clinical biomarkers because of its critical role in immune responses, metabolism, communication with cells and the extracellular matrix in almost all tissues and organs in the human body. The dynamic properties of blood give rise to the possibility that subtle changes occurring within the body, such as changes associated with a disease process or in response to an injury such as hypoxia, may leave 'fingerprints' in the blood (Taurino *et al.*, 2010). MiRNAs play a key role in cellular functions and can act as the 'fingerprints' associated with understanding disease pathogenesis and mechanisms such as ischaemia. Transcriptional profiling of miRNAs from whole blood has become a useful

tool in research and diagnosis. Profiling allows the examination of regulatory pathways affected by changes in miRNAs by comparing the level of miRNAs expressed in a given disease state or in response to severe stimuli such as ischaemia reperfusion injury (Tomic *et al.*, 2005).

Global miRNA expression changes identified by miRNA profiling have been described in numerous diseases such as heart disease, cancer and Alzheimer's disease. The finding that some miRNAs were expressed in a tissue-specific fashion was confirmed in an early study by Lagos-Quintana et al. (2001), showing that miR-1, miR-122a, and miR-124a expression was restricted to myocardium, liver and brain respectively. Further studies have continued to examine tissue-specific expression of miRNAs, including miR-1, were found to be expressed exclusively in the human heart (Ludwig *et al.*, 2016; Mitchelson & Qin, 2015). Further studies have confirmed the early work by Lagos-Qunitana et al. (2001) with tissue specific expression of miR-122a and miR-124 confirmed to be specifically expressed by the liver and brain respectively (Landgraf *et al.*, 2007; Ludwig *et al.*, 2016)

In an effort to identify miRNAs that were enriched or specifically expressed within a particular tissue, Ludwig et al. (2016) determined the distribution of miRNAs across human tissues. A tissue specific index (TSI) was designed to define the distribution of miRNAs. The TSI is a quantitative, graded scalar measure for the specificity of expression of a miRNA with respect to different organ. The values range from 0 to 1, with scores close to 0 represent miRNAs expressed in many or all tissues and scores close to 1 miRNAs expressed only one specific tissue. Specifically, the TSI for a miRNA *j* is calculated as:

$$\mathrm{tsi}_j = \frac{\sum_{i=1}^{N}(1 - x_{j,i})}{N - 1},$$

where N corresponds to the total number of tissues measured and $\chi_{j,I}$ is the expression intensity of tissue i normalized by the maximal expression of any tissue for miRNA *j*. The majority of miRNAs measured fell in a middle TSI range. MiRNAs specific for a single tissue were designated a TSI of > 0.85 while housekeeping miRNAs, found in an abundance of

tissues, a TSI of <0.5. The authors observed many different miRNA and miRNA families were predominantly expressed in certain tissues (Ludwig *et al.*, 2016).

Three principle methods are used to measure the expression levels of miRNAs including qRT-PCR, microarray and NGS. Next generation sequencing is the method of choice to discover and identify miRNAs and other small RNA subsets. The primary advantage of NGS is that it does not require prior knowledge of target miRNAs and therefore is not limited to studies of only known miRNAs. Next generation sequencing provides a vast amount of data and is currently the best platform for novel miRNA discovery. Next generation sequencing, using Illumina (Illumina, San Diego, CA, USA) technology, involves the preparation of cDNA libraries from a representation of human RNA sequences. The libraries are denatured and bound at one end to a solid surface coated with adaptor oligonucleotides. The free end of each fragment 'bends over' and hybridises to a complimentary adaptor on the surface, which initiates complimentary strand synthesis. Multiple cycles of this solid-phase amplification followed by denaturation, create clusters of over 1000 copies of single strand DNA molecules (van Dijk *et al.*, 2014).

Preliminary experiments found NGS reactions were incompatible with the serum samples collected from cardiac surgery patients (see APPENDIX G). Heparin is known to interfere with the reverse transcription process involved in library preparation for NGS (Moldovan *et al.*, 2014). Despite the successful use of heparinase prior to qRT-PCR to allow PCR amplification, cDNA could not be generated hence library preparation for NGS could not proceed (Results showing failed cDNA generation shown in APPENDIX G). Next generation sequencing uses a unique set of probes, primers and enzymatic steps to generate cDNA hence the method of cDNA generation for NGS is different to the method used to generate cDNA for qRT-PCR. An alternative method capable of interrogating the expression of small RNAs was therefore used.

The OpenArray system (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) was selected to examine the expression of multiple miRNAs simultaneously to determine if there was a miRNA signature release during CPB. The OpenArray technology is based on

high throughput TaqMan relative quantification RT-PCR, using TaqMan primers. High throughput PCR allows scaling of the PCR reaction from the microliter down to the nanolitre. Miniaturisation of the PCR dramatically reduces the required volume of reagents and samples, reducing reagent cost and conserving clinical samples. The OpenArray system utilises microscope slide-sized plates containing 3 072 through-holes 300 μm in diameter and 300 μm deep to which known miRNAs assays are bound. The advantage of high throughput qRT-PCR is that it allows the simultaneous amplification of 754 known miRNAs, however this is also a major limitation as the number of miRNAs that can be detected are biased towards the inclusion of commonly expressed miRNAs. The panel is also limited as it does not comprehensively measure other subsets of small RNAs. A limited number of small RNAs including snoRNAs, snRNA, and tRNAs are routinely included on the plate as internal controls.

Using the QuantStudio 12K Flex OpenArray Real-Time PCR System, circulating miRNA profiles were investigated to determine if there is a global release of miRNAs during and following CPB. This chapter also investigates if there is a common signature of hypoxically regulated miRNAs released during this time. In addition, highly expressed miRNAs were identified and potential sources of release investigated. Pathway analyses of the highly expressed miRNAs was conducted to gain understanding of molecular changes that occur during and following CPB. To date, this is the first study of comprehensive circulating miRNA expression profiling during CPB and the early postoperative period.

6.2 Aims and Hypotheses

Hypothesis: There is a global release of microRNAs during and after CPB.

Aim: To determine if there is a generalised release of small RNAs during and following CPB.

Hypothesis: Hypoxically regulated miRNAs are released during CPB remaining elevated in the early postoperative period.

Aim: To examine if the miRNAs preferentially released during or following CPB are hypoxically regulated.

Hypothesis: There is a release of miRNAs from specific organs and tissues during CPB.

Aim: To determine tissue of origin for highly expressed miRNAs released during and following CPB.

6.3 Methods Overview

Patient inclusion

Due to the unknown effect of CPB on miRNA levels and to maintain a homogenous cohort limiting potential gender variability in miRNA levels, ten male patients undergoing elective CABG surgery with CPB at FMC were studied (cohort C). The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee (SAC HREC SAC HREC 202.13), and written informed consent was obtained. Blood was obtained at 3 time points including a preoperative sample (prior to skin incision), 30 min following initiation of CPB and postoperatively prior to skin closure. Arterial blood (10 mL) was collected from the radial arterial catheter. For serum separation, 6 mL was aliquoted into a silicone coated blood collection tube (BD Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ).

RNA extraction from serum

RNA was extracted from serum using the miRCURY™ RNA Isolation Kits for Biofluids (Exiqon, Copenhagen, Denmark) and treated with heparinase I according to the method outline in Chapter 2 pg. 54. *(Isolation of small RNA from serum for Bioanalyzer analysis and high-throughput real time PCR).* Serum was selected to maintain consistency with samples taken from the same tertiary hospital as cohort A patients (Chapter 3). The final RNA pellet was resuspended in 6ul of RNase free water.

High-throughput qRT-PCR

The Taqman™ OpenArray™ Human Advanced MicroRNA Panel was used for high-throughput miRNA profiling (ThermoFisher Scientific, Waltham, MA, USA). Reactions were

carried out according to the standard protocol as outlined in Chapter 2 pg. 55-57. High-throughput data generated by OpenArray was analysed by Expression Suite Software version 1.0.1 (ThermoFisher Scientific, Waltham, MA, USA) as described in Chapter 2 pg. 59.

6.4 Results

The median age of male patients undergoing CABG with CPB cardiac surgery was 64, with a median time on CPB of 77.5 min and a median X clamp time of 43 min. Median ICU stay was 41.9 h and length of stay 6 days. According to the RIFLE criteria, 3 patients developed AKI postoperatively. Of the 3 patients that developed AKI, 1 patient developed RIFLE class R, 1 patient developed RIFLE class I and one patient developed RIFLE class F requiring readmission to ICU for continuous renal replacement therapy (RRT).

Table 6.4.1 Preoperative, Procedural and Outcome characteristics of the 10 patients undergoing cardiopulmonary bypass- ***Cohort C***

	n=10
Baseline	
Age, years	64 (56-72)
BMI	29.8 (34.6-22.6)
COPD	1 (10%)
Previous cardiac surgery	0
Diabetes	2 (20%)
Hypertension	8 (80%)
Congestive heart failure	0
Smoking history	7 (70%)
Ejection fraction < 30%	0
Emergency surgery	0
Cerebrovascular disease	1 (10%)
Myocardial infarction	9 (90%)
Preoperative Hb, g/L	148 (115-159)
Preoperative creatinine, µmol/L	87.5 (75-107)
Procedure	
CPB duration, min	77.5 (41-117)
X clamp time	43 (24-85)
CPB Naso temperature, min ^{O}C	34.3 (33.9-34.65)
CPB MAP, avg mmHg	62.2 (56.99-69.45)
CPB Hb, min g/L	99 (87-113)
CPB Hb, max g/L	106.5 (89-118)
CPB blood glucose, min mmol/L	6.4 (5.4-9.2)
CPB blood glucose, max mmol/L	8.1 (5.6-16.5)
Outcome	
AKI	3 (30%)
Highest post op creatinine (µmol/L)	105 (66-222)
ICU time (hr)	41.9 (20.6-125.9)
Length of stay (days)	6 (5-12)
Mortality	0

Data expressed as median and range. Baseline characteristics defined by the Australian and New Zealand Society of Cardiac and Thoracic Surgeons national database. Acute Kidney Injury (AKI) defined by RIFLE. All CPB related data accessed from Australian and New Zealand Collaborative Perfusion Registry. Body Mass Index (BMI), Chronic Obstructive Pulmonary Disease (COPD), Haemoglobin (Hb), Cardiopulmonary Bypass (CPB), Nasopharngeal (Naso), Mean Arterial Pressure (MAP), Intensive Care Unit (ICU).

MicroRNA expression profiles differ in serum measured preoperatively, during cardiopulmonary bypass and postoperatively

The expression levels of 754 human miRNAs were compared in serum samples taken preoperatively, during CPB and postoperatively. Of the 754 assays, 561 miRNAs were detectable in both the preoperative and CPB samples, 46 miRNAs were detectable in the preoperative sample only and 88 miRNAs only in the CPB sample. Fifty-nine miRNAs were undetectable in either sample. Undetectable samples were defined by Expression Suite Software v 1.1 (ThermoFisher Scientific, Waltham, MA, USA) as a Cq > 35 PCR cycles and a low amplification threshold of $\leq$ 1.2, indicating poor efficiency of the PCR reaction. The samples meeting these criteria are not included in subsequent analysis.

Of the 561 miRNAs detected, the levels of 363 miRNAs were increased during CPB and levels of 198 miRNAs were decreased during CPB when compared to the preoperative expression (Figure 6.4.1).

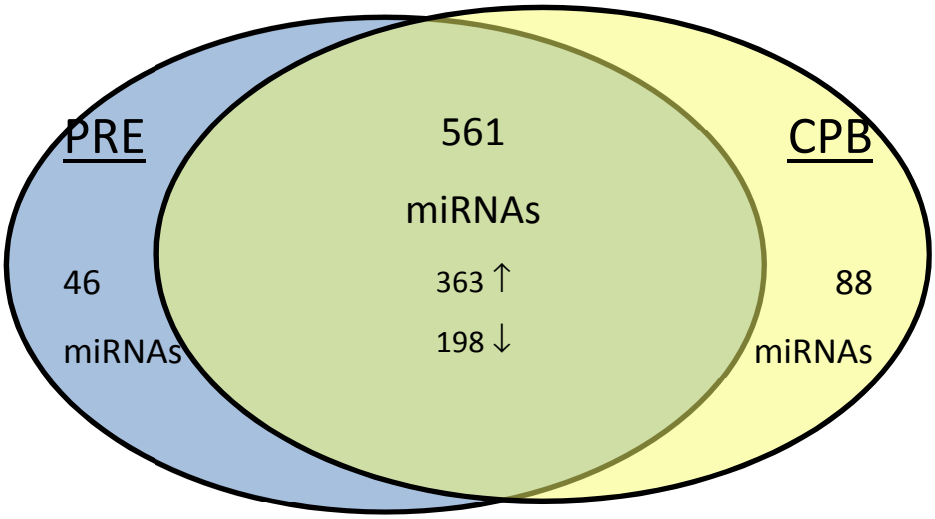


Figure 6.4.1 Venn diagram showing the number and overlap of differentially expressed miRNAs measured preoperatively (PRE) and during cardiopulmonary bypass (CPB).
The area inside the closed curve (green) represents the number of miRNAs expressed both preoperatively and during CPB and whether miRNAs levels increased (↑) or decreased (↓) during CPB when compared to preoperative levels. Region outside the curve represents miRNAs isolated to preoperative (blue) or CPB period (yellow).

In both the preoperative and postoperative samples 562 miRNAs were detectable, 48 were detected only in the preoperative sample, while 101 were isolated to the postoperative sample. Forty-three miRNAs were undetectable. When compared to preoperative levels the levels of 388 miRNAs were increased postoperatively and levels of 174 miRNAs were decreased (Figure 6.4.2).

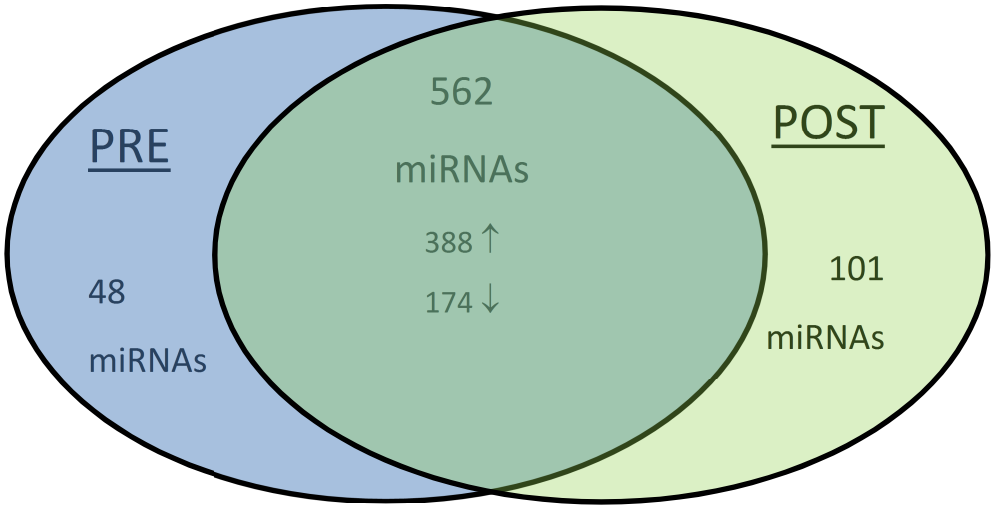


Figure 6.4.2 Venn diagram showing the number and overlap of differentially expressed miRNAs detected preoperatively (PRE) and postoperatively (POST) following cardiac surgery with cardiopulmonary bypass.

The area inside the closed curve (dark green) represents the number of miRNAs expressed both preoperatively and postoperatively and whether miRNAs levels increased (↑) or decreased (↓) postoperatively when compared to preoperative levels. Region outside the curve represents miRNAs isolated to preoperative (blue) or postoperative period (light green).

Fold change expression in microRNAs measured during cardiopulmonary bypass

For each miRNA a fold-change in expression was calculated as the ratio of the average expression in CPB and postoperative sample versus the average expression in preoperative samples (Chapter 2 pg. 58). Fold change analysis allows the determination of changes in expression levels during CPB and postoperatively when compared to baseline preoperative expression levels. For all miRNAs that were successfully amplified, there was a generalised increase during CPB with an average 1.5-fold change, with increased miRNA levels measured during CPB when compared to preoperative levels.

Analysis of the dataset showed 134 miRNAs increased expression by 0.5 to 2-fold and 73 miRNAs increased expression by 2 to 4-fold. A 5 to 10-fold change in expression was observed for 37 miRNAs. MiRNA-518b and miR-593 expression increased greater than 10-fold during CPB, this change however was not statistically significant as the levels of miR-518b and miR-593 measured preoperatively and during CPB were undetermined in 8 patients. Of the two patients where amplification of the miRNAs was detected, one of the results recorded was inconclusive due to the high Ct value (> 35 PCR cycles) and low amplification threshold (< 1.2). (See Appendix H for full list of miRNA fold changes)

Sixty-three miRNAs showed a 0.5 to 2-fold downregulation in expression during CPB, while 20 miRNAs decreased in expression 2 to 4-fold. Expression levels of 10 miRNAs decreased 5 to 10-fold during CPB while miR-575 showed the greatest fold change of > 10-fold, however the change was not statistically significant as expression was only detected in one of the 10 patients (Table 6.4.2).

Table 6.4.2 MicroRNA fold changes measured during CPB

Fold Change	**# of miRNAs UPREGULATED**	**# of miRNAs DOWNREGULATED**
>10	2	1
5-10	37	10
2-4	73	20
0.5-2	134	63

A volcano plot was used to visualise changes in the large open array data set. It plots fold-change versus significance on the x and y axes respectively. The y axis is the negative log of the p value, highly significant points with low p values appear towards the top of the plot. The log (base 2) of the fold change is plotted on the x axis. The log of the fold change is used so that changes in both directions become equidistant from the centre. The larger the magnitude in fold change in expression of a miRNA, the more extreme its point will lie on

the x axis. The more significant the difference, the smaller the p-value and thus the higher the point will lie on the y axis. Analysis showed 9 miRNAs are significantly upregulated during CPB and one miRNA was significantly downregulated (Figure 6.4.1).

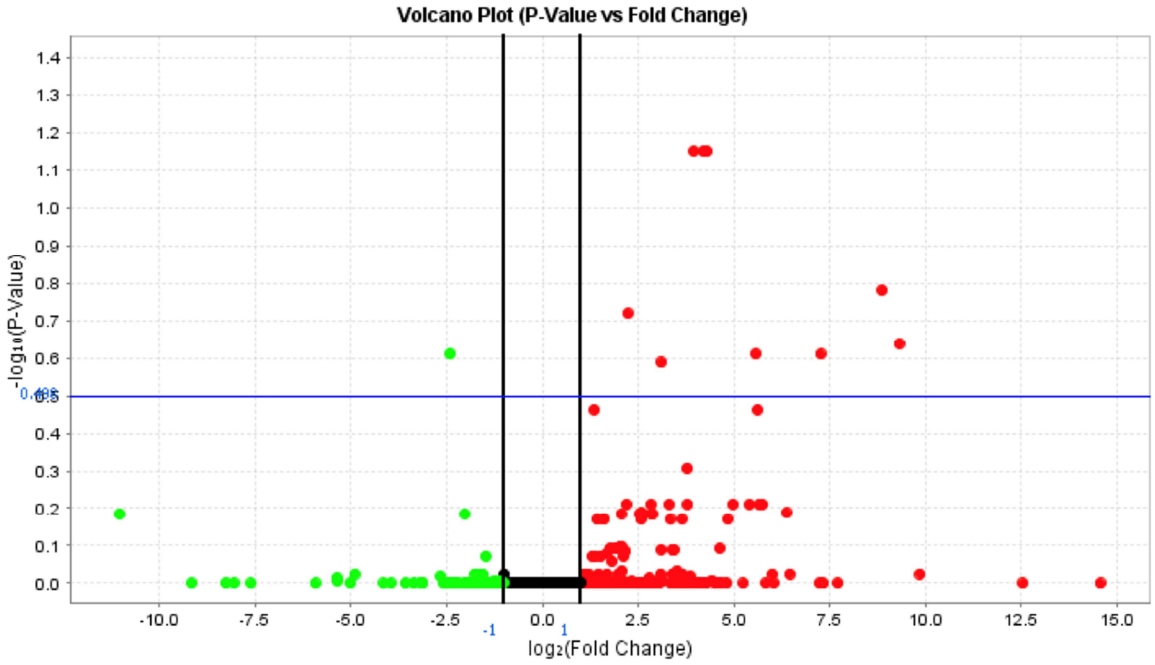


Figure 6.4.3 Volcano plot displaying Fold Change (log_2) of miRNAs in serum measured during CPB versus preoperatively.

Each dot represents an individual miRNA. The black vertical line represents a fold change boundary of 2 and blue horizontal line represents adjusted p-value for FDR for multiple comparisons where p = 0.05. Dots above the blue line having $p < 0.05$ and dots below $p > 0.05$. Black dots represent fold change less than 2. Red dots represent miRNAs with a fold change greater than 2 and an upregulation in expression during CPB. Green dots represent miRNAs with a fold change of greater than -2 and a downregulation in expression during CPB. miRNAs highly upregulated during CPB are identified in the top right quadrant and include miR-218, -1260, -1290, -520c-3p, -662, 138-2-3p, -1225-3p, -939-3p and -133a. Average fold change was 1.5.

Fold change expression in microRNAs measured postoperatively

When comparing postoperative serum miRNA levels to preoperative levels, the expression of 135 miRNAs were upregulated by 0.5 to-2 fold. Sixty-three miRNAs showed a 2 to 4-fold increase in expression postoperatively. Thirty-five miRNAs showed upregulation in expression with a fold-change of 5 to 10. For all miRNAs that were successfully amplified,

there was a generalised increase postoperatively with an average 1.2-fold change increase in miRNA levels measured during CPB when compared to preoperative levels. (See Appendix H for full list of miRNA fold changes)

Fifty-nine miRNAs showed a 0.5 to 2-fold downregulation in expression postoperatively, while 17 miRNAs expression decreased 2 to 4-fold. Expression levels of 6 miRNAs decreased 5 to 10-fold postoperatively. MiR-575 showed the greatest fold change during CPB and remained elevated in the postoperative period. However, the change in miR-575 expression was not statistically significant as levels were only detected in 1 of the 10 patients. Levels of miR-575 were undetermined in 9 patients preoperatively and during CPB (Table 6.4.3). Volcano plot analysis showed eight miRNAs were significantly upregulated postoperatively (Figure 6.4.2).

Table 6.4.3 MicroRNA fold-change measured postoperatively

Fold Change	**# of miRNAs UPREGULATED**	**# of miRNAs DOWNREGULATED**
>10	1	0
5-10	35	6
2-4	63	17
0.5-2	135	59

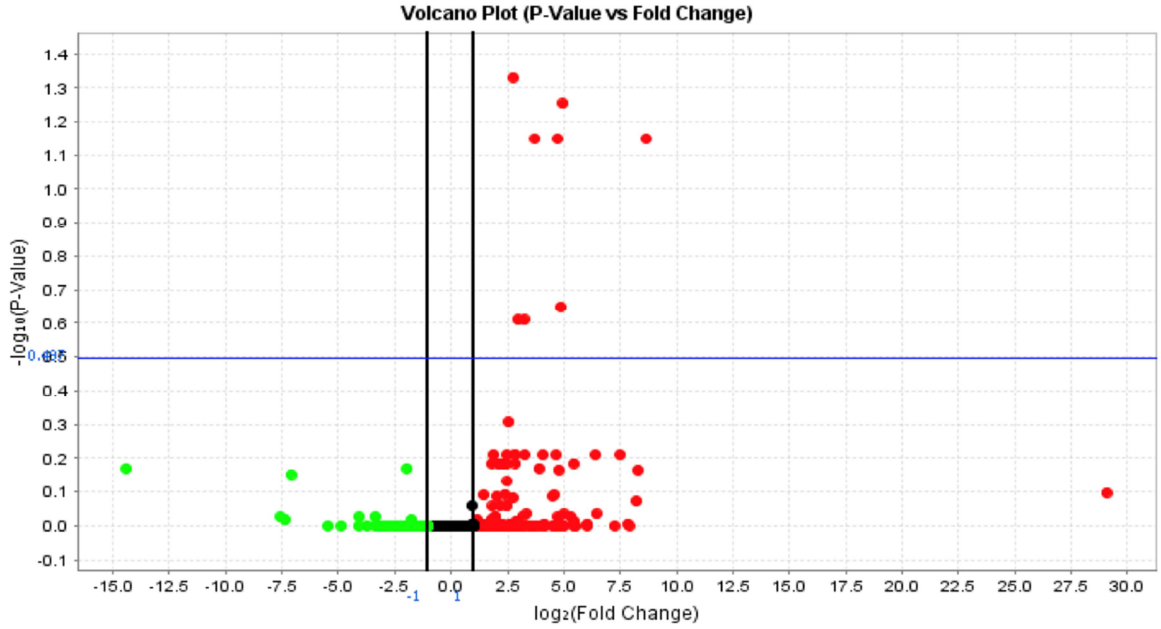


Figure 6.4.4 Volcano plot displaying Fold Change (log_2) of miRNAs in serum measured postoperatively versus preoperatively.

Each dot represents an individual miRNA. The black vertical line represents a fold change boundary of 2 and blue horizontal line represents adjusted p- value for FDR for multiple comparisons where p = 0.05. Dots above the blue line having $p < 0.05$ and dots below $p > 0.05$. Black dots represent fold change less than 2. Red dots represent miRNAs with a fold change greater than 2 and an upregulation in expression postoperatively. Green dots represent miRNAs with a fold change of greater than 2 and a downregulation in expression postoperatively. miRNAs highly upregulated postoperatively are identified in the top right quadrant and include miR-218, -143, -133a, 133b, -1259, -1-3p, -31, and -378-3p. Average fold change was 1.2.

Different miRNAs are significantly increased during and following cardiopulmonary bypass

The changes in miRNA levels measured during CPB and postoperatively were considered to be significant when compared to preoperative levels if the fold change was ≥ 2 and adjusted p-value for FDR for multiple comparisons was $p < 0.05$. Nine miRNAs were significantly upregulated during CPB including miR-218, miR-1260, miR-1290, miR-520c-3p, miR-662, miR-138-2-3p, miR-1225-3p, miR-939-3p and miR-133a. MiR-218 and miR-133a continued to be significantly upregulated in the postoperative period together with miR-143, miR-133b, miR-1, miR-31 and miR-378a-3p. The miRNA sequence of miR-1259 overlaps an annotated snoRNA, snoRNA12. SnoRNAs are a class of small RNAs that primarily guide chemical modifications of other classes of RNAs including rRNAs and tRNAs (Skreka *et al.*, 2012). As a snoRNA, miR-1259 was not included for further examination. Levels of miR-98 were

significantly downregulated during CPB. While miR-98 was detected in 7 patients preoperatively, levels were undetermined due to undetectable levels in 8 patients during CPB. Due to the number of undetermined samples, the changes in miR-98 levels were not significant and were also not included for further analysis.

There were two types of change in expression during and following CPB, those miRNAs which increased during CPB and remained increased and those that reached their peak level postoperatively. Expression levels of 7 miRNAs, including miR-662, miR-1260, miR-1290, miR-520c, miR-138, miR-1225, miR-939, peaked during CPB and remained elevated above preoperative levels following CPB. The expression of 7 miRNAs, including miR-218, miR-133a, miR-133b, miR-143, miR-1, miR-31, miR-378, whilst increased during CPB, reached their peak expression postoperatively (Figure 6.4.3).

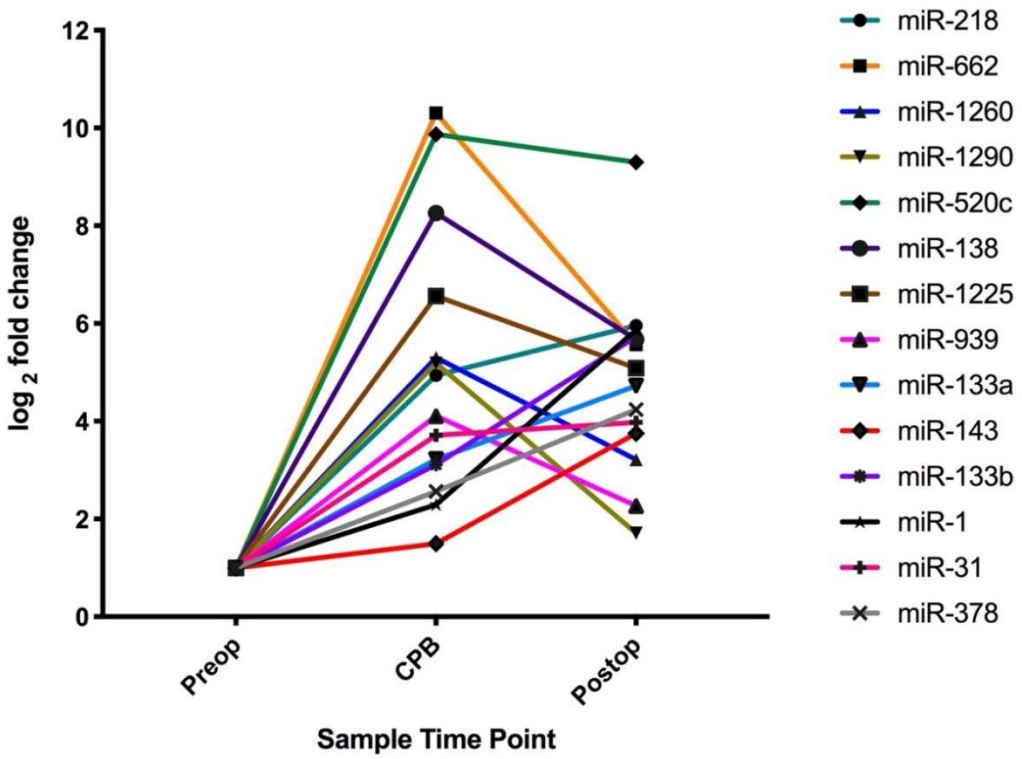


Figure 6.4.5 Fold changes of highly induced miRNAs measured during CPB and postoperatively when compared to preoperative levels.
X axis represents sample time point, Y axis represents log_2 fold change from preoperative levels. Expression levels of 7 miRNAs peaked during CPB and remained elevated above preoperative levels following CPB. The expression of 7 miRNAs, whilst increased during CPB, reached their peak expression postoperatively.

Analysis of small RNAs

Analysis of the limited number of other small RNAs available on the OpenArray Panel including snRNA, snoRNA, rRNA and tRNA established that these representatives of other RNA subsets were not significantly induced during or following CPB. The snRNA, U6 was detected in all patients during and following CPB however upregulation from preoperative levels was less than 0.5 and was not considered significant. The presence of snoRNAs including miR-1201, RNU 44, RNU 48 and miR-1259 were undetermined with no significant amplification threshold reached during or following CPB. The ribosomal RNA, miR-1826 was also undetermined in all samples with amplification levels below $\leq$ 1.2. Transfer RNA represented by miR-1274a was detected in all patients however the fold change was $\leq$ 1 during and following CPB and did not reach significance. A fold change $\geq$ 2 from preoperative levels reflected a substantial change in miRNA level and was considered a significant fold change for subsequent analysis.

Tissue of origin and functional role of highly expressed microRNAs

Investigation of the significantly upregulated miRNAs during and following CPB, using Tissue Atlas and the NCBI gene database, found highly upregulated miRNAs had a high TSI for the heart, central nervous system and systemic inflammatory response (Figure 6.4.4). Investigation of cell signalling pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis discovered the highly expressed miRNAs influence pathways including the PI3K/Akt/mTOR, an intracellular signal transduction pathway that promotes metabolism, proliferation, cell survival, growth and angiogenesis in response to extracellular signals (Kanehisa *et al.*, 2019). Many of these miRNAs have also been shown to be upregulated by hypoxia and inflammation (Table 6.4.4).

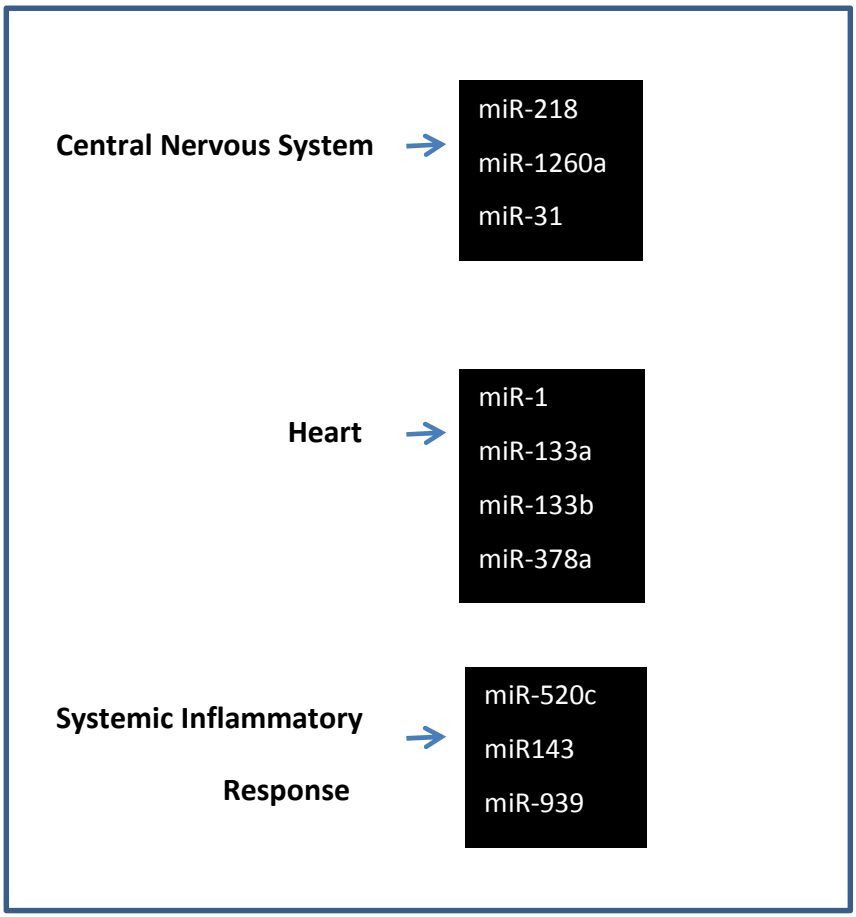


Figure 6.4.6 Tissue of origin of highly upregulated miRNAs measured during and following CPB.

Highly expressed miRNAs during and following CPB. These may be specific for release from the myocardium, central nervous system or due to induction of the systemic inflammatory response.

Table 6.4.4 Fourteen highly upregulated microRNAs expressed during cardiopulmonary bypass and postoperatively. Timing of upregulation, significance of fold change, potential tissue of origin of release and functional annotation.

miRNA	Timing of upregulation	Fold change increase	p value	# of patients upregulated	Tissue of origin	Tissue Specificity Index	Functional Annotation
hsa-miR-218	CPB Post	3.941 4.951	< 0.0001 < 0.0001	9 10	Brain Spinal Cord	0.92	* Upregulation under hypoxic conditions(Fang *et al.*, 2017) * Upregulation in renal cell carcinoma – promotion of HIF pathway (Zhang *et al.*, 2016) * Protective role in inflammation and COPD-increased expression levels in bronchial airway epithelium (Conickx *et al.*, 2017) * Upregulation during propofol administration (Xu *et al.*, 2015)
hsa-miR-133a	CPB Post	2.223 3.723	0.001 < 0.0001	10 10	Muscle Myocardium	0.95	* Downregulation in cardiac fibrosis (Renaud *et al.*, 2015) * Marker for MI and coronary artery stenosis (Wang *et al.*, 2013) * Intercellular communication in heart failure (Bang *et al.*, 2015) * Upregulation suppresses apoptosis in cardiomyocytes (Li *et al.*, 2015) * As a marker of hypertension (Parthenakis *et al.*, 2016)

hsa-miR1260a	CPB	4.299	< 0.0001	10	Brain Spinal cord Nerve	0.85	* Increased expression in abdominal aortic aneurysmal tissue – functional role in vascular smooth muscle formation (Cheuk & Cheng, 2014)
hsa-miR-1290	CPB	4.187	< 0.0001	10	Colon Kidney Lung	0.78	* Knockdown studies – inhibited differentiation and induced proliferation, over expression – slowing of cell cycle, change in expression levels of crucial cell cycle proteins (Yelamanchili *et al.*, 2014) * Upregulation postponed cytokinesis and activated the Wnt signaling pathway (Wu et al)
hsa-miR-520c-3p	CPB	8.872	0.001	7	Spleen Liver	0.51	* Increased expression – reduced IL-8 expression – role in inflammation (Tang *et al.*, 2017)
hsa-miR-662	CPB	9.306	0.002	10	Brain Kidney Lymph node	0.33	* Increased expression in serum of patients with congestive heart failure (Cakmak *et al.*, 2015)
hsa-miR-138-2-3p	CPB	7.257	0.003	8	Brain Kidney Stomach	0.32	* Induced by hypoxia – mediator of endothelial cell dysfunction. HIF1-α activation is required for hypoxia-induced expression of miR-138 (Sen *et al.*, 2013) * Overexpression inhibited hypoxia-induced endothelial cell proliferation and induced cell cycle arrest at the G1 stage (Zhou *et al.*, 2017)
hsa-miR-1225-3p	CPB	5.563	0.003	9	Kidney Stomach Small intestine	0.78	* Overexpression inhibits cell proliferation via repression of insulin receptor substrate 1 (Zheng *et al.*, 2016) * Regulates expression of markers of adult renal progenitor cells including CD133 and PAX2 and important genes in repair mechanisms of renal progenitor cells (Sallustio *et al.*, 2013)

hsa-miR-939	CPB	3.106	0.003	7	Spleen Kidney Stomach	0.52	* Down regulated in patient with complex pain syndrome. Targets proinflammatory genes including IL-6, TNFα and nitric oxide synthase 2. In vitro studies - overexpression results in decreased IL-6, TNFα and nitric oxide synthase 2. Pathway analysis – plays a critical role in regulation of inflammatory mediators (McDonald *et al.*, 2016) * Role in angiogenesis. Downregulated in patients with CAD with a sufficient coronary collateral circulation compared a poor coronary collateral circulation (Hou *et al.*, 2017) * Downregulates CD2-associated protein which plays an essential role in maintaining podocyte integrity and reducing proteinuria) (Huang *et al.*, 2016)
hsa-miR-143	Post	2.750	< 0.0001	9	Nerve Bladder Colon	0.81	* Cell signaling pathway, response to oxidative stress (Gomes *et al.*, 2018) * Key role in vascular smooth muscle hemostasis – down regulation following restenosis (Yu *et al.*, 2017) * Decreased levels in asthma patients – suppress proliferation and protein deposition in bronchial smooth muscle (Cheng *et al.*, 2016) * Serum expression increased in patients with SIRS (Han *et al.*, 2016)
hsa-miR-133b	Post	4.732	< 0.0001	9	Myocardium Muscle	0.96	* Largely released from the heart – plasma levels correlate with myocardial expression (Garcia *et al.*, 2013) * Overexpression inhibits cardiomyocyte injuries caused by viral myocarditis, inhibits release of cytokines TNFα and IL-6 (Zhang *et al.*, 2017)

							* Targets β-adrenergic receptors on myocardium, reducing signaling (Mitchelson & Qin, 2015) * Overexpression suppressed PI3K, Akt and TOR signaling pathways (Wang *et al.*, 2017) * Suppresses key factors of angiogenesis (Soufi-Zomorrod *et al.*, 2016)
hsa-miR-1259	Post	8.657	< 0.0001	7			* Also known as small nucleolar RNA – subclass of small non-coding RNAs (miR sequence for miR1259 overlaps) * Included in open array for normalization but it has been reported that snoRNA expression is as variable as miRNA expression in numerous disease states (Gee *et al.*, 2011; Warner *et al.*, 2018).
hsa-miR-1	Post	4.866	0.002	8	Myocardium Muscle	0.97	* Elevated plasma levels predict heart failure following MI (Zhang *et al.*, 2013) * Regulatory role in arrhythmias – affects cardiac conduction, repolarisation, calcium channel activity (Liao *et al.*, 2016) * Serum and urine levels of miR-1 increased following CPB- changes induced by CPB or myocardial damage (Zhou *et al.*, 2013) * In vitro – strongly downregulated in hypoxia. Downregulation due to hypoxia leads to enhanced cell proliferation. Pulmonary artery hypertension – overexpression reduces pulmonary vasculature remodeling (Sysol *et al.*, 2018)
hsa-miR-31	Post	2.976	0.038	9	Brain Spinal Cord Thyroid	0.48	* Activates the HIF pathway by targeting FIH, an inhibitor of HIF pathway. HIF normally activated under hypoxic conditions but mediated FIH takes

							place under normoxia (Liu *et al.*, 2010) * Increased expression, decreased neutrophil adhesion to endothelial cells, feedback control of inflammation (Suarez *et al.*, 2010)
hsa-miR-378a-3p	Post	3.237	0.049	7	Muscle Myocardium Bladder Colon	0.80	* Patients with aortic stenosis had lower levels of miR-133, miR-1 and miR-378, is an independent predictor for left ventricular hypertrophy and regulates MAPK signaling pathway (Chen *et al.*, 2014) * Promotes cardiac repair following MI, upregulation promotes angiogenesis, novel endogenous repair mechanism activated in acute MI (Templin *et al.*, 2017)

Expression of microRNAs associated with red blood cells, platelets and acute kidney injury

Due to the increase in small RNAs observed during and following CPB, miRNAs enriched in RBC and platelets were investigated as possible sources of general miRNA release (Kirschner *et al.*, 2011). Expression levels of miRNAs specific for RBC including miR-451, miR-92a and, miR-486 were not significantly altered during or following CPB. There was a 2-fold increase in miR-16 postoperatively, consistent with the results of Chapter 3, however the increase was not statistically significant.

MiRNAs associated with AKI, determined from prior studies, were also investigated. A 4-fold increase in miR-494 was observed during CPB and a 5-fold increase in miR-155 was observed during the same period. The results however were not statistically significant due to patient variability with only 6 of the 10 patients showing expression of these miRNAs and due to the small number of patients in the cohort with 3 of the 10 patients developing AKI. A two-fold induction of miR-210 was observed during CPB which continued into the immediate postoperative period with a 2.7-fold induction observed following CPB (Table 6.4.5). These results are in keeping with the changes observed in Chapters 3, 4 and 5.

Table 6.4.5 Fold changes of microRNAs specific for red blood cells, platelets and acute kidney injury during and following cardiopulmonary bypass

	CPB vs Preop fold change	**Post op vs Preop fold change**
RBC		
miR-451	-1.2	0.44
miR-16	1.39	2.33
miR-92a	-0.13	0.26
miR-486-5p	0.96	0.91
Platelets		
miR-126	0.71	0.03
miR-223	-0.33	-0.49
miR-339	0.88	0.07
AKI		
miR-21	0.08	2.43
miR-210	2.10	2.73
miR-192	1.64	2.36
miR-205	2.82	2.48
miR-494	4.05	2.98
miR-155	5.73	1.30
miR-146a	1.73	1.61

MicroRNA expression and pathway analysis

Target prediction analysis of the most highly expressed miRNAs was performed using the DNA Intelligent Analysis (DIANA)-mirPath v 2.0. DIANA-miRPath v2 utilises miRNA targets predicted with high accuracy based on DIANA-microT-CDS and/or experimentally verified targets from TarBase v8 (Karagkouni *et al.*, 2018). Following exclusion of cancer–related pathways, the highly expressed miRNAs upregulated during CPB, were notably enriched in 7 miRNA-mediated KEGG pathways (Kanehisa *et al.*, 2019) (Table 6.4.6). Ten pathways, and their most significantly predicted gene targets, were identified by the upregulated miRNAs expressed following CPB (Table 6.4.7).

Kyoto Encyclopedia of Genes and Genomes pathway analysis of the highly expressed miRNAs including miR-1290, miR-143, miR-138, miR- 520c and miR-133b, were found to regulate gene expression of the hippo, PI3K-Akt, AMPK and p53 signalling pathways are primarily upregulated during and following CPB. These pathways play a key role in cellular signalling and processes including the response to cellular stress. Interestingly, the HIF-1 signalling pathway was found to be significantly affected by 7 of the 14 highly abundant miRNAs during and following CPB. Seven of the highly expressed miRNAs including miR-218, miR-1260, miR-1290, miR-143, miR-133b, miR-31, miR-378 regulate expression of 31 genes in the HIF pathway (Figure 6.4.5).

Table 6.4.6 Highly expressed microRNAs during cardiopulmonary bypass and their associated microRNA-mediated KEGG pathways

KEGG pathway	p-value	# genes	#miRNAs
Hippo signalling	0.00003	41	6
AMPK signalling	0.00009	33	5
p53 signalling	0.0003	22	5
ErbB signalling	0.006	18	5
Wnt signalling	0.02	30	5
Insulin signalling	0.02	31	6
HIF-1 signalling	0.03	23	3

Table 6.4.7 Highly expressed miRNAs following CPB and their associated miRNA-mediated KEGG pathways

KEGG pathway	p-value	# genes	#miRNAs
p53 signalling	0.000001	31	5
Hippo signalling	0.00003	40	4
Cell cycle	0.00004	42	5
mRNA surveillance	0.0007	31	5
Arrhythmogenic right ventricular cardiomyopathy	0.001	16	3
mTOR signaling	0.002	21	5
PI3K-Akt signalling	0.003.	79	5
HIF-1 signalling	0.005	31	5
AMPK signalling	0.006	37	5
Adrenergic signalling in cardiomyocytes	0.04	28	5

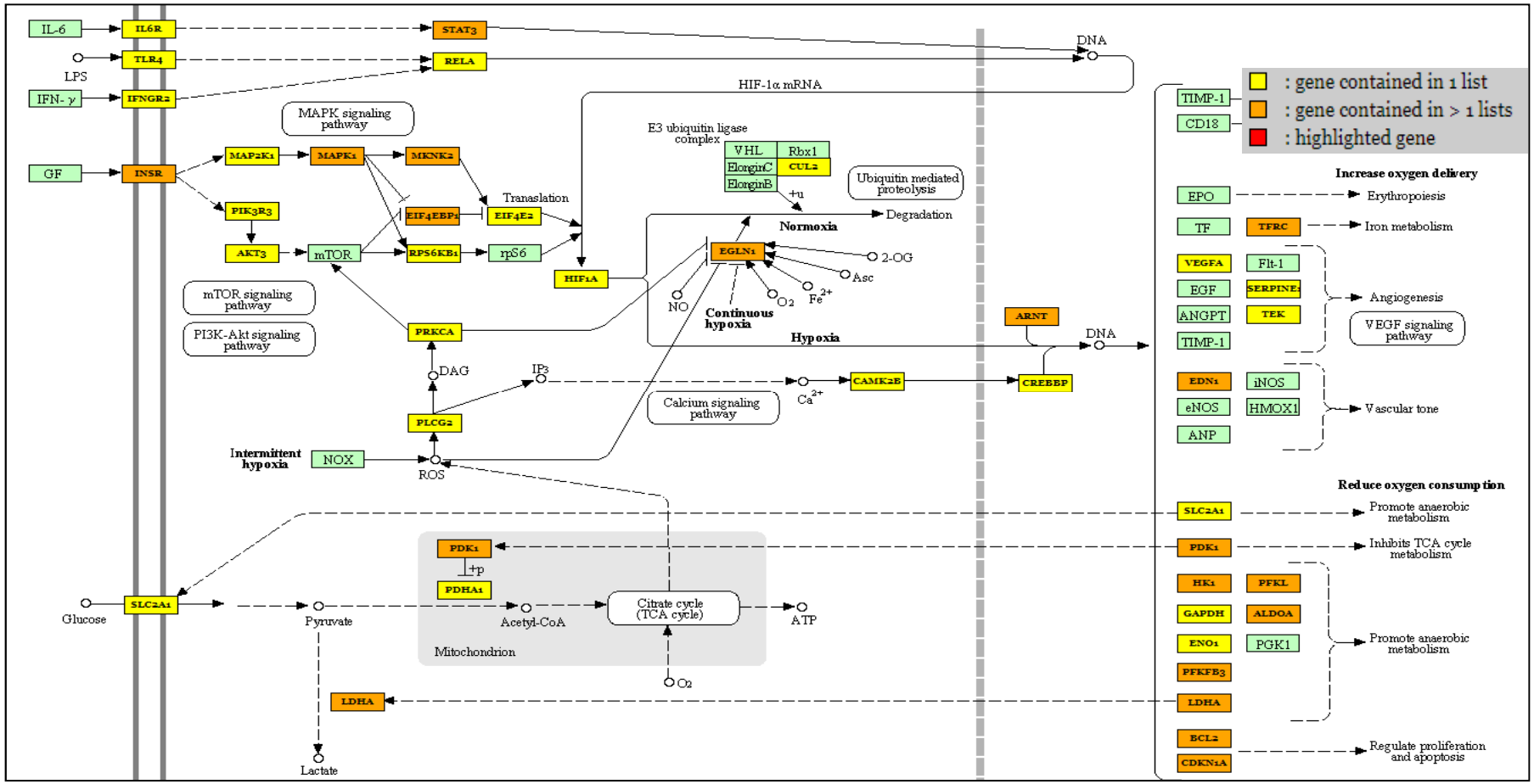


Figure 6.4.7 MiRNAs upregulated during and following CPB and their effect on the HIF signalling pathway.

Investigation using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis discovered thirty-one genes in the hypoxia inducible factor signalling pathway are affected by 7 of the significantly increased miRNAs measured during and following cardiopulmonary bypass. Genes affected by one miRNA are highlighted in yellow, genes affected by more than one miRNA are highlighted in orange (Kanehisa et al., 2019). Reprinted with permission from the authors.

6.5 Discussion

The results of this chapter provide novel evidence of differential miRNA expression profiles during and following CPB. The result of this chapter also confirms the results of Chapter 3 and 4 that miR-210 levels are increased during and following CPB. Examination of the differentially expressed miRNAs suggest the heart may potentially be the source of release of the most abundantly circulating miRNAs. Remarkably, many of the highly expressed miRNAs measured during and following CPB are upregulated in response to hypoxia.

Serum microRNA are differentially expressed in patients during and following cardiopulmonary bypass

With the use of OpenArray technology, the expression levels of 754 miRNAs were investigated in serum samples collected from 10 male patients undergoing CABG. Samples taken preoperatively, during CPB and immediately postoperatively demonstrated 561 circulating miRNAs were differentially expressed during CPB and 562 miRNAs were differentially expressed postoperatively when compared to preoperative levels. The magnitude of change differed among miRNAs.

To investigate gender and sample time-related variations, Mooney et al. (2015) used the Open Array QuantStudio 12K Flex to profile plasma microRNA levels in male and female healthy volunteers. Inspection of the circulating miRNA profiles revealed that the vast majority of miRNAs were stable across a variety of sample time points and between male and female individuals. The authors concluded that the OpenArray platform was suitable for circulating miRNA profiling and biomarker discovery (Mooney *et al.*, 2015). While NGS is the preferred method for discovery of novel miRNAs, high throughput qRT-PCR is a highly specific and sensitive method of miRNA expression profiling. The major limitation for high throughput qRT-PCR is that it does not allow for unbiased miRNA discovery as the miRNAs investigated are preselected.

Levels of other small RNAs were also investigated including a limited number of snRNAs, snoRNA, rRNA, and tRNA. SnRNAs including U6 and snoRNAs including RNU 44 and RNU48 are included on the OpenArray panel to act as internal controls. There were no significant

differences in expression levels of these representative small RNAs measured during or following CPB when compared to preoperatively levels. The release of miRNAs observed following high throughput qRT-PCR may help identify the dense band observed at the miRNA nucleotide length following Bioanalyzer analysis. The dense band observed following Bioanalyzer analysis could also represent tRNA fragments (10-45 nt lengths) which can be increased under hypoxic conditions including AKI. Periods of oxidative stress, including hypoxia, induces a direct conformational change in tRNA structure that promotes subsequent tRNA fragmentation reflecting cell damage (Mishima *et al.*, 2014). However, due to the limited number of other small RNAs assays available on the OpenArray plates, the upregulation of other small RNAs, including tRNA fragments could not be confirmed. Further investigations could be carried to help identify other small RNA subsets released during CPB. Other methods of deep sequencing exist and preliminary experiments need to be carried out to determine which methods are viable for small RNA determination during CPB.

Plausible mechanisms for the generalised 1.5-fold upregulation of miRNA observed during and following CPB include miRNA release from RBC due to haemolysis, platelet activation or consumption, SIRS or a global effect caused by hypoxia and the insult of the surgical procedure. Levels of platelet rich miRNAs including miR-223 were found to be upregulated in the circulation of patients receiving anti-platelet therapy for acute coronary syndrome. A study by Kaudewitz et al. (2016) of 125 patients with a history of acute coronary syndrome reported levels of platelet reactivity were altered in patients on anti-platelet therapy. Levels of miR-126 were upregulated in patients receiving anti-platelet therapy with miR-126 directly and indirectly affecting platelet receptor expression and platelet aggregation (Kaudewitz *et al.*, 2016). These studies highlight possible mechanism of generalised miRNA release during CPB associated with haemolysis and dysregulation of platelet function as investigation into miRNAs highly expressed by RBCs and platelets found there was no significant upregulation of these miRNAs during and following CPB. Further investigation of the most significantly upregulated miRNAs was carried out to determine their potential functional role and tissue of origin.

Hypoxically regulated microRNAs are preferentially increased during and following cardiopulmonary bypass

The mechanisms which determine the level of circulating miRNAs under physiological conditions and in response to pathological stimuli remains to be elucidated and several possibilities should be taken into consideration. The levels of circulating miRNAs may be released from apoptosing cells following disruption of the plasma membrane, release from cells exposed to stressful conditions including hypoxia, modulation of miRNAs synthesis and degradation processes which occur in the cell and uptake of circulating miRNAs by a recipient cell (D' Alessandra *et al.*, 2010).

Hypoxia triggers a distinct program of responses aimed at relieving tissue hypoxia and removing irreversibly damaged cells. These responses include endothelial cell proliferation, migration, and angiogenesis and also growth arrest and apoptotic cell death (Semenza, 2010b). A study by King et al (2012) reported that hypoxia enhanced the release of exosomes containing elevated levels of miR-210 in breast cancer cell lines exposed to moderate (1% O_2) or severe (0.1%) hypoxia. The authors suggested that the process may be mediated by the HIF oxygen sensing pathway with exosomal miRNAs promoting hypoxic signaling. The preferential release of hypoxically regulated miRNAs during and following CPB may be in response to the pathological stimuli induced by hypoxia during CPB or due to hypoxically triggered exosomal release.

Further examination of the miRNAs that met the selection criteria ($\log_2$ fold change ≥ 2, FDR $P \leq 0.05$) identified 14 miRNAs that were significantly upregulated in the circulation during and following CPB. Investigation of these 14 miRNAs significantly upregulated during and following CPB, using the resources of the Gene database (National Centre for Biotechnology Information) discovered the upregulated miRNAs are primarily associated with release by hypoxia and inflammation.

Expression levels of miRNAs, including miR-1 miR-218 and miR-138, were found to be significantly upregulated during and following CPB. These miRNAs are reported to be significantly upregulated under hypoxic conditions. Sen et al. (2013) reported that hypoxia-

induced miR-138 targets S100 calcium-binding protein A1, a calcium-binding protein highly expressed in cardiomyocytes where it is vital for orchestrating calcium ion fluxes required for optimal excitation-contraction coupling. Expression of miR-138 regulates S100 calcium-binding protein A1 in a hypoxia dependent manner.

In vitro experiments carried out by Fang et al. (2017) reported that hypoxia significantly increased the expression level of miR-218. Knockdown of HIF-1α by RNA interference using siRNAs significantly decreased the expression of miR-218 indicating that hypoxia up-regulates the expression of miR-218 through HIF-1α. In a study of 20 patients undergoing cardiac surgery with CPB, Zhou et al. (2013) reported serum and urine levels of miR-1 increased following CPB. The authors suggested the release of miR-1, which strongly correlated with serum troponin I levels, may result from direct cardiac injury due to surgical trauma and manipulation of the heart or from molecular mechanisms induced by the effects of CPB. The results of this chapter confirm the findings of Zhou et al. (2013) and extend the findings by suggesting a potential molecular mechanism for the upregulation of hypoxia induced miRNAs in the circulation during and following CPB.

Other miRNAs significantly upregulated during and following CPB included miR-31, miR-520c-3p, miR-939, miR-143 and miR-133b. These miRNAs are known to regulate multiple genes playing a critical regulatory role in a network of proinflammatory mediators such as IL-6, TNF-α and nitric oxide synthase 2. A study by Han et al. (2016) reported miR-143 was elevated in the serum of patients with SIRS compared with normal controls. MiR-143 is known to influence cell signaling pathways and is involved in the cellular response to oxidative stress (Gomes *et al.*, 2018). In children with viral myocarditis it was reported by Zhang et al. the overexpression of miR-133b reduced myocardial injury, inhibiting the proliferation of cardiomyocytes and the release of cytokines TNF-α and IL-6 (Zhang *et al.*, 2017). Data from a study carried out by McDonald et al. (2016) suggested that miR-939 may regulate multiple proinflammatory genes and that downregulation of miR-939 in patients suffering complex regional pain syndrome contributes to an increase in expression of circulating levels of proinflammatory cytokines including IL-6. The upregulation of miRNAs

associated with regulating the inflammatory response may potentially reflect a protective molecular response to the SIRS elicited by CPB.

MicroRNAs associated with acute kidney injury were upregulated in patients with cardiac-surgery associated acute kidney injury

MicroRNAs associated with AKI including miR-21, miR-210, miR-192, miR-205, miR-494, miR-155 and miR-146a were upregulated during and following CPB. Lorenzen et al. (2011) reported miR-210 was upregulated in patients with CSA-AKI and miR-210 levels predicted mortality. MiR-21 levels increase following AKI caused by ischaemia reperfusion injury with circulating levels of miR-21 shown to correlated with AKI severity, in-hospital mortality and the need for postoperative renal replacement therapy (Du *et al.*, 2013). Levels of miR-494 were found to be increased in patients with AKI, with upregulation of miR-494 contributing to the development of AKI following ischaemia reperfusion (Lan *et al.*, 2012).

The results of this study report miR-494 was upregulated 4-fold during CPB while miR-155 expression increased over 5-fold during the CPB period. MicroRNA-210 levels increased more than 2-fold during and following CPB, supporting the results of Chapter 3 where a two-fold increase in miR-210 expression was observed during and following CPB. Despite the fold changes observed, the increased expression levels of the miRNAs associated with AKI were not statistically significant. Further examination of the data discovered, due to patient variability and the method used to calculate the mean Cq of each miRNA, patients who developed CSA-AKI consistently had lower Cq values than the mean, with lower Cq values translating to higher expression levels. One limitation of the Expression Suite software is that it uses the arithmetic mean of each patients Cq value to calculate a mean Cq for each miRNA. The mean used to report average Cq values therefore does not reflect individual patient variability. Due to the small sample size and small number of patients developing CSA-AKI, no conclusion can be drawn and further investigation into miRNAs associated with AKI in a larger cohort would be needed.

Levels of increased circulating microRNAs are released from the heart during and following cardiopulmonary bypass

The Human miRNA Tissue Atlas was used to find the potential source of release of the highly upregulated miRNAs. The Tissue Atlas provides knowledge of expression pattern of miRNAs in different tissue and tissues that express specific miRNAs (Ludwig *et al.,* 2016). Ludwig et al. (2016) determined the abundance of 1997 miRNAs in 61 tissue biopsies of different organs from two individuals collected post-mortem. One thousand three hundred and sixty-four miRNAs were discovered in at least one tissue and 143 were present in each tissue. Each miRNA is designated a TSI, a quantitative graded scalar measure for the specificity of expression of a miRNA with respect to different organs. TSI values range from 0 to 1 with scores close to 0 representing miRNAs expressed in many or all tissues and scores close to 1 representing miRNAs expressed in only one specific tissue and hence are tissue specific miRNAs.

Cardiopulmonary bypass elicits many complex changes during the perioperative period due to alterations in blood flow and exposure of blood constituents to the extracorporeal circuit. Non-physiological flow to major organs, haemolysis of RBC and destruction of platelets are potential sources of generalised miRNA release during CPB. Results of this study discovered the most significantly upregulated miRNAs during and following CPB including, miR-1, miR-133a, and miR-133b, are primarily released by the myocardium. As reported by Tissue Atlas, miR-1, miR-133a and miR-133b all have a TSI of greater than 95%. The high TSI of miR-1, miR-133a and miR-133b indicate the increased levels measured in the circulation during and following CPB may reflect increased release of these miRNAs from the myocardium.

The expression of miRNAs specific to skeletal and cardiac muscle are commonly referred to as myomiRs. MyomiRs, include miR-1, miR 133a, miR-133b, miR-206 and miR-499, are involved in the control of muscle homeostasis by coordinating both myoblast proliferation and differentiation (Coenen-Stass *et al.*, 2016). Circulating levels of tissue-specific myomiRs have been found to be increased patients with CAD and following MI. Wang et al. (2013) reported that miR-133a is released into the peripheral circulation from injured myocardium, with circulating miR-133a levels positively correlating with the severity of coronary artery

stenosis. In a mouse model it was found that changes in plasma level of miRNAs were associated with reciprocal changes in their cardiac expression, miR-1, miR-133a, miR-133b and miR-499-5p (D' Alessandra *et al.*, 2010)

Garcia et al. (2013) reported miR-133b is largely released from the heart with circulating levels correlating with myocardial expression. MiR-133a, a marker for MI and degree of coronary artery stenosis, has also been identified to be involved in intercellular communication in heart failure patients (Bang *et al.*, 2015). Levels of miR-1 were also reported to be elevated in the circulation of patients following MI, with levels predicting heart failure. Zhou et al. (2013) investigated serum and urine levels of miR-1 following CPB and reported levels were increased in the circulation and urine with changes potentially induced by CPB or myocardial damage. This study reported upregulation of tissue specific miRNAs primarily released by the heart. The cause of specific miRNA release has yet to be identified but is suggestive of release from the myocardium potentially as a response to the ischaemic insult of aortic cross clamping and cardioplegic arrest.

Levels of increased circulating microRNAs are indicative of upregulation of hypoxia pathways during and immediately following cardiopulmonary bypass.

This study also investigated the potential functional role of highly expressed miRNAs and their predicted gene targets. Using DIANA-TarBase v8, a reference database devoted to the indexing of experimentally supported miRNA targets and the KEGG database, numerous cell signaling pathways, including the HIF pathway, were predicted to be regulated by the miRNAs significantly upregulated during and following CPB (Kanehisa *et al.*, 2010; Karagkouni *et al.*, 2018),

Pathway analysis and target prediction are predominately applied to and are hence biased towards cancer data sets to find driver genes and pathways, to propose cancer mechanisms and biomarkers, and to identify key regulators of cancer-related gene networks. Pathway analysis and target prediction investigation has extended beyond cancer research and is widely used for a variety of disease and pathological states in order to gain a greater understanding of the molecular mechanisms involved. The simplest pathway and network

analysis provides a high-level summary of pathways affected, whereas more complex methods provide detailed hypotheses about affected cellular mechanisms (Creixell *et al.*, 2015). The Mutation Consequences and Pathway Analysis working group of the International Cancer Genome Consortium (ICGC) has developed standard procedures for the analysis of cancer genome data generated by the ICGC (Hudson *et al.*, 2010).

Simple pathway analysis was carried out to identify networks and molecular systems that are enriched by the miRNAs significantly upregulated during and following CPB. Enrichment analysis identifies genes in pathways that are present in a gene list more frequently than expected by chance. The gene sets are collected from databases such as KEGG, enabling the generation of diagrams of enriched pathways with colours highlighting the genes of interest (see Figure 6.4.5) (Kanehisa *et al.*, 2010).

Following the exclusion of cancer biased pathways numerous cell signaling pathways were identified to be enriched by genes influenced by the highly expressed miRNAs identified in this study. The hippo and the PI3K-Akt signaling pathways have been identified to promote cardiomyocyte proliferation and survival *in vivo* (Lin *et al.*, 2015). Receptors of the ErbB signal through many pathways including the Akt pathway to regulate cellular processes of proliferation, differentiation and apoptosis (Hynes & MacDonald, 2009).The p53 network was also found to be highly regulated during and following CPB. The p53 signaling network is activated upon exposure to stressful stimuli and has a major role in the cells response to these stresses. The p53 pathway is involved in regulating apoptosis, cell cycle and DNA repair. Hypoxia has also been described to be a p53 inducer. While the direct mechanisms are not fully understood it has been shown that pVHL protein, which is a transcriptional target of HIF-1 and therefore induced under hypoxia, could enhance p53 translation (Galban *et al.*, 2003). One of the main common features of both the p53 and HIF pathway is that both are able to promote cell survival and adaptation to a mild stress as well as to induce cell death when the cell is confronted by a more severe stress (Sermeus & Michiels, 2011).

The HIF pathway, activated by low oxygen concentration, was also found to be significantly enriched during and following CPB. Of the highly expressed miRNAs, miR-218, miR-1260

and miR-1290 were upregulated during CPB, and miR-143, miR-218, miR-133b, miR-31 and miR-367 upregulated following CPB, all influence genes within the HIF pathway. The results suggest the activation of cell signaling pathways in response to cellular stress, including hypoxia, may serve a protective role to promote cell survival or an adaptation mechanism to manage the stresses of CPB by relieving hypoxia and removing irreversibly damaged cells.

6.6 Study Limitations

This study is limited by the small number of patients enrolled in the trial, and the development of AKI in 3 patients providing a heterogenous sample. The use of the Taqman OpenArray Human microRNA Panel did not allow for discovery of novel miRNAs and miRNAs assays on panel are known. The use of Expression suite software, recommended for use by the manufacturer of the OpenArray, was limited by its statistical capabilities and the generation of the mean Cq value for each miRNA. The limitation of enrichment analysis such as KEGG pathway analysis is that it does not take into account the interaction between genes and proteins and assumes that genes in the list occur independently. Despite the limitations, enrichment analysis continues to evolve and allows an understanding of the functional consequences of the altered gene set.

6.7 Conclusions

To date this is the first study to provide novel evidence of global changes in circulating miRNAs during and following CPB. This study found there is a differential release of miRNAs during and following CPB. MiRNAs significantly upregulated during and after CPB are induced by hypoxia and the inflammatory response shedding light onto the possible molecular effects of CPB. Surprisingly the highly upregulated miRNAs released during and following CPB were not due to a generalised release of miRNAs associated with platelets activation or due to RBC destruction. Examination of miRNAs with high circulating levels suggested the heart as the source of release of the most abundantly circulating miRNAs during CPB. Pathway analysis discovered numerous cell signalling pathways are enriched by the miRNAs that are increased in the circulation during and following CPB with predicted upregulation of biological pathways involving responses to cellular stress and hypoxia.

Major findings:

- There is a global release of miRNAs during and after CPB
- There is a specific release of miRNAs from the heart, CNS and SIRS during and following CPB
- Hypoxically regulated miRNAs are released during CPB remaining elevated into the early postoperative period

Future studies should involve validation of highly expressed miRNAs in a larger cohort and further investigation into release other subsets of small RNAs during CPB. Determination of the functional role and tissue of origin of highly expressed miRNAs during CPB will give further insight into the molecular effects of CPB and alteration of physiological states. A greater understanding of miRNA release during CPB will also provide further insight into effects of changes in conduct of CPB. Further investigation and validation of miRNAs associated with AKI during CPB may reveal a marker or panel of biomarkers to identify patients at risk of developing AKI postoperatively, providing an opportunity to alter the management of CPB when intervention is still feasible.

CHAPTER 7: DISCUSSION

The purpose of the work described in this thesis was to examine whether it was possible to isolate and measure circulating and urinary miRNAs during and following CPB, to determine whether there are changes in levels of miRNA during CPB and into the early postoperative period, to determine if there are specific miRNAs released during CPB, to identify whether hypoxically regulated miRNAs are released during CPB, if they are indicative of renal hypoxia and if they ultimately predict AKI.

Alterations in circulating and urinary microRNA-210 during and following cardiac surgery with cardiopulmonary bypass

The results of Chapter 3 demonstrated circulating and urinary miRNAs could be successfully isolated and measured in patients undergoing cardiac surgery with CPB (cohort A n = 10). Similarly, successful measurements were also achieved in a larger cohort of patients (cohort B n = 40) and are described in Chapter 4. The results of Chapter 3 demonstrated levels of circulating miR-210 were significantly increased during CPB. In both patient cohorts circulating levels of hypoxically regulated miR-210 were significantly increased immediately following CPB and returned to baseline levels within 4 h postoperatively. The addition of crystalloid fluid and hence the effects of haemodilution on circulating levels of miRNAs may explain the lack of significant increase in miR-210 measured during CPB in the larger cohort (cohort B). Significantly higher amounts of crystalloid fluid were administered intraoperatively to cohort B patients undergoing cardiac surgery with CPB when compared to cohort A patients.

The significant increase in miR-210 levels demonstrated immediately following CPB in this study are in keeping with those of Emanueli et al. (2016) who found plasma concentrations of miRNAs including miR-210, miR-1, miR-133a, miR-133b, miR-208a, miR-208b and miR-24 significantly increased during the perioperative period following termination of CPB and immediately after heparin reversal. These miRNAs were selected as they have previously been found to be increased in the blood acutely following MI and were investigated to determine a correlation with cardiac troponin, the current biomarker of myocardial damage.

The results of this thesis have confirmed the finding of Emanueli et al. (2016) of a significant release of hypoxically regulated miR-210 in the immediate postoperative period and have extended it to demonstrate the release of miRNAs during the CPB period.

The source of the elevated levels of miR-210 during CPB is uncertain. MiRNAs including miR-210 may be released from the heart following ischaemia and reperfusion that occurs due to aortic cross clamping and cardioplegic arrest. Cross clamping the aorta, to isolate the heart from the systemic circulation, and inducing electromechanical chemical cardioplegic arrest to stop the beating heart are known to cause a variable degree of perioperative myocardial hypoxia and injury (Hausenloy *et al.*, 2012). MiR-210 is known to be upregulated in hypoxic cardiomyocytes through Akt- and p53- dependent pathways and exerts cytoprotective effects in response to oxidative stress by potentially reducing mitochondrial reactive oxygen species production (Mutharasan *et al.*, 2011). Mechanical manipulation of the heart may also contribute to miR-210 release as demonstrated by the significant increase in miR-210 levels following positioning of the Octopus during OPCABG described in Chapter 3. The increase in miR-210 levels during and following cardiac surgery with CPB was of a greater magnitude than the miR-210 release observed during OPCABG suggesting miR-210 release is not solely due to mechanical disruption and grafting of the heart.

MiRNA-210 is a major hypoxia-induced miRNA with multiple lines of evidence of a role in the response to an ischaemic insult. *In vitro* studies have shown miR-210 is upregulated in H9c2 cells, derived from a cardiomyocyte cell line, under hypoxic conditions (Feng *et al.*, 2018). *In vivo* studies have shown miR-210 improves angiogenesis, inhibits apoptosis and improves cardiac function in a murine model of myocardial infarction (Hu *et al.*, 2010). Increased circulating levels of miR-210 have been also reported in patients with AKI following cardiac surgery. In a study of 77 patients with AKI admitted to the ICU, from medicine (n = 29), general surgery (n = 20) and following cardiac surgery with CPB (n = 28), Lorenzen et al. (2011) showed circulating miR-210 levels significantly increased 2-fold in patients with AKI when compared to healthy controls. MiR-210 levels also predicted mortality, with multivariate Cox regression analysis finding miR-210 level an independent predictor of survival. The authors suggested miR-210 may serve as a novel biomarker for

AKI, reflecting pathophysiological changes on a cellular level. The increased levels of miR-210 levels demonstrated here during and following CPB may be indicative of the hypoxic/ischaemic insult during CPB not only on the myocardium but also on the kidneys, supporting miR-210 as a potential marker of AKI.

There has been very limited study of the changes in urinary miRNAs following cardiac surgery with CPB. A study by Zhou et al. (2013) measured levels of miR-1 in both serum and urine of patients undergoing cardiac surgery with CPB and reported that urinary levels of miR-1 were 50% lower than serum levels. Studies into other clinical disease states such as idiopathic nephrotic syndrome also reported lower levels of miRNAs measured in urine when compared to serum. Levels of urinary and circulating miRNAs also differed depending on the miRNA being measured (Luo *et al.*, 2013). The results of chapter 3 and 4 are consistent with studies in the literature with mean concentrations of miRNAs measured in urine detectable but at substantially lower concentrations than in serum and plasma.

The discovery of novel urinary miRNAs during CPB may help identify patients at risk of developing AKI as unique urinary miRNA profiles have been found to be associated with different renal diseases. To date, several small clinical studies have found a correlation between the urinary expression of miRNAs and clinical and histological parameters in numerous kidney diseases including diabetic nephropathy and lupus nephritis (Argyropoulos *et al.*, 2013; Guan *et al.*, 2012). MiR-210 levels have been investigated as a potential biomarker in tumours of the urinary tract including transitional and renal cell carcinoma. Following NGS of urine samples from patients with transitional cell carcinoma, a statistically significant difference was found for miR-210 levels. Urine levels of miR-210 were reported to be six-fold higher in patients with transitional cell carcinoma when compared to healthy controls. The increased urinary miR-210 levels were reported to be attributed to hypoxia (Geva *et al.*, 2017).

Following correction of urinary dilution with urine creatinine in this study, a 200% increase in urinary miR-210 levels was observed during CPB in cohort A patients, with elevated levels extending into the early postoperative period. Results in cohort B patients were not as

marked though with a lesser 60% increase in miR-210 levels measured during CPB. Plausible explanations for the difference in urinary miR-210 levels reported between the two cohorts are the increased volume of fluids administered, the increased use of diuretics and a higher urine output, reflected in increased urine volume and lower urinary creatinine values, reported in cohort B patients undergoing cardiac surgery. Measurement of urinary biomarkers during the CPB is complicated due to the effects of urinary dilution. Friedrich et al. (2017) reported a significant decrease of urinary NGAL at the beginning of CPB, whereas a significant increase of urinary NGAL was seen after 120 min of CPB. The authors suggested the initial urinary NGAL decrease was probably caused by haemodilution at the induction of anaesthesia, though there was no description of CPB circuit used, prime volume or constituents. There is no consensus on the optimal amount of urine output during CPB, thus the measurement of urine output or urinary biomarkers for the development of AKI remains uncertain (Parolari *et al.*, 2012).

The results of Chapter 5 found that in patients who developed AKI, there was a 70% increase in urinary miR-210 levels measured during CPB when compared to patients who did not develop AKI. While these increases were not statistically significant, it is conceivable that the increase in urinary miR-210 levels during CPB may reflect a localised cellular response to hypoxia. Further investigation is needed in a larger cohort to determine the significance of urinary miR-210, its potential as a marker of AKI and to determine its cellular source. The issue with measurement of urinary miRNAs is that they can be shed from cells originating from the kidney or passively filtered from the circulation (Trionfini & Benigni, 2017). Future studies could investigate the source of urinary miR-210 and other miRNAs during CPB and whether they originate for the kidney or are passively filtered from the circulation.

Regardless of the biological sample used or the differences in perioperative procedures, differential release of circulating miR-210 were demonstrated during and immediately following CPB with return to baseline levels in the early postoperative period. To determine if the increased miRNA levels observed during and following CPB were due to a global unspecific release of miRNAs Bioanalyzer analysis was carried out. Bioanalyzer analysis

reported in Chapter 3 found the total concentration of small RNAs increased 10-fold during CPB, which continued into the immediate postoperative period. Due to the significant increase in small RNAs observed, further investigation with a different, independent method of expression profiling, high throughput qRT-PCR, was carried out. The results, as described in chapter 6, demonstrated there was a modest generalised increased release of miRNAs but also a release of specific miRNAs during CPB. The release of other small RNAs including tRNA fragments may have also contributed to the dense band observed on Bioanalyzer analysis, however due to the limitations of OpenArray, this could not be confirmed. Further investigation and validation into miRNAs and other small RNA subsets specifically released during CPB may provide insight into the molecular effects of CPB, potentially identifying a unique miRNA signature in response to CPB or identify markers of hypoxia or AKI during CPB.

Does microRNA-210 expression correlate with renal oxygenation during cardiopulmonary bypass?

The studies in Chapter 5 explored if miR-210 expression correlated with renal hypoxia as assessed by uPO_2, a predictive marker of AKI following CPB. Previous work from the MMC collaborators had found that the nadir uPO_2 on CPB correlated with an increased incidence of AKI post cardiac surgery (Zhu *et al.*, 2018). The results of Chapter 5 demonstrated, in patients who developed AKI postoperatively, the lowest uPO_2 recorded during CPB significantly correlated with circulating miR-210 levels measured during CPB. This is the first study to report that levels of circulating miR-210 correlated with urinary hypoxia, with urinary hypoxia reflecting hypoxia in the renal medulla. *In vivo* studies of hypoxic kidney injury found miR-210 levels were upregulated significantly in the injured kidney, verifying the involvement of miR-210 in the molecular response of the hypoxic kidney (Liu *et al.*, 2017). *In vitro* studies found miR-210 overexpression caused significant inhibition of the HIF-1α pathway with attenuation of hypoxia induced renal tubular cell apoptosis by targeting HIF-1 directly and suppressing HIF-1 pathway activation (Liu *et al.*, 2017). The results of this thesis suggest that the mechanism of increased circulating and urinary miR-210 levels is at least in part due to hypoxic induction in the kidney.

Is microRNA-210 a predictive marker of acute kidney injury following cardiac surgery with cardiopulmonary bypass

As described in Chapter 5, differential miRNA expression profiles were discovered in patients who did and did not develop AKI following cardiac surgery with CPB. In patients who developed AKI postoperatively, there was a significant increase in circulating miR-210 levels measured in the immediate postoperative period. Patients who did not develop AKI also had a significant increase in miR-210 expression levels postoperatively, though the increase was less marked. MiR-210 levels were also detectable in the urine of both patients that developed AKI and patients that did not develop AKI.

Haemodilutional anaemia is associated with the development of AKI and mortality in patients undergoing cardiac surgery with CPB (Karkouti *et al.*, 2005). Anaemia-induced tissue hypoxia may be a central mechanism of organ injury and morbidity during acute haemodilution (Shander *et al.*, 2011). While cohort B patients who did not develop AKI postoperatively received more fluid intraoperatively than patients who did develop AKI postoperatively, their Hb levels remained significantly higher than patients who did develop AKI. Acute haemodilutional anaemia during CPB leads to a reduction in Hb concentrations with low Hb concentrations associated with increased AKI and mortality (Swaminathan *et al.*, 2003). Univariate analysis reported in Chapter 5 found the minimum Hb on CPB to be predictive of AKI. The increased levels of circulating and urinary miR-210 observed in patients who did develop AKI and the significantly reduced Hb levels reported during CPB is suggestive of renal tissue hypoxia during CPB.

In light of the significant correlation between miR-210 levels and lowest uPO_2 measured on CPB, further exploration of the potential predictive role of miR-210 expression levels measured during CPB and the development of AKI was then carried out. Although levels of miR-210 increased during CPB, its association with AKI did not reach statistical significance. It is possible that with a larger sample size, the findings may be consistent with the results reported by Aguado-Fraile et al. (2015) who identified and validated a set of miRNAs including miR-210 measured preoperatively and postoperatively as a potential marker of CSA-AKI. As previously discussed Lorenzen et al. (2011) also reported elevated levels of

plasma miR-210 in AKI patients and identified miR-210 as a strong independent predictor of survival in AKI.

This is the first study to report the upregulation of circulating and urinary miR-210 levels during the CPB period, a known time of insult. Further studies of miR-210 expression in a larger patient cohort with varying severities of AKI are needed to fully elucidate whether miR-210 is a predictive marker of AKI following CPB. At present there is no point of care testing rapid enough to determine miRNA levels and affect patient care (Dave *et al.,* 2019.) Future studies could involve the development of non PCR based tests for rapid point of care testing for miRNA detection to allow results to guide a perfusionist to optimise patient care (Rissin *et al.,* 2017).

Alterations in circulating and urinary microRNA-16 during and following cardiac surgery with cardiopulmonary bypass

In Chapter 3, circulation levels of the haemolysis associated miR-16, were shown to be differentially expressed during the CPB period and into the immediate postoperative period, returning to baseline levels within 4 hr postoperatively. While increased levels of miR-16 were reported in the immediate postoperative period, in the larger patient cohort the result was not statistically significant. Plausible explanations for the differences observed can potentially be attributed to the haemodilution of circulating miRNAs with the addition of crystalloid fluid during the perioperative period having a dilutional effect on the miRNAs measured.

Urinary miR-16 levels increased by 2000% in the urine of patients in the immediate postoperative period in patient cohort A. Results from cohort B were not as marked with surprisingly no statistically significant changes in mean urinary miR-16 levels measured during or following CPB. The greater number of outliers reported in the in the larger patient cohort indicate there was greater patient variation in urinary miR-16 levels. Lower urine creatinine values were also measured in cohort B patients, indicative of a greater degree of urine dilution.

Haemolysis can significantly affect plasma levels of RBC-derived miRNAs including miR-16 (Kirschner *et al.*, 2011). Haemolysis is a known consequence of CPB. Cardiopulmonary bypass related haemolysis is caused by mechanical shear stress within the extracorporeal circuit including turbulent passage through the arterial pump, oxygenator, reservoir, filters and the use of cardiotomy suction. The increased miR-16 levels reported in cohort A patients may be attributable to the use of roller arterial pumps. Trials reporting clinical outcome measures from comparisons between centrifugal pumps and roller pump are controversial (Hansbro *et al.*, 1999) with conflicting results reporting the effects of the two pump types on transfusion, platelet count, plasma free Hb and clinical outcomes (Saczkowski *et al.*, 2012). The absence of a significant increase in plasma free Hb during CPB in cohort A patients may reflect the poor sensitivity of free Hb to detect haemolysis at low levels of red cell damage. The increase in miR-16 levels reported in cohort A patients may be a result of low level haemolysis which is not reflected in free plasma haemoglobin levels.

Does haemolysis underlie microRNA release during cardiac surgery with cardiopulmonary bypass?

The correlation between plasma free Hb and miR-16 levels observed in this thesis most plausibly reflects exposure of the blood to the extracorporeal circuit and use of cardiotomy suction with resultant haemolysis. The correlation of miR-16 to levels of plasma free Hb reinforce miR-16 as a potential marker of haemolysis. As reported in chapter 3 and chapter 4, levels of miR-16 significantly correlated with levels of plasma free Hb levels. These results are supported by the literature with levels of miR-16 reported to increase with increased percentage of free haemoglobin present in plasma (Kirschner *et al.*, 2013).

Studies including those by Kirschner et al. (2011), McDonald et al. (2011) and Pritchard et al. (2012) have identified not only miR-16 but, miR-451, miR-486-5p and miR-92a as being the most highly abundant miRNAs associated with RBCs. Following haemolysis circulating levels of these specific miRNAs were increased. Surprisingly, results from high throughput profiling described in Chapter 6 found miR-451, miR-486-5p and miR-92a were not significantly upregulated during or following CPB with a less than one-fold change when compared to preoperative levels. High throughput qRT-PCR did demonstrate levels of miR-

16 were upregulated over two-fold following CPB, in keeping with the results in chapter 3, however the increase was not statistically significant. This is the first study reporting the correlation between free haemoglobin and levels of circulating miR-16 during CPB, implicating not only is an increase in miR-16 observed during sample processing and collection but also *in vivo* as low level haemolysis induced by CPB can significantly affect levels of circulating miRNAs.

Technical challenges and limitations of measurement of miRNAs during and following cardiac surgery with CPB

Choice of biological samples for microRNA analysis

The presence of miRNAs in blood and urine, together with their changes in expression in various pathological conditions, suggests these extracellular miRNAs might be informative biomarkers of disease and pathological states. In the clinical setting, both plasma and serum are used for extracellular miRNA detection and in this thesis both have been studied. Many studies comparing miRNA levels in serum and plasma side-by-side find little or no difference in miRNA quantification (Chen *et al.*, 2008; D' Alessandra *et al.*, 2010). Wang et al. (2012) reported higher total miRNA concentration in serum for specific miRNAs including miRNAs released upon platelet activation. However for the more abundant miRNAs, including miR-16, the concentrations between serum and plasma were the same. Thus, plasma and sera generally have similar miRNA expression patterns except in specific instances where significant differences between these biological fluids are apparent and so direct comparisons between the two biological fluids cannot always be made (Moldovan *et al.*, 2014).

While serum was collected for the pilot study into miRNA expression levels during CPB, plasma collected for a separate study (Cohort B), was used for investigation in chapters 4 and 5. Due to the potential subtle changes in expression patterns between plasma and serum, no direct comparisons can be made between the expression profiles of the two biological samples. While there remains no consensus on the ideal biological fluid, serum or plasma, to use for miRNA measurement consistency needs to be maintained to ensure the

differences in miRNA levels measured is not due to differences in miRNA levels between biological fluids. Results of a side-by-side comparison study between plasma and serum from Cohort A patients found higher levels of miR-210 and miR-16 in plasma when compared to serum. However, this study also found perioperative volume administration during CPB can also impact on levels of circulating miRNAs regardless of biological fluid sampled.

Effect of institutional protocols on microRNA levels

There remains a paucity of data on the role of miRNAs in cardiac surgery and their expression during the CPB period. The focus of published studies has been on cardiac specific miRNAs measured before and after CPB, neglecting the period of CPB. CPB presents a challenging period for biomarker determination due to the variety of sources of miRNA release caused by the stress of surgery, anesthetic drugs administered and CPB associated factors of MAP, arterial pump flow, Hct, transfusion, haemodilution, haemolysis and non-pulsatile flow. Differences in institutional protocols for anaesthetic and perfusion management of the patient during the perioperative period make comparisons between institutions difficult.

Differences in circulating and urinary miRNAs demonstrated between the two patient cohorts may have been attributable to differences in anaesthetic and perfusion practices. The effects of haemodilution could potentially explain the differences in miRNA levels observed between the two patient cohorts. On average, cohort A patients undergoing cardiac surgery with CPB received 1300 mL of CPB circuit prime and an additional 425 mL of crystalloid fluid during the CPB period. With an average adult circulating blood volume of 5000 mL, the addition of 1725 mL may have diluted circulating miRNAs by 35%. Cohort B patients undergoing cardiac surgery received an average of 1600 mL from the prime of the CPB circuit and 2250 mL of crystalloid fluid during the perioperative period which may have diluted circulating miRNAs by 70%. The haemodilution of cohort B patients may potentially explain the absence of increased circulating and urinary miRNA levels during CPB.

The results of this thesis have shown differences in perfusion methods and protocols can impact markedly on the levels circulating and urinary miRNAs and so perfusion practices need to be taken into consideration when examining biomarkers in patients undergoing cardiac surgery with CPB.

Interference of heparin and use of heparinase

The CPB period presents a potentially challenging situation for RNA determination because patients are systemically given large doses of heparin. Due to the level of systemic heparinisation required to safely conduct CPB, heparin is an unavoidable drug that can cause interference of miRNA analysis by qRT-PCR. Heparin is known to inhibit the reverse transcriptase and polymerase enzymes used in PCR (Al-Soud & Radstrom, 2001). In this study, treatment of serum and plasma samples with heparinase to degrade heparin, as reported by Wang et al, allowed successful PCR amplification and determination of miRNA levels (Wang *et al.*, 2009). Interestingly, due to the renal clearance of heparin, this study found all urine samples needed to be treated with heparinase for successful qRT-PCR amplification.

The only other study measuring circulating miRNAs during CPB was by Yang et al. (2015). They reported changes in cardiac specific miRNAs including miR-1, miR-208a and miR-499 levels in patients undergoing valve replacement but did not address the issue of the effect of heparin on miRNA detection by PCR. To maintain consistency in sample handling, samples without heparin were also treated with heparinase. Investigations were carried out to ensure the addition of heparinase itself did not alter miRNA expression levels. Preliminary investigations were also carried out to ensure the addition of heparin did not alter levels of circulating miRNAs by the *in vitro* addition of heparin at various concentrations to whole blood. These results are in keeping with study by Martino et al. (2012) reporting circulating levels of miRNAs were unaffected by heparin in patients undergoing dialysis.

Next generation sequencing is currently the gold standard for expression profiling for novel miRNA discovery. Due to the inability to successfully generate cDNA for NGS library

preparation, an alternative method of interrogating miRNA expression was used. The results of this study have shown that high throughput qRT-PCR allowed the amplification of known miRNAs. Exploration of other methods to avoid heparin interference with miRNA analysis could be further explored. The NanoString platform allows multiplexing analysis of up to 800 RNAs, similar to high throughput qRT-PCR. Unlike qRT-PCR, NanoString technology works by directly capturing, imaging and counting fluorescent barcodes allowing quantification of miRNAs. At the time of this study, there are no reports using samples contaminated with heparin and NanoString technology. Preliminary investigations would need to be carried out to determine if profiling utilising NanoString technology would be successful.

Controls for microRNA analysis during and after cardiac surgery with cardiopulmonary bypass

In this thesis, miRNA expression by qRT-PCR was performed by determining the relative quantification of each miRNA. Relative quantification uses the expression level of a target miRNA as a ratio of its Cq value to that of a control miRNA. There remains a lack of consensus for optimal control for quantification of miRNAs and characterising miRNA expression in plasma, serum and urine. Using relative quantification is challenging when a universal and constant internal reference miRNA has not been found to date (H. Schwarzenbach *et al.*, 2015).

Other potential internal reference markers include use of other small RNAs such as snRNAs and snoRNAs. Small RNAs, including RNU48 and RNU 44 were included in the high-throughput qRT-PCR OpenArray Panel as internal controls. However, studies suggest the use of snRNAs and snoRNAs as controls introduce bias when quantifying miRNA expression because they are not the same class as miRNAs and hence do not reflect the biochemical character of miRNA molecules in regards to transcription, processing and tissue-specific expression patterns. The efficiency of extraction, reverse transcription and PCR amplification of snRNAs and snoRNAs may differ from that of miRNAs (Benz *et al.*, 2013). It has been argued that it is best to quantify miRNAs with reference genes belonging to the same RNA class (Moldovan *et al.*, 2014). A solution to the lack of a suitable internal control is the use of an external control such as the addition of a spike-in synthetic miRNA.

Due to the lack of established internal reference miRNAs, some studies have only carried out quantification to external miRNAs. Exogenous, synthetic non-human miRNAs are added to the sample to allow for relative quantification of miRNAs, as well as estimating the efficiency of miRNA extraction and the reverse transcription step, ensuring miRNA quantification is not affected by the technical variability that may be introduced at different analysis steps (H Schwarzenbach *et al.*, 2015). In this study, an external miRNA, cel-miR-54, corresponding to a *C. elegans* miRNA was used as an external control. Plant miRNA, ath-miR-159a corresponding to *Arabidopsis thaliana* miRNA, was used as an external control for high throughput qRT-PCR experiments. A disadvantage of using external spike-in controls is that while experimental variability can be eliminated, it does not correct for the quality of the sample (Moldovan *et al.*, 2014). Collection, preparation or storing of biological samples including plasma, serum and urine may result in changes in miRNAs levels caused by miRNA degradation. The effect of degradation was minimised in this study, as all samples were prepared in an identical manner, as described in the methods with minimal variations in collection, preparation and correct storage until analysis. The results of this thesis have shown miRNAs were able to be successfully measured and quantified using an external control in patients undergoing cardiac surgery during the CPB period.

Is there a global release of small RNAs during cardiac surgery?

The results of Chapter 3 showed there was an increase in miR-210 and miR-16 during the perioperative period. This result was confirmed in a larger cohort study in Chapter 4. Due to the miR-210 and miR-16 release observed, investigations were carried out to determine if there was a global release of small RNAs or specific release in response to the insult of CPB.

Off pump coronary artery bypass grafting

In order to determine whether a global release of miRNAs occurred due to the insult of cardiac surgery, miRNA levels were measured in patients undergoing OPCABG. The results of Chapter 3 demonstrated that levels of miR-210 were significantly elevated during placement of the mechanical stabilising device and grafting of the heart. MiR-16 levels remained unaffected during OPCABG. The increased miR-210 levels measured during

OPCABG may be attributed to the manipulation and mechanical disruption to the heart during the procedure or due to hypoxia induced during manipulation of the myocardium for grafting. The results of Chapter 6 reported miRNAs potentially specific to the heart including miR-1, miR-133a and miR-133b were released during CPB and, along with hypoxically regulated miR-210, could potentially be released from the myocardium. The level of miR-210 release observed during OPCABG was of a lesser magnitude than observed during CPB. These results demonstrate the miRNA release observed during cardiac surgery with CPB were not solely attributable to the stresses of cardiac surgery on the heart. To further investigate the release of miRNAs and other small RNAs during and following CPB, analysis with the Bioanalyzer was performed.

The release of small RNAs during cardiac surgery with cardiopulmonary bypass

Following the differential release profiles of miR-210 and miR-16 demonstrated in Chapters 3 and 4, analysis of serum samples was carried out on the Bioanalyzer. The small RNA chip, used for high resolution analysis of small RNAs sized between 6 and 150 nt lengths, can determine RNA quality and quantity for further downstream experiments including high throughput RT-PCR. Bioanalyzer analysis identified the presence of not only miRNAs, but also slightly longer nt RNAs possibly revealing a small RNA repertoire including miRNAs, piRNAs, tRNAs, snoRNA and snRNA as was reported in the study by Umu et al. (2018) profiling serum samples utilising the Bioanalyzer.

In the preoperative sample and samples taken postoperatively at 4, 6, 8, 10,12 and 24 hr, a band of length 40-60 nt was observed consistent with the presence of tRNA and tRNA-derived fragments (Gebetsberger & Polacek, 2013). In samples taken during the CPB period a strong band of 18-21 nt in length, indicative of a possible miRNA release. To further interrogate the constituents of the small RNA release observed on Bioanalyzer analysis, and to confirm if a global release of miRNAs occurs during CPB, methods for miRNA profiling of all miRNAs were undertaken.

Next generation sequencing is used for the identification and characterization of novel miRNAs. Following RNA extraction, treatment with heparinase and overnight RNA

precipitation, the production of cDNA, the first step in the process of sample preparation for NGS, could not successfully be generated. Potential issues included interference caused by trace amounts of anaesthetic agents in the serum collected and the increased volume of heparinase used to neutralise heparin in a larger starting volume of serum which may have affected RNA quality due to the production of the breakdown product, uronic acid. A review of the literature could not find any examples of NGS sequencing in the presence of heparin. The failure of NGS demonstrates the importance of preliminary investigations carrying out novel experimental methods on samples containing heparin due to the unknown effect of the anticoagulant. Due to the inability to generate cDNA for NGS, alternate methods of large scale miRNA profiling were undertaken.

Due to the success of miRNA detection using qRT-PCR in Chapters 3 and 4, high throughput qRT-PCR was carried out. High throughput RT-PCR allows the simultaneous profiling of many hundred miRNAs and works on same platform as qRT-PCR with same amount of starting material. While NGS is the preferred method for discovery of novel miRNAs, high throughput qRT-PCR is a highly specific and sensitive method of miRNA expression profiling. Unlike NGS, high through-put RT-PCR does not allow for novel miRNA discovery as the miRNAs being investigated are known and does not allow the identification of other small RNA subsets. The high throughput qRT-PCR OpenArray Human miRNA Panel allowed for the profiling of 754 known miRNAs and other small RNAs including snRNAs, snoRNA, rRNA, mRNA and tRNA. This is the first study to determine miRNA expression using high throughput RT-PCR on patients during and following CPB.

The results presented in Chapter 6 of high throughput qRT-PCR of serum collected from a homogenous cohort of 10 male patients undergoing isolated CABG confirmed differential release profiles of miRNAs during and following CPB. A two-fold increase in miR-210 levels during CPB and a 2.7-fold increase following CPB supported the results of Chapter 3 and 4. A two-fold increase in miR-16 following CPB as observed in the results of Chapter 3 was also confirmed. High throughput qRT-PCR revealed 14 miRNAs that were highly upregulated in 7 or more of the 10 patients, potentially identifying a specific miRNA signature may be

released during CPB. Future studies involve the validation of these highly expressed miRNAs and investigation into their casual relevance.

Are specific microRNAs released during cardiac surgery with cardiopulmonary bypass?

The high throughput qRT-PCR results indicate that the release of miRNAs during CPB is potentially global with greater than 50% of miRNAs detected during and following CPB having a less than a two-fold change during and following CPB. Surprisingly, miRNAs associated with haemolysis, likely contributors to miRNA levels due to the haemolysis associated with CPB, were not significantly upregulated greater than 2-fold during or following CPB.

The results of Chapter 6 demonstrated there is potentially a release of specific miRNAs during CPB. Nine miRNAs were significantly upregulated in all patients during CPB including miR-218, miR-1260, mirR-1290, miR-520c-3p, miR-662, miR-138-2-3p, miR-1225-3p, miR-939-3p and miR-133a. MiR-218 and miR-133a continued to be significantly upregulated in the postoperative period together with miR-143, miR-133b, miR-1, miR-31 and miR-378a-3p. Validation and further investigation into these miRNAs may identify a unique miRNA signature for CPB. The discovery of novel miRNAs is likely to be required to further identify other miRNAs of interest as the use of high throughput qRT-PCR is limited to the profiling of known miRNAs. Identification of key miRNAs may give further insight and improved understanding of the molecular mechanism of CPB. Future investigations to gain further understanding of the Bioanalyzer analysis are needed. This includes the identification and verification of other small RNA subsets, including tRNA fragments.

What is the tissue of origin of microRNAs released during cardiac surgery with cardiopulmonary bypass?

The results in this thesis provide evidence that differential release profiles of circulating and urinary miR-210 and miR-16 occurred during and following CPB. Elucidation of the tissue of origin of miRNAs released and potential target cells of circulating miRNAs would increase insight into potential pathophysiological relevance of miRNA release during CPB.

Although it is not possible to unequivocally assign the origin of detected miRNAs, investigation of the highly expressed miRNAs identified by high throughput qRT-PCR identified significantly upregulated miRNAs whose expression is largely or wholly myocardial. Interestingly miRNAs miR-1, miR-133a and miR-133b were demonstrated to be highly expressed during and following CPB. Tissue localisation studies were carried out by Ludwig et al. (2016) to determine the tissue of origin of miRNAs and variations in abundance of miRNAs across tissues. The tissue specificity index, a quantitative, graded scalar measure for the specificity of expression of a miRNA with respect to different organs, found miR-1, miR-133a and miR-133b to have a tissue specific index of 0.95 indicating these miRNAs may be specific for the myocardium (Ludwig *et al.*, 2016). MiR-133a and miR-133b are primarily released into the peripheral circulation from the injured myocardium and have also been identified to be involved in intercellular communication in heart failure patients. MiR-133a and miR-133b were found to be significant diagnostic and/or prognostic markers across different cardiovascular disease progression stages (Bang *et al.*, 2015; Navickas *et al.*, 2016; Wang *et al.*, 2013). Future large-scale studies could further investigate the release of the myocardial specific miRNAs and determine if they are released in response to the hypoxic insult of CPB and ischaemic arrest.

Which signalling pathways are altered during cardiac surgery with cardiopulmonary bypass?

Pathway analysis of the highly expressed miRNAs identified by high throughput qRT-PCR, as described in Chapter 6, suggests a hypoxic response is initiated during CPB and continues into the immediate postoperative period. Using pathway analysis tools, investigation into the functional role of highly expressed circulating miRNAs and their predicted gene targets confirmed numerous cell signaling pathways, including the HIF pathway, were regulated by miRNAs during and after CPB. This is the first study to suggest a hypoxic release of miRNAs in response to CPB. The hippo and the PI3K-Akt signaling pathways were also identified to be regulated by the highly expressed miRNAs. The hippo and the PI3K-Akt signaling pathways have been found to promote cardiomyocyte proliferation and survival in vivo (Lin *et al.*, 2015). The p53 network was also found to be highly regulated during and following CPB. The p53 signaling network is activated upon exposure to stressful stimuli and has a

major role in the cellular response to these stresses. The p53 pathway is involved in regulating apoptosis, cell cycle and DNA repair.

Interestingly, many of the highly expressed miRNAs in this study were also involved in upregulation of inflammatory pathways. The use of CPB results in activation of the immunological response with the upregulation of inflammatory mediators including cytokines, IL-6, IL-8 and TNFα (Day & Taylor, 2005) (Trager *et al.*, 2016)). Pathway analysis supports the upregulation of inflammatory pathways including the insulin signaling pathway at a molecular level. The insulin signaling pathway is closely linked with dysregulation of transcriptional mediated pathways including activation of proinflammatory stress mediators (Rehman & Akash, 2016).

Transcriptional profiling using NGS and microarray has been carried out on left ventricular tissue, right atrial tissue and skeletal muscle samples to examine responses to CPB at a gene level (Ruel *et al.*, 2003) (Muehlschlegel *et al.*, 2015). These studies found CPB and cardioplegic arrest induced a distinct pattern of change in specific pathways such as up-regulation of inflammation and transcription activators, apoptotic genes and stress genes with up-regulation in targets relating to regulation reactive oxygen species production, apoptosis and cytokine production. (Ruel *et al.*, 2003). Interestingly there was a significant up-regulation in the gene ontology category involved in vasculature development (Muehlschlegel *et al.*, 2015). Hypoxia and the HIF pathway is a known important component of the homeostatic mechanisms that link vascular oxygen supply to metabolic demand thus regulating angiogenesis suggesting a potential role for hypoxically regulated miR-210 release during and following CPB.

Further studies into the complex interaction of pathways and networks initiated by CPB could be carried out to determine if the release of miRNAs during CPB is a protective or pathological effect. Future studies in computational hierarchical clustering of miRNAs and pathway analysis based on the interaction levels of highly expressed miRNAs using Gene Ontology enrichment network analysis could give further insight into pathway regulation

during CPB. Future investigations into the role of hypoxia and associated highly expressed miRNAs need to be validated with luciferase reporter gene studies.

Are other microRNAs increased during cardiopulmonary bypass due to hypoxia or associated with acute kidney injury

The results from chapter 6 revealed that miRNAs associated with AKI including miR-21, miR-210, miR-192, miR-205, miR-494, miR-155 and miR-146a, were upregulated during and following CPB. The results of this study report miR-494 was upregulated 4-fold during CPB while miR-155 expression increased over 5-fold during the CPB period. MiR-210 levels increased more than 2-fold during and following CPB, supporting the results of Chapter 2. Numerous studies have identified upregulation of these miRNAs associated with AKI.

Lorenzen et al. (2011) reported miR-210 was upregulated in patients with CSA-AKI with miR-210 levels predicting mortality. Du et al. (2013) demonstrated miR-21 levels increased following AKI caused by ischaemia reperfusion injury with circulating levels of miR-21 shown to correlated with AKI severity and hospital mortality and the need for postoperative renal replacement therapy. MiR-192 was found to be enriched in the kidneys with levels in tissue, blood and urine levels increased during AKI (Kito *et al.*, 2015). Levels of miR-494 were found to be increased in patients with AKI, with upregulation of miR-494 contributing to the development of AKI following ischaemia reperfusion (Lan *et al.*, 2012). Saikumer et al. (2012) reported miR-155 was a major regulator of inflammation with levels found to be highly regulated following tubular injury induced by ischaemia reperfusion injury.

Despite the fold changes observed, the increased expression levels of the miRNAs associated with AKI investigated in this thesis, including miR-21, miR-210, miR-192, miR-205, miR-494, miR-155 and miR-146a, were not statistically significant. The inter-patient variability in patient miRNAs levels measured, the small sample size and the small number of patients who developed AKI (n = 3) may contribute to the lack of statistical significance observed. A larger cohort study may help identify a panel of miRNAs to identify the development of AKI during CPB. Future studies could examine the expression of tissue

specific and hypoxia specific miRNAs in a larger cohort, to determine if a specific miRNA signature can be identified.

Further investigation into highly expressed miRNAs to determine their tissue of origin and to determine if miRNAs are released from specific tissue in response to CPB or released as a form of cell to cell communication is needed. A panel of serum miRNAs to predict AKI could be developed to identify patients at risk during CPB. High-throughput qRT-PCR or other methods of novel miRNA discovery could be used to identify highly expressed miRNAs. Future work should focus on large-scale studies involving miRNA profiling of urine with high throughput RT-PCR. Identification of highly expressed urinary miRNAs may provide insight into the causal relevance of increased miRNA levels and identify biomarkers for AKI during CPB. Further validation of the highly expressed miRNAs associated with hypoxia and AKI need to be explored. Measurement and validation of a potential panel of AKI biomarkers, including those suggested by Aguado-Fraile et al. (2015) including, miR-101, miR-127, miR-126, miR-26b, miR-29a, miR-146a, miR-27a, miR-93 and miR-10a may aid in the early detection and identification of patients at risk of AKI development.

The results of experiments carried out in this thesis demonstrate that differentially expressed circulating miRNAs present informative markers that are able to begin to unlock the potential molecular mechanisms affected by CPB. Developing miRNAs into an accurate and useful tool for the development of AKI during CPB will require an extensive phase of validation with multiple replication studies. Due to the paucity of studies of miRNA expression during CPB, more work is required to increase data transparency, such as adherence to the MIQE guidelines when using qRT-qPCR, and detailed description of perioperative management of the patient to allow better comparison of miRNA expression data.

The expression of circulating and urinary miRNAs in patients undergoing cardiac surgery utilising CPB has not previously been examined in detail. The results of this thesis present the first comprehensive investigation into miRNAs expression levels during cardiac surgery with CPB with circulating and urinary miRNAs successfully isolated and measured during CPB

and into the early postoperative period, with differential patterns of miRNA release observed.

Hypoxically regulated miR-210 levels were found to be increased during CPB and into the early postoperative period. Levels of miR-210 measured during CPB significantly correlated with levels of uPO2 a predictive marker of AKI suggesting a hypoxic release of miR-210 release during CPB. Further investigation and validation of miR-210, and other miRNAs associated with hypoxia, as predictive marker of AKI needs to be carried out in a larger cohort. The results of this thesis also found specific miRNAs, with higher circulating levels measured during CPB, were associated with molecular pathways including cell signalling pathways and the HIF pathway. Larger studies could be carried out observing the relationship between perioperative factors including MAP, DO_2, arterial pump flow and Hct with circulating miR-210 levels, hypoxia and the development of AKI. Thus, current studies into the role of modifiable perioperative CPB factors, including ideal arterial pump flow, DO_2 and Hct, and the development of AKI could be complemented with measurement of miR-210 levels.

The results of this thesis provide additional evidence of hypoxically regulated miRNA release, dysregulation of hypoxia related molecular pathways and provide evidence of a potential hypoxia response to CPB which has not be previously shown. Further investigation and validation of highly expressed miRNA during CPB and following CPB to determine their biological significance and functions and integrated pathway analysis is required. Future studies could also extend to examination miRNA levels of other patient populations including patients undergoing extracorporeal membrane oxygenation and patients with a left ventricular assist device. Perfusion is moving towards evidence based clinical practice with clinical outcomes observed following changes in clinical management. Multicentre trials would require uniform perfusion protocols to facilitate multisite trials and assessment of novel changes to practice or novel predictive tests. A greater understanding of molecular effects of CPB may help guide future directions of CPB conduct and management with changes in conduct of CPB, including MAP maintained during CPB, pump flow, Hct, transfusion, DO_2 and RBF, complemented with investigation into molecular effects of

changes. In turn, this knowledge may improve perfusion management, and serve as a tool for examining effects of changes in management, potentially reducing the incidence of CSA-AKI thus leading to improved clinical outcomes.

Appendix A: Reagents and Kits

Reagent	Catalogue number	Supplier
Chloroform		Chem-supply, Gillman, SA, Aust
DMEM Medium		ThermoFisher Scientific, Waltham, MA
Ethanol		Chem-supply, Gillman, SA, Aust
Heparinase Buffer **Tris-HCl** **Sodium chloride** **Calcium chloride**		 Chem-supply, Gillman, SA, Aust Chem-supply, Gillman, SA, Aust Chem-supply, Gillman, SA, Aust
Heparinase I	H2519	Sigma-Aldrich®, St Louis, MO
Isopropanol		Chem-supply, Gillman, SA, Aust
miRCURY™ RNA Isolation Kits for Biofluids	300112	Exiqon, Copenhagen, Denmark
RNase Inhibitor	M0307L	New England Biolabs® Inc, Ipswich, MA
RPMI-1640 Medium	30-2001	ATCC, Manassas,VA, USA
Taqman RT kits	4366596	Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA
TaqMan Universal PCR Master Mix no AmpErase UNG	4324018	Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA
QS Taqman OpenArray Human miRNA panel	4470187	Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA
Taqman OpenArray real time master mix	4462159	Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA
OpenArray Accessories Kit	4469576	Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA
OpenArray Tips Loader	4457246	Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA
OpenArray 384 well sample plates	4406947	Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA
Megaplex RT and PreAmp - Human	4444750	Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA
Taqman PreAmp master mix	4391128	Applied Biosystems, Foster City, CA, USA
TRIzol Reagent		ThermoFisher Scientific, Waltham, MA
TRIzol LS Reagent		ThermoFisher Scientific, Waltham, MA
Urine Exosome RNA Isolation Kit	47200	Norgen Biotek Corp, Thorold, ON, Canada
Tissue Culture plates - 6 well	CNG3506	Adelab Scientific, Thebarton, SA, Aust

Appendix B: Equipment and Software

Equipment	Supplier
Aligent 2100 Bioanalyzer	Aligent Technologies, Santa Clara, CA
Dry block heater	Thermoline L+M, Sydney, NSW, Aust
Expression Suite Software v.1.0.1	ThermoFisher Scientific, Waltham, MA
GraphPad Prism 8	GraphPad Software Inc, La Jolla, CA
Microcentrifuge 5424	Eppendorf, Hamburg, Germany
Nanodrop 8000 Spectrophotometer	ThermoFisher Scientific, Waltham, MA
Roche/Hitachi Modular Analyser	Hitachi High-Technologies Corp., for Roche Diagnostics GmbH, Tokyo, Germany
Rotorgene Q	Qiagen®, Foster City, CA
Q-gene	
QuantStudio 12K Flex Real-Time PCR System	ThermoFisher Scientific, Waltham, MA
STATA statistical software, version	StataCorp, TA, USA

Appendix C: Taqman Assays

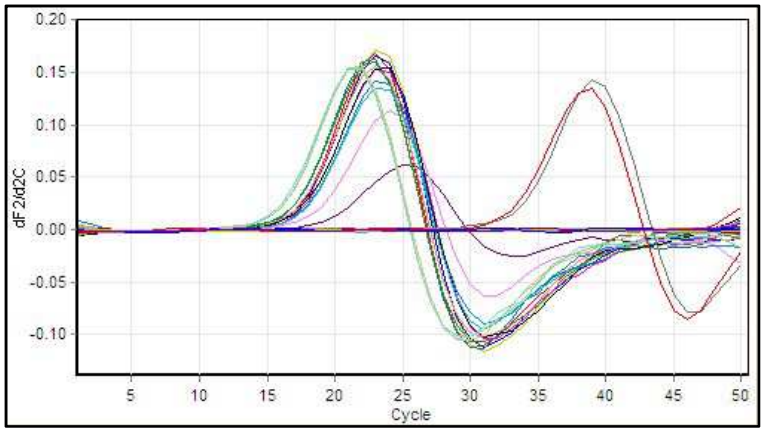
Taqman assays	Assay ID	Supplier
hsa-miR-16-5p	000391	ThermoFisher Scientific, Waltham, MA
hsa-miR-210-3p	000512	ThermoFisher Scientific, Waltham, MA
cel-miR-54-3p	001361	ThermoFisher Scientific, Waltham, MA
ath-miR-159a	000338	ThermoFisher Scientific, Waltham, MA

Appendix D: Cell lines and Oligos

Cell Line	Catalogue number	Supplier
RCC4 (VHL deficient)	03112702	Sigma-Aldrich®, St Louis, MO
RCC4 plus VHL	03112703	Sigma-Aldrich®, St Louis, MO
293 [HEK 293]	ATCC CRL-1573	ATCC, Manasssas, VA, USA

Oligo duplex	Sequence	Supplier
Arabadopsis thaliana	5”UUUGGAUUGAAGGGAGCUCUA	Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA.
Caenorhabditis elegans	5”UACCCGUAAUCUUCAUAAUCCGAG	Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA

Appendix E: Preliminary Experiments



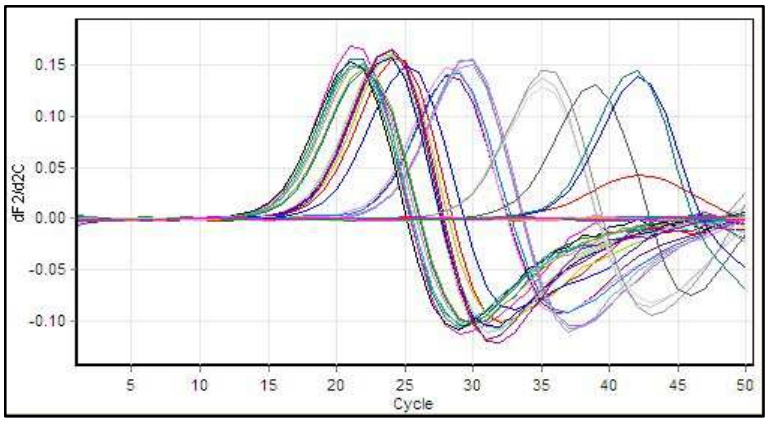
No.	Colour	Name	Take Off	Amplification
1		VHL + 16	18.6	1.76
2		VHL + 16	18.7	1.75
3		VHL + 16	18.6	1.68
4		serum + heparinase	18.6	1.68
5		serum + heparinase	18.4	1.69
6		serum + heparinase	18.9	1.74
7		heparin+ heparinase	18.1	1.66
8		heparin+ heparinase	18.4	1.70
9		heparin+ heparinase	18.1	1.71
10		20ul heparin+ heparinase	18.8	1.67
11		20ul heparin+ heparinase	18.6	1.69
12		20ul heparin+ heparinase	18.9	1.68
13		serum no heparinase	18.3	1.75
14		serum no heparinase	18.1	1.69
15		serum no heparinase	18.2	1.80
16		heparin no heparinase	32.1	-0.04
17		heparin no heparinase	17.3	0.70
18		heparin no heparinase	16.1	0.09
19		20ul no heparinase	42.1	0.13

No.	Colour	Name	Take Off	Amplification
20		20ul no heparinase	41.1	0.11
21		20ul no heparinase	44.0	0.06
22		VHL + no RT	42.0	0.00
23		VHL + no RT	37.0	0.20
24		VHL + no RT	40.3	0.13
25		serum + heparinase no RT	24.3	0.04
26		serum + heparinase no RT	20.1	0.06
27		serum + heparinase no RT	20.0	0.00
28		serum no heparinase no RT	22.3	0.25
29		serum no heparinase no RT	26.7	0.00
30		serum no heparinase no RT	26.1	0.00
31		mm1+ 16+ water	37.5	0.06
32		mm1+ 16+ water	37.1	0.07
33		mm1+ 16+ water	38.1	0.08
34		mm2 + water	31.5	-0.01
35		mm2 + water	38.3	-0.01
36		mm2 + water	11.1	0.39

APPENDIX E1: Comparative Quantification report and take off graph for effect of *in vitro* heparin administration and neutralisation with heparinase

Results of experiment carried out to determine effect of 2.5 µL heparinase on large doses of heparin mimicking doses administered for CPB. Venous blood was collected (30 mL) from a healthy volunteer and divided into 3 silicone coated blood collection tubes (BD, Vacutainer, Becton Dickenson and Company, Franklin Lakes, NJ) for serum separation. Five µL of heparin was added to one tube (low dose heparin) and 20 µL was added to a second tube (high dose heparin). Following serum separation, RNA extraction with TriZol LS, heparinase treatment of all samples and overnight RNA precipitation, qRT-PCR was carried out. Results shown as take of graph for amplification and table displaying Cq value (take off) and amplification of PCR.

1-3 show successful amplification of PCR with hsa-miR-16 detected using RNA extracted from RCC4 cell line (positive control);
4-5 serum with no heparin + heparinase treatment;
7-9 addition of 5µL heparin + heparinase treatment,
10-12 addition of 20µL of heparin + heparinase treatment.
Cq values within range of 18.1-18.9 and amplification $\geq$ 1.65 – indicating successful PCR detection and amplification of hsa-miR-16 in all samples.
13-15 show successful amplification of serum with no heparinase treatment – note Cq values within range of serum no heparin with heparinase treatment.
16-18 addition of 5µL of heparin without heparinase treatment and
19-21 addition of 20µL of heparin without heparinase treatment – note wide range of take-off Cq and amplification of PCR $\leq$ 1.65 indicating failure of PCR amplification due to interference of heparin.
22-36: negative controls to ensure no contamination of RT-PCR reagents used – Low amplifications indicate no product detected for PCR amplification.



No.	Colour	Name	Take Off	Amplification
1		VHL + miR16	19.9	1.64
2		VHL + miR16	19.6	1.70
3		VHL + miR16	20.6	1.73
4		pre heparin + heparinase miR16	24.7	1.83
5		pre heparin + heparinase miR16	24.7	1.72
6		pre heparin + heparinase miR16	24.6	1.74
7		CPB + heparinase miR16	17.0	1.64
8		CPB + heparinase miR16	17.2	1.72
9		CPB + heparinase miR16	16.8	1.75
10		6 hr post + heparinase miR16	16.9	1.76
11		6 hr post + heparinase miR16	16.7	1.72
12		6 hr post + heparinase miR16	17.0	1.75
13		24 hr post + heparinase miR16	19.1	1.70
14		24 hr post + heparinase miR16	19.3	1.71
15		24 hr post + heparinase miR16	18.9	1.67
16		pre heparin no heparinase miR16	24.9	172
17		pre heparin no heparinase miR16	24.9	1.78
18		pre heparin no heparinase miR16	25.0	1.77
19		CPB no heparinase miR16	26.1	-0.01
20		CPB no heparinase miR16	34.1	0.09
21		CPB no heparinase miR16	17.8	0.26

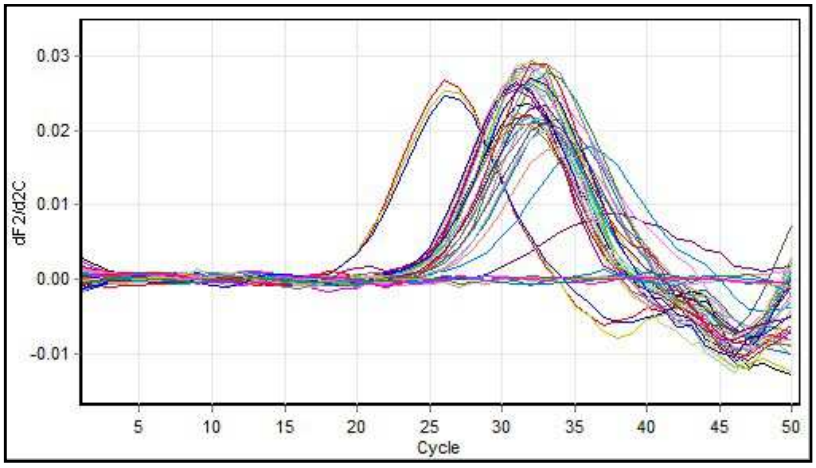
No.	Colour	Name	Take Off	Amplification
22		6 hr post no heparinase miR16	17.6	1.73
23		6 hr post heparinase miR16	17.5	1.74
24		6 hr post heparinase miR16	17.6	1.73
25		24 hr post no heparinase miR16	19.4	1.76
26		24 hr post no heparinase miR16	19.5	1.74
27		24 hr post no heparinase miR16	19.4	1.70
28		pre CPB + heparinase miR16 no RT	28.7	0.24
29		pre CPB + heparinase miR16 no RT	38.0	0.56
30		pre CPB + heparinase miR16 no RT	36.5	0.88
31		pre CPB no heparinase miR16 no RT	38.6	0.44
32		pre CPB no heparinase miR16 no RT	37.7	0.08
33		pre CPB no heparinase miR16 no RT	44.1	0.08
34		mm1 +water	37.1	0.12
35		mm1 +water	43.1	0.62
36		mm1 +water	37.2	1.84
37		mm2 + water	25.5	0.30
38		mm2 + water	43.1	-0.08
39		mm2 + water	27.1	0.04

APPENDIX E2: Comparative Quantification report and take off graph for effect of *in vivo* administration of heparin administration for CPB and neutralisation with heparinase

Results of experiment carried out to determine effect of 2.5 µL heparinase on heparin administered for CPB. Venous blood was collected (20 mL) at 4 time points

1. prior to heparinisation; 2. during CPB (following systemic heparinisation (30 000IU)); 3. 6 hr post CPB following neutralisation of heparin with protamine post CPB; and 4. 24 hr post CPB. Blood was divided into 2 silicone coated blood collection tubes (BD, Vacutainer, Becton Dickenson and Company, Franklin Lakes, NJ) for serum separation. Following serum separation, RNA extraction with TriZol LS, one sample from each time point was treated with heparinase while the 2nd sample was not. qRT-PCR was carried out. Results shown as take of graph for amplification and table displaying Cq value (take off) and amplification of PCR.

1-3 show successful amplification of PCR with hsa-miR-16 detected using RNA extracted from RCC4 cell line (positive control);
4-5 serum collected pre heparinisation + heparinase treatment;
7-9 during CPB + heparinase treatment,
10-12 6 hr post CPB + heparinase treatment;
13-15 24 hr post CPB. Cq values within range of with little variation between technical replicates. Amplification $\geq$ 1.65 – indicating successful PCR detection and amplification of hsa-miR-16 in all samples.
16-18 shows successful amplification of pre heparinisation serum with no heparinase treatment – note Cq values within range of pre heparinisation serum with heparinase treatment (no. 4-6) thus indicating use of heparinase itself, in the absence of heparin, does not alter miRNA levels.
19-21 during CPB with no heparinase treatment– note wide range of take-off Cq and amplification of PCR $\leq$ 1.65 indicating failure of PCR amplification due to interference of heparin.
22-24 6 hr post CPB and No: 25-27 24 hr post CPB – heparin cleared from circulating allowing successful amplification of PCR. Addition of heparinase confirming miRNA levels not altered by absence of heparin.
28-39 negative controls to ensure no contamination of RT-PCR reagents used – Low amplifications indicate no product for PCR amplification.



No.	Colour	Name	Take Off	Amplification
1		VHL -	21.4	1.71
2		VHL -	21.0	1.70
3		VHL -	21.4	1.69
4		pre + heparinase	28.2	1.65
5		pre + heparinase	28.4	1.66
6		pre + heparinase	28.6	1.69
7		PS + heparinase	28.2	1.71
8		PS + heparinase	28.4	1.69
9		PS + heparinase	28.1	1.65
10		CPB +heparinase	26.0	1.66
11		CPB +heparinase	26.3	1.69
12		CPB +heparinase	26.4	1.64
13		RW + heparinase	25.9	1.68
14		RW + heparinase	26.0	1.65
15		RW + heparinase	25.9	1.68
16		post op + heparinase	26.0	1.79
17		post op + heparinase	26.3	1.70
18		post op + heparinase	26.4	1.68
19		4 hr + heparinase	28.1	1.68
20		4 hr + heparinase	27.9	1.65
21		4 hr + heparinase	28.1	1.63
22		6 hr + heparinase	27.9	1.71
23		6 hr + heparinase	28.0	1.73

No.	Colour	Name	Take Off	Amplification
24		6 hr + heparinase	27.8	1.75
25		8 hr + heparinase	27.3	1.78
26		8 hr + heparinase	27.4	1.75
27		8 hr + heparinase	27.5	1.73
28		10 hr + heparinase	27.0	1.69
29		10 hr + heparinase	26.9	1.68
30		10 hr + heparinase	26.9	1.70
31		12hr + heparinase	27.5	1.70
32		12hr + heparinase	27.6	1.68
33		12hr + heparinase	27.5	1.72
34		18 hr + heparinase	27.5	1.64
35		18 hr + heparinase	27.9	1.65
36		18 hr + heparinase	27.4	1.68
37		24 hr + heparinase	27.1	1.69
38		24 hr + heparinase	27.1	1.68
39		24 hr + heparinase	27.0	1.66
40		mm1	38.8	0.00
41		mm1	43.1	0.00
42		mm1	40.1	0.00
43		mm2	10.8	0.00
44		mm2	45.3	0.00
45		mm2	26.0	0.00

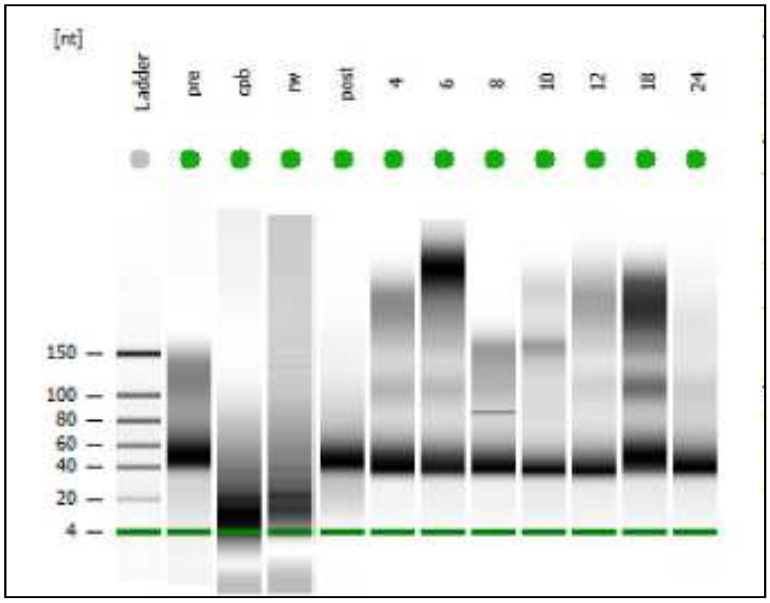
APPENDIX E3: Comparative Quantification report and take off graph to observe effect of sternotomy on miRNA levels.

Results of experiment carried out to determine effect of sternotomy on miRNA levels. Venous blood was collected (20 mL) at 12 time points: Prior to heparinisation (pre); Post sternotomy (PS); 10 min following initiation of CPB (CPB); following CPB (post); 4 hr post op; 6 hr post op; 8 hr post op; 10 hr post op; 12 hr post op; 18 hr post op; 24 hr post op.

Blood was divided into 2 silicone coated blood collection tubes (BD, Vacutainer, Becton Dickenson and Company, Franklin Lakes, NJ) for serum separation. Following serum separation, RNA extraction with TriZol LS and treatment with heparinase qRT-PCR was carried out. Results shown as take off graph for amplification and table displaying Cq value (take off) and amplification of PCR.

1-3 show successful amplification of PCR with hsa-miR-210 detected using RNA extracted from RCC4 –VHL cell line (positive control)
4-5 preoperative sample + heparinase treatment
7-9 post sternotomy sample + heparinase treatment
10-12 CPB sample + heparinase treatment
13-15 Rewarming sample + heparinase treatment
16-18 Postoperative sample + heparinase treatment
19-21 4 hr postoperative sample + heparinase treatment
22-24 6 hr postoperative sample + heparinase treatment
25-27 8 hr postoperative sample + heparinase treatment
28-30 10 hr postoperative sample + heparinase treatment
31-33 12 hr postoperative sample + heparinase treatment
34-36 18 hr postoperative sample + heparinase treatment
37-39 24 hr postoperative sample + heparinase treatment
Cq values within range of 18.1-18.9 and amplification $\geq$ 1.65 – indicating successful PCR detection and amplification in all samples. Post sternotomy samples have similar Cq values to preoperative samples- indicating increase in miRNA levels during CPB is not due to insult of median sternotomy.
39-45: negative controls to ensure no contamination of RT-PCR reagents used – Low amplifications indicate no product detected for PCR amplification.

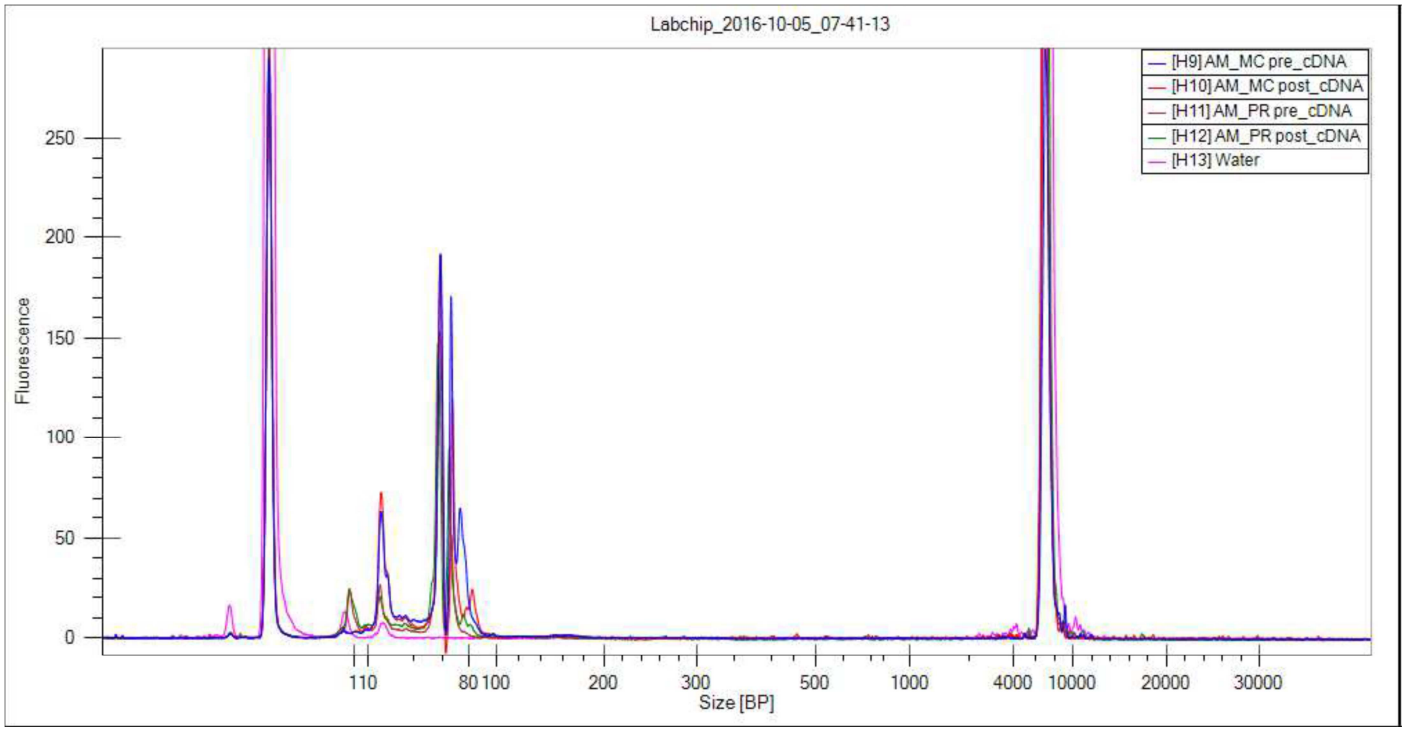
Appendix F: Bioanalyzer result



APPENDIX F: Example of Bioanalyzer gel electrophoresis result

Arterial blood collected from a patient undergoing cardiac surgery with CPB. Samples collected preoperatively, during CPB and postoperatively. RNA extracted from serum using miRCURY RNA Isolation kits for Biofluids (Exiqon, Copenhagen, Denmark). Analysis carried out with Aligent 2100 Bioanalyzer. Bioanalyzer analysis uses a microfluidics-based platform for sizing and quantification of small RNAs. A ladder (1st channel) is included in each run to determine nucleotide (nt) length. MiRNAs are typically 18-25 nt length, tRNA 40-60 nt length and tRNA fragments 10-45 nt.

Appendix G: Next Generation Sequencing – Lab Chip traces



Failed lab chip trace for next generation sequencing preparation.

NGS library preparation begins with generation of cDNA. Successful generation of cDNA is confirmed on the lab chip prior to samples proceeding onto size selection through the Pippin preparation. The peaks observed in the figure are primers used in the reaction. The absence of any other peaks indicates inhibition of the generation of cDNA in patient samples collected prior to CPB, heparinised samples collected during CPB and following neutralisation of heparin with protamine following termination of CPB.

Appendix H: Taqman Open Array Human MicroRNA Panel

CPB Fold Change Distribution – Upregulation

Δ FOLD CHANGE

0.5 - 2	2 - 4	4 – 10	➢ 10
mmu-miR-491	hsa-miR-708	hsa-miR-218-1	hsa-miR-518b
hsa-miR-99a	hsa-miR-218	hsa-miR-662	hsa-miR-593
hsa-miR-483-5p	has-miR-1271	hsa-miR-520c-3p	
hsa-miR-410	hsa-miR-519a	hsa-miR-101	
hsa-miR-33a	hsa-miR-655	hsa-miR-935	
hsa-miR-26a-1	has-miR-1255B	hsa-miR-138-2	
hsa-miR-299-3p	hsa-miR-135b	hsa-miR-135b	
hsa-miR-494	hsa-miR-31	hsa-miR-513C	
hsa-miR-183	has-miR-1183	has-miR-665	
hsa-miR-490	hsa-miR-206	hsa-miR-582-5p	
hsa-miR-370_	hsa-miR-605	hsa-miR-302a	
hsa-miR-646	hsa-miR-136	hsa-miR-220b	
has-miR-1291	hsa-miR-331-5p	hsa-miR-220b	
hsa-miR-744	hsa-miR-522	hsa-miR-155	
hsa-miR-10b	mmu-miR-615	hsa-miR-596	
has-miR-664	hsa-miR-887	has-miR-1285	
hsa-miR-618	hsa-miR-564	hsa-miR-1225-3p	
hsa-miR-766	hsa-miR-19b-1	hsa-miR-573	
hsa-miR-659	hsa-miR-125b-1	hsa-miR-587	
hsa-miR-486-3p	hsa-miR-584	hsa-miR-302c	
hsa-miR-202	has-miR-1825	hsa-miR-1208	
hsa-miR-200b	hsa-miR-874	hsa-miR-190b	
hsa-miR-519c	has-miR-1275	hsa-miR-92a-1	
hsa-miR-382	hsa-miR-571	hsa-miR-872	
hsa-miR-28-3p	has-miR-1303	hsa-miR-628-5p	
hsa-miR-30a-3p	hsa-miR-939	hsa-miR-524	
hsa-miR-574-3p	hsa-miR-432	hsa-miR-485-5p	
hsa-miR-326	hsa-miR-21	hsa-miR-454	
hsa-miR-377	hsa-miR-148a	hsa-miR-1260	
has-miR-1253	hsa-miR-342-5p	mmu-miR-124a	
hsa-miR-125b	hsa-miR-668	hsa-miR-377	
hsa-miR-485-3p	hsa-miR-616	hsa-miR-1290	
hsa-miR-150	hsa-miR-99b	hsa-miR-127-5p	
hsa-miR-181a-2	hsa-miR-92b	hsa-miR-141	
hsa-miR-95	hsa-miR-597	hsa-miR-521	
hsa-miR-654	hsa-miR-205	hsa-miR-636	

hsa-miR-591	has-miR-1233	hsa-miR-520a	
hsa-miR-342-3p	hsa-miR-34b		
hsa-miR-222	hsa-miR-1227		
hsa-miR-671-3p	hsa-miR-424		
hsa-miR-1	has-miR-1247		
hsa-miR-320	hsa-miR-549		
hsa-miR-661	hsa-miR-608		
hsa-miR-625	hsa-miR-517c		
hsa-miR-139-3p	hsa-miR-34a		
hsa-miR-346	hsa-miR-454		
hsa-miR-146b-3p	hsa-miR-373		
hsa-miR-720	has-miR-191		
hsa-miR-551b	mmu-miR-134		
hsa-miR-450a	hsa-miR-378		
hsa-miR-34a	hsa-miR-214		
hsa-miR-433	has-miR-1276		
hsa-miR-886-3p	hsa-miR-183		
hsa-miR-139-5p	hsa-miR-770-5p		
hsa-miR-324-3p	hsa-miR-548c-5p		
hsa-miR-184	hsa-miR-367		
hsa-miR-16-1	hsa-miR-593		
hsa-miR-10a	hsa-miR-133a		
has-miR-1274A	hsa-miR-204		
hsa-miR-324-5p	hsa-miR-200a		
hsa-miR-140-3p	has-miR-1289		
hsa-miR-152	hsa-miR-520g		
hsa-miR-99a	hsa-miR-548b-5p		
hsa-miR-639	hsa-miR-29c		
hsa-miR-650	hsa-miR-133b		
hsa-miR-24-2	hsa-miR-518d		
has-miR-320B	hsa-miR-657		
hsa-miR-431	hsa-miR-638		
hsa-miR-296	hsa-miR-31		
hsa-miR-486	hsa-miR-212		
hsa-miR-576-3p	hsa-miR-224		
hsa-miR-145	hsa-miR-193b		
hsa-miR-361	hsa-miR-643		
hsa-miR-213			
hsa-miR-132			
hsa-miR-425-5p			
hsa-miR-539			
hsa-miR-27			
hsa-miR-744			

hsa-miR-409-3p hsa-miR-106b hsa-miR-141 hsa-miR-339-3p has-miR-1179 hsa-miR-210 hsa-miR-500 hsa-miR-339-5p hsa-miR-645 hsa-miR-22 hsa-miR-432 hsa-miR-345 hsa-miR-423-5p hsa-miR-497 hsa-miR-146b hsa-miR-363 hsa-miR-196b hsa-miR-27a hsa-miR-126 hsa-miR-93 hsa-miR-656 hsa-miR-648 hsa-miR-425 hsa-miR-191 hsa-miR-384 hsa-miR-361-3p hsa-miR-219-2-3p hsa-miR-532-3p hsa-miR-487b hsa-miR-146a hsa-miR-18b has-miR-1274B hsa-miR-484 hsa-miR-29b hsa-miR-532 hsa-miR-100 hsa-miR-186 hsa-miR-30d hsa-miR-126 hsa-miR-29a hsa-miR-942 hsa-miR-330 hsa-miR-675			

hsa-miR-30a-5p hsa-miR-24 hsa-miR-194 hsa-miR-130a hsa-miR-193a-5p hsa-miR-548d-5p hsa-miR-941 hsa-miR-203 hsa-miR-148b hsa-miR-199a hsa-miR-211 hsa-miR-590-5p			

CPB Fold Change Distribution – Down Regulation

Δ FOLD CHANGE

0.5 - 2	2 - 4	4 – 10	➢ 10
hsa-miR-149 hsa-miR-885-5p mmu-miR-93 hsa-miR-431 hsa-miR-450b-3p mmu-miR-374-5p hsa-miR-21 hsa-miR-15a hsa-miR-614 hsa-miR-429 hsa-miR-30b hsa-miR-340 hsa-miR-20b hsa-miR-20a hsa-miR-505 hsa-miR-148a hsa-miR-106b hsa-miR-1298 hsa-miR-26b hsa-miR-144 hsa-miR-892b hsa-miR-302b hsa-miR-592 hsa-let-7e	hsa-miR-29b-2 hsa-miR-142-5p hsa-miR-29a hsa-miR-380-3p hsa-miR-335 hsa-miR-595 hsa-miR-371-3p hsa-miR-642 mmu-miR-96 hsa-miR-98 hsa-miR-362 hsa-miR-630 hsa-miR-1249B hsa-miR-654-3p hsa-miR-17 hsa-miR-629 hsa-miR-517b hsa-miR-518c hsa-miR-337-3p hsa-miR-1286	has-miR-548 hsa-miR-802 hsa-miR-888 hsa-miR-553 hsa-miR-601 has-miR-1262 hsa-miR-541 hsa-miR-519b-3p hsa-miR-518e hsa-miR-548a-5p	hsa-miR-575

hsa-let-7d hsa-miR-340 hsa-miR-30d hsa-miR-598 hsa-miR-365 hsa-miR-625 hsa-miR-9 hsa-let-7g hsa-miR-569 hsa-miR-26a hsa-miR-362-3p hsa-miR-15b hsa-miR-28 hsa-miR-144 hsa-let-7f hsa-miR-30c mmu-miR-140 hsa-miR-148b hsa-miR-301b mmu-let-7d hsa-miR-520h hsa-miR-190 hsa-miR-338-3p hsa-miR-216b mmu-miR-451 hsa-miR-199a-3p hsa-miR-543 hsa-miR-889 hsa-miR-629 hsa-miR-9#_B hsa-miR-142-3p hsa-miR-409-5p hsa-miR-18a hsa-miR-27b hsa-miR-122 mmu-miR-379 hsa-miR-199b hsa-miR-363 hsa-miR-18a			

Postoperative Fold Change Distribution – Upregulation

Δ FOLD CHANGE

0.5 - 2	2 - 4	4 - 10	➢ 10
hsa-miR-95	hsa-miR-513C	hsa-miR-1259	hsa-miR-518b
hsa-miR-9	hsa-miR-584	hsa-miR-520c-3p	
hsa-miR-1255B	hsa-miR-133a	hsa-miR-524	
hsa-miR-486-3p	hsa-miR-550	hsa-miR-135b	
hsa-miR-517a	hsa-miR-935	hsa-miR-1179	
mmu-miR-124a	hsa-miR-296-3p	hsa-miR-665	
hsa-miR-29b	hsa-miR-377	hsa-miR-520a	
hsa-miR-10b	hsa-miR-1248	hsa-miR-136	
hsa-miR-431	hsa-miR-191	mmu-miR-615	
hsa-miR-363	hsa-miR-183	hsa-miR-1267	
hsa-miR-519c	hsa-miR-378	hsa-miR-302a	
hsa-miR-501	hsa-miR-582-5p	hsa-miR-202	
hsa-miR-214	hsa-miR-597	hsa-miR-559	
hsa-miR-1208	hsa-miR-141	hsa-miR-10a	
hsa-miR-424	hsa-miR-31	hsa-miR-330-5p	
hsa-miR-548b-5p	hsa-miR-450a	hsa-miR-96	
hsa-miR-135b	hsa-miR-183	hsa-miR-548a-5p	
hsa-miR-573	hsa-miR-335	hsa-miR-872	
hsa-let-7c	hsa-miR-31	hsa-miR-218	
hsa-miR-193b	hsa-miR-146b-3p	hsa-miR-647	
hsa-miR-571	hsa-miR-1285	hsa-miR-1	
hsa-miR-654	hsa-miR-596	hsa-miR-708	
hsa-miR-591	hsa-miR-23b	hsa-miR-373	
hsa-miR-887	hsa-miR-125b	hsa-miR-455-3p	
hsa-miR-367	hsa-miR-342-5p	hsa-miR-218-1	
hsa-miR-433	hsa-miR-143	hsa-miR-133b	
hsa-miR-181a-2	hsa-miR-127-5p	hsa-miR-138-2	
hsa-miR-199b	hsa-miR-671-3p	hsa-miR-518d	
hsa-miR-657	hsa-miR-125b-1	hsa-miR-662	
hsa-miR-650	hsa-miR-206	hsa-miR-454	
hsa-miR-141	hsa-miR-200b	hsa-miR-520h	
hsa-miR-668	hsa-miR-490	hsa-miR-92b	
hsa-miR-139-3p	hsa-miR-224	hsa-miR-33a	
hsa-miR-1227	hsa-miR-205	hsa-miR-23b	
hsa-miR-566	hsa-miR-145	hsa-miR-1225-3p	
hsa-miR-497	hsa-miR-302c		
has-miR-1254	hsa-miR-605		
hsa-miR-212	hsa-miR-200a		

hsa-let-7a hsa-miR-455 hsa-miR-483-5p hsa-miR-193a-5p hsa-miR-548d-5p hsa-miR-661 hsa-miR-410 hsa-miR-891a hsa-miR-190 hsa-miR-107 hsa-miR-643 hsa-miR-425-5p hsa-miR-889 hsa-miR-595 hsa-miR-155 hsa-miR-618 hsa-miR-29c hsa-miR-509-5p hsa-miR-99a hsa-miR-545 hsa-miR-1247 hsa-let-7g hsa-miR-203 hsa-miR-886-3p hsa-miR-375 mmu-let-7d mmu-miR-499 hsa-miR-100 hsa-miR-675 hsa-miR-195 has-miR-664 hsa-miR-302b hsa-miR-152 hsa-miR-326 hsa-miR-130a hsa-miR-645 hsa-miR-30a-3p hsa-miR-220c hsa-miR-409-5p hsa-miR-511 hsa-miR-181c hsa-miR-532 hsa-miR-1291	hsa-miR-325 mmu-miR-491 hsa-miR-485-5p hsa-miR-874 hsa-miR-16-1 hsa-miR-656 hsa-miR-1303 hsa-miR-521 hsa-miR-17 hsa-miR-1260 hsa-miR-636 hsa-miR-758 hsa-let-7a hsa-miR-1275 hsa-miR-204 hsa-miR-564 hsa-miR-361-3p hsa-miR-517c hsa-miR-520d-5p hsa-miR-34a hsa-miR-549 hsa-miR-1271 hsa-miR-520e hsa-miR-24-2 hsa-miR-25		

hsa-miR-22 hsa-miR-875-5p hsa-miR-382 hsa-miR-542-3p hsa-let-7f hsa-miR-210 hsa-miR-629 hsa-miR-500 hsa-miR-488 hsa-miR-186 hsa-miR-27b hsa-miR-638 hsa-miR-648 hsa-miR-320 hsa-miR-486 hsa-miR-188-3p hsa-miR-34a hsa-miR-593 hsa-miR-136 hsa-miR-423-5p hsa-miR-132 hsa-miR-429 hsa-miR-222 hsa-miR-27a hsa-miR-335 hsa-miR-1233 mmu-miR-96 hsa-miR-323-3p hsa-miR-365 hsa-miR-655 hsa-miR-10b hsa-miR-1238 hsa-miR-211 hsa-miR-299-3p hsa-miR-92a-1 hsa-miR-101 hsa-miR-302d hsa-miR-18a hsa-miR-30a-5p hsa-miR-1825 hsa-miR-346 hsa-miR-140-3p hsa-miR-384			

hsa-miR-660 hsa-miR-548c-5p hsa-miR-30d hsa-miR-18b hsa-miR-663B hsa-miR-598 hsa-miR-28-3p hsa-miR-769-5p hsa-miR-370 hsa-miR-215 hsa-miR-345			

Postoperative Fold Change Distribution – Down Regulation

Δ FOLD CHANGE

0.5 - 2	2 - 4	4 – 10	➢ 10
hsa-miR-181a hsa-let-7g mmu-miR-140 hsa-miR-221 hsa-miR-378 hsa-miR-331-5p hsa-miR-425 hsa-miR-145 hsa-miR-127 hsa-miR-9 hsa-let-7e hsa-miR-128a hsa-miR-339-5p hsa-miR-30c hsa-miR-422a hsa-miR-26b hsa-miR-193b hsa-miR-202 hsa-miR-1286 hsa-miR-199a-3p hsa-miR-625 hsa-miR-200c hsa-miR-376c hsa-miR-432 hsa-miR-374	hsa-miR-199a hsa-miR-569 hsa-miR-431 hsa-miR-301b hsa-miR-522 hsa-miR-512-5p hsa-miR-196b hsa-miR-454 hsa-miR-553 hsa-miR-450b-3p hsa-miR-1249 hsa-miR-135a hsa-miR-770-5p hsa-miR-34c hsa-miR-551b hsa-miR-1263 hsa-miR-630	hsa-miR-487a hsa-miR-219-2-3p hsa-miR-519b-3p hsa-miR-517b hsa-miR-802 hsa-mIR-1262	hsa-miR-575

hsa-miR-142-3p hsa-miR-30b hsa-miR-409-3p hsa-miR-301 hsa-miR-191 hsa-miR-376a mmu-miR-495 hsa-miR-148a hsa-miR-543 hsa-miR-642 hsa-miR-411 hsa-miR-505 hsa-miR-27a hsa-miR-181c hsa-miR-144 hsa-miR-15b hsa-miR-125a-5p hsa-miR-628-3p hsa-miR-216b hsa-miR-340 mmu-miR-374-5p hsa-miR-20a hsa-miR-184 hsa-miR-18a hsa-miR-190b hsa-miR-28 hsa-miR-377 hsa-let-7d hsa-miR-151-5p hsa-miR-149 hsa-miR-26b hsa-miR-340 hsa-miR-142-5p has-miR-1183			

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