Effect of remote ischaemic preconditioning in cardiac dysfunction and end-organ injury following cardiac surgery with cardiopulmonary bypass in children: A translational approach investigating clinical outcome and myocardial molecular biology.

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Abstract

Congenital heart disease (CHD) is the most common birth defect, causing an important rate of morbidity and mortality. Treatment of CHD requires surgical correction in a significant percentage of cases which exposes patients to cardiac and end organ injury. Cardiac surgical procedures often require the utilisation of cardiopulmonary bypass (CPB), a system that replaces heart and lungs function by diverting circulation into an external circuit. The use of CPB can initiate potent inflammatory responses, in addition a proportion of procedures require a period of aortic cross clamp during which the heart is rendered ischaemic and is exposed to injury. High O_2 concentrations are used during cardiac procedures and when circulation is re-established to the heart which had adjusted metabolically to ischaemia, further injury is caused in a process known as ischaemic reperfusion injury (IRI).

Several strategies are in place in order to protect the heart during surgery; however injury is still caused, having detrimental effects in patients at short and long term.

Remote ischaemic preconditioning (RIPC) is a technique proposed as a potential cardioprotective measure. It consists of exposing a remote tissue bed to brief episodes of ischaemia prior to surgery in order to activate protective pathways that would act during CPB, ischaemia and reperfusion.

This study aimed to assess RIPC in paediatric patients requiring CHD surgical correction with a translational approach, integrating clinical outcome, marker analysis, cardiac function parameters and molecular mechanisms within the cardiac tissue.

A prospective, single blinded, randomised, controlled trial was conducted applying a RIPC protocol to randomised patients through episodes of limb ischaemia on the day before surgery which was repeated right before the surgery started, after anaesthesia induction.

Blood samples were obtained before surgery and at three post-operative time points from venous lines, additional pre and post-bypass blood samples were obtained from the right atrium. Myocardial tissue was resected during the ischaemic period of surgery. Echocardiographic images were obtained before the surgery started after anaesthetic induction and the day after surgery, images were stored for later off line analysis.

PICU surveillance data was collected including ventilation parameters, inotrope use, standard laboratory analysis and six hourly blood gas analyses.

Pre and post-operative quantitation of markers in blood specimens included cardiac troponin I (cTnI) and B-type natriuretic peptide (BNP), inflammatory mediators including interleukins IL-6, IL-8, IL-10, tumour necrosis factor (TNF-α), and the adhesion molecules ICAM-1 and VCAM-1; the renal marker Cystatin C and the cardiovascular markers asymmetric dymethylarginine (ADMA) and symmetric dymethylarginine (SDMA).

Nitric oxide (NO) metabolites and cyclic guanosine monophosphate (cGMP) were measured before and after bypass.

Myocardial tissue was processed at baseline and after incubation at hyperoxic concentration during four hours in order to mimic surgical conditions. Expression of genes involved in IRI and RIPC pathways were analysed including heat shock proteins (HSPs), toll like receptors (TLRs), the transcription factors nuclear factor κ-B (NF- κ-B) and hypoxia inducible factor 1 (HIF-1). The participation of hydrogen sulfide enzymatic genes, apelin and its receptor were explored.

There was no significant difference according to group allocation in any of the echocardiographic parameters. There was a tendency for higher cTnI values and inotropic score in control patients post-operatively; however this was not statistically significant. BNP presented no significant difference according to group allocation.

Inflammatory parameters tended to be higher in the control group, however only TNF- α was significantly higher. There was no difference in levels of Cystatin C, NO metabolites, cGMP, ADMA or SDMA.

RIPC patients required shorter PICU stay; all other clinical and laboratory analysis presented no difference related to the intervention.

Gene expression analysis revealed interesting patterns before and after incubation. No differences were found according to RIPC or control allocation.

This study provided with valuable descriptive information on previously known and newly explored parameters in the study population. Demographic characteristics and the presence of cyanosis before surgery influenced patterns of activity in several parameters; numerous indicators were linked to the degree of injury suffered by the myocardium.

RIPC did not reduce markers of cardiac injury or improved echocardiographic parameters and it did not have an effect on end organ function; some effects were seen in inflammatory

responses and gene expression analysis. Nevertheless, an important clinical outcome indicator, PICU length of stay was reduced suggesting benefit from the intervention.

Larger studies with more statistical power could determine if the tendency of lower injury and inflammatory markers linked to RIPC is real. The present results mostly support findings of larger multicentre trials which have reported no cardiac benefit from RIPC in paediatric cardiac surgery.

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Dedication

This work is dedicated to my parents Carlos and Virginia, who have loved, missed me and supported me. You have given me the best example of effort and integrity and any and every achievement in my life I owe to you.

Author´s Declaration

I declare that, except where explicit reference is made to the contribution of others, the composition of this thesis and the work described within it was carried out entirely by myself and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signed ……..

Maribel Verdesoto

October 2016

Abbreviations

°C	degree centigrade
ACD	α-crystallin domain
ADMA	asimmetric dimethylarginine
ADP	adenosine diphosphate
Ag	antigen
AKI	acute kidney injury
Akt	protein kinase B
AlkPhos	alkaline phosphatase
ALT	alanine transaminase
AMP	adenosine monophosphate
APJ	apelin receptor
APLN	apelin
APTT	partial thromboplastin time
AS	academic supervisor
ASD	atrial septal defect
ASK1	apoptosis signal-regulating kinase 1
AST	aspartate transaminase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AV	aortic valve

AVSD	atrioventricular septal defect
B_0	zero standard
BAK	bcl-2 homologous antagonist/killer
BAX	bcl-2-like protein 4
Bcl-XL	B-cell lymphoma-extra large
BNP	brain natriuretic peptid
BSA	bovine serum albumin
Ca^{2+}	calcium
CABG	coronary artery bypass graft
CAM	cellular adhesion molecule
cAMP	cyclic adenosine monophosphate
CAT	cysteine aminotransferase
CBS	cystathionine β-synthase
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CH	concentration of hydrogen
CHD	congenital heart disease
CI	chief investigator
CK	creatin-kinase
CK-MB	creatin-kinase cardiac isoenzyme
Cl	chloride
cm/s	centimetres per second

CMIA	chemiluminescent microparticle immunoassay
CO_2	carbon dioxide
CoA	aortic coarctation
CONSORT	consolidated standards of reporting trials
COX-2	cyclooxygenase-2
CPB	cardiopulmonary bypass
CRP	C reactive protein
CRYAB	small heat shock protein B5
CSE	cystathione γ-lyase (CTH)
Ct	threshold cycle
CTE	C-terminal extension (small heat shock proteins)
CTH	cystathione γ-lyase (CSE)
cTn	cardiac troponin
cTnI	cardiac troponin I
cTnT	cardiac troponin T
CuZnSOD	copper-zinc superoxide dismutase
CXCR4	C-X-C chemokine receptor type 4
DDAH	dimethylarginine dimethylaminohydrolase
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECMO	extracorporeal membrane oxygenation
ecSOD	extracellular superoxide dismutase

EDTA	ethylenediaminetetraacetic acid
EF	ejection fraction
ELISA	enzyme-linked immunosorbent assay
eNOS	endogenous nitric oxide synthase
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
EUFS	fluorescence sensitivity setting
EUROCAT	European Surveillance of Congenital Anomalies
FBS	foetal bovine serum
Fe	iron
FS	fractional shortening
FtH	iron sequestering protein ferritin H chain
g	gram
GLUT	glucose transporter
GMP	guanosine monophosphate
GPCR	G protein coupled receptors
h	hours
H^+	hydrogen
H_2S	hydrogen sulphide
H_2SO_4 2N	sulfuric acid solution
Hb	haemoglobin
HCO_3	bicarbonate

HCT	haematocrit
HIF-1	hypoxia inducible factor 1
HIV	human immunodeficiency virus
HLHS	hypoplastic left heart syndrome
HO-1	heme oxygenase 1
HSP	heat shock protein
HSPB7	cardiovascular small heat shock protein
HUGO	human genome organisation
ICAM-1	intercellular adhesion molecule-1
ICU	intensive care unit
IL	interleukin
IM	internal membrane (mitochondria)
iNOS	inducible nitric oxide synthase
IQ	interquartile
IRI	ischaemic reperfusion injury
IV	intravenous
JAK	janus kinase
JDP	J-domain proteins
K^+	potassium
kDa	kilodalton
kg	kilogram
LDH	lactate dehydrogenase
LDL	light density lipoprotein

LPA	left pulmonary artery
LV IVRT	left ventricle isovolumetric relaxation time
LVOT	left ventricle outlet tract
M	molar
MAPCA	multiple aortopulmonary collateral arteries
MAPK	mitogen-activated protein kinases
mAU	milliAU (anson unit)
MCH	mean corpuscular haemoglobin
MCV	mean corpuscular volume
M-FiO_2	mean fraction of inspired oxygen
mg	milligram
MHC	major histocompatibility complex
miRNAs	micro RNA
mitoK_{ATP}	mitochondrial ATP sensitive potassium channel
ml	millilitre
mM	millimol
MMA	monomethyl-L-arginine
mmHg	millimetres of mercury
MMP	metalloproteinase
MnSOD	manganese superoxide dismutase
MPA	main pulmonary artery
M-Pmean	mean airway pressure

MPTP	mitochondrial permeability transition pore
mRNA	messenger ribonucleic acid
MV E/A ratio	mitral inflow E to A velocities ratio
MV LAT E/E'	lateral mitral annulus E to E´ ratio
MV LAT S	lateral mitral annulus systolic velocity
MV SEP S	septal mitral annulys systolic velocity
MVSEP E/E'	septal mitral annulus E to E´ratio
MYD-88	myeloid differentiation primary response gene 88
Na^+	sodium
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NBD	nucleotide binding domain (heat shock proteins)
NBS	sodium/bicarbonate symporter
NEF	nucleotide exchange factor
NF-κB	nuclear factor kappa B
ng	nanogram
NGAL	neutrophil gelatinase-associated lipocalin
NHE	sodium-hydrogen pump exchanger
NHS	National Health Service
NICOR	National Institute for Cardiovascular Outcomes Research
nM	nanomolar
nmol	nanomol

nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NSB	non-specific binding
NTD	N terminal domain
NTproBNP	N-terminal pro-brain natriuretic peptide
O_2	oxygen
O_2SAT	oxygen saturation
OD	optical density
OM	outer membrane (mitochondria)
PA	pulmonary artery
PAF	platelet activating factor
PBS	phosphate-buffered saline
PCO_2	partial pressure of carbon dioxide
PCR	polymerase chain reaction
PDA	patent ductus arteriosus
PDE	phosphodiesterase
PECAM-1	platelet-endothelial cell adhesion molecule-1
pg	picogram
pH	potential hydrogen
PHD	prolyl hydroxylases
PI	principle investigator

PI3K	phosphoinositide-3-kinase
PICU	paediatric intensive care unit
PK	protein kinase
pmol	picomol
PO	post-operative
PRMT	protein arginine methyltransferase enzymes
PT	prothrombin time
PV	pulmonary valve
RBC	red blood cell
RCT	randomised controlled trial
RD	reagent diluent
RHSC	Royal Hospital for Sick Children
RIPC	remote ischaemic preconditioning
RISK	reperfusion injury salvage kinase
RLU	relative light units
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
RV	right ventricle
S	standard
SAGE	serial analysis of gene expression

SBD	substrate binding domain (heat shock proteins)
SDF-1α	stromal cell-derived factor-1
SDMA	symmetric dimethylarginine
sHSP	small heat shock protein
SIRS	systemic inflammatory response syndrome
SOD	superoxide dismutase
ß-ME	beta-mercaptoethanol
STAT	signal transducers and activators of transcription
TCPC	total cavopulmonary connection
TGA	transposition of the great arteries
TLR	toll like receptor
TMB	tetramethyl benzidine
TNF-α	tumour necrosis factor α
TOF	tetralogy of Fallot
TV ANT S	tricuspid annular systolic velocity
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VSD	ventricular septal defect
WBC	white blood cell
µg	microgram
µl	microliter

Chapter 1. Introduction

1.1 Heart – Basic concepts on anatomy and function

The heart is the centre of the circulatory system, responsible for maintaining pulmonary and systemic circulation; it is located in the mediastinum between the lungs and is enclosed by the three pericardium layers, an external fibrous layer, and two serous parietal and visceral layers, the parietal layer is in contact with the myocardium and is also called epicardium.

Myocardium makes up the walls of the four chambers constituting the heart, it has a similar structure to skeletal muscle, with sarcomeres acting as a contraction unit with myosin, actin and troponin playing an important role involving the generation of action potential through the flow of sodium and potassium ions.

The internal structures of the heart are lined by endocardium, a smooth thin layer structurally similar to endothelium in order to prevent abnormal blood clotting.

The heart is divided into four chambers, two upper relatively thin walled chambers called left and right atria and two lower, thicker chambers, left and right ventricles. The base of the heart is composed by atria and vessels while ventricles form the apex.

As seen on *Figure 1.1* the left and right sides of the heart are separated by the septum and atria and ventricles are separated by valves, the right atrium and ventricle are separated by the tricuspid valve, consisting of three cusps of endocardium strengthened by connective tissue, while the left atrium is separated from the left ventricle by the bicuspid or mitral valve, made of two cusps. Papillary muscles originate in both ventricles and attach to their respective valvular cusps through chordae tendineae, strands of fibrous connective tissue, in order to prevent valvular prolapse. The atria receive blood into the heart which passes to the ventricles in a motion controlled by the bicuspid and mitral valves, when the ventricles contract the pressure is higher within the ventricles and the valves close in order to prevent backflow.

The cardiovascular system includes the heart acting as a pump and vasculature involving two circulatory networks: the systemic and pulmonary circulation. Physiologically the heart is divided into two synchronised pumps; the left heart pumps blood through the systemic circulation and the right heart propels the pulmonary circulation.

The right atrium (RA) receives unoxygenated blood returning from the general circulation through the superior vena cava from the upper body and the inferior vena cava from the lower body. From the RA blood flows through the tricuspid valve into the right ventricle (RV). When the RV contracts, the pressure is higher within the ventricle and the tricuspid valve closes in order to prevent backflow; the blood is pumped through the pulmonary artery, passing through the semilunar pulmonary valve consisting of three flaps and located at the junction between RV and pulmonary artery; it is forced open by contraction of the RV to then prevent backflow when the ventricle relaxes to start the pulmonary circulation where blood will be oxygenated.

Oxygenated blood returns from the lungs through four pulmonary veins to the left atrium (LA), passes through the mitral valve to the left ventricle (LV) with thicker walls than the RV, the RV pumps blood through the aorta to the general circulation after passing through the aortic semilunar valve.

Anatomical information obtained from Weinhaus and Roberts, 2009; Thiriet, 2014.

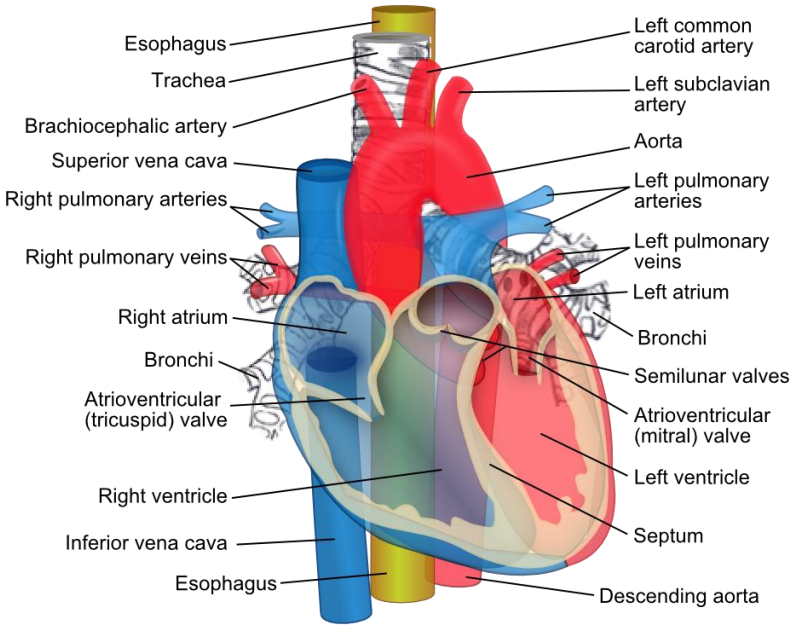

Figure 1.1. Heart anatomy. The image shows a frontal section of the heart, representing internal structures. Major vessels and their branches are shown. Image obtained at https://commons.wikimedia.org/wiki/File:Relations_of_the_aorta,_trachea,_esophagus_and_other_heart_structures.png, attribution: ZooFar; reproduced under creative commons license.

1.2 Congenital Heart Disease

Congenital heart disease (CHD) is defined as the presence of heart disease at birth as a consequence of abnormal intrauterine development; the types of possible structural and functional alterations are wide and the adaptive mechanisms resulting from them may cause complications of their own (reviewed by Adelman, 2011).

1.2.1 Incidence

CHD is the most common birth anomaly presenting with an incidence of 9 in 1000 live births. Incidence worldwide has increased over recent decades, probably due to better diagnostic resources. Geographical variability is marked presenting the highest incidence in Asia, followed by European countries (van der Linde *et al*, 2011).

Data collected from 16 European countries through the population based European Surveillance of Congenital Anomalies system (EUROCAT) revealed that 36000 children present with CHD at birth and 3000 further cases are diagnosed and terminated during pregnancy, suffer late foetal death or early neonatal death within the European Union every year with an overall incidence of 8 per 1000 births regardless of the aetiology (Dolk *et al*, 2011).

Incidence figures vary significantly depending on the account of different CHD diagnosis and the degree of the defect identified reaching a much larger incidence when smaller defects, which may never require medical treatment, are taken into account (Hoffman and Kaplan, 2002).

The United Kingdom (UK) presents a congenital defect incidence of 20.8 per 1000 live births; cardiovascular anomalies account for 30% of these cases (Tennant *et al*, 2010), representing a significant cause of childhood morbidity and mortality with an incidence of 5.6 per 1000 live births (Townsend *et al*, 2013).

Although the incidence of CHD has dropped over a 30 year period in the UK, the number of cardiac surgical procedures had a 60% increase during the last decade due to significant improvement in surgical techniques, bypass utilization and cardiac protective measures during surgery. Paediatric mortality caused by CHD dramatically dropped by 83% from 1979 to 2008 (Townsend *et al*, 2013), leading to a survival rate of 89.5% after 20 years in

representative areas (Tennant *et al*, 2010). For the first time a majority of adult patients are living with CHD presenting nevertheless with a lower quality of life even after having the defects surgically corrected (Townsend *et al*, 2013).

The majority of serious CHDs manifest in the early life of the child and can be identified promptly when adequate medical services are accessible thus allowing early treatment. The National Health Service (NHS) in the United Kingdom provides prenatal and paediatric cardiac assessment. After establishing the diagnosis, CHD management varies according to the type and severity of the defect comprising observation, pharmacologic treatment, non-surgical interventionism and corrective surgery. The choice of management is determined according to cardiac centres and medical criteria.

The latest data available corresponds to the financial year 2014/2015 during which 5532 cardiac surgical procedures were performed in children in the United Kingdom. From these, 4546 surgeries used cardiopulmonary bypass (CPB). During the same year 190 CPB interventions were performed at the Royal Hospital for Sick Children (RHSC), the national paediatric cardiac centre for Scotland (The National Institute for Cardiovascular Outcomes Research – NICOR).

1.2.2 Aetiology of Congenital Heart Disease

Cardiac development starts approximately 15 days after fecundation; the process involves several pathways and detailed processes which could be disrupted and result in CHD not only while the foetal heart is formed, but until circulation is established after birth.

A multifactorial aetiology of CHD, where genetic and environmental factors interact was hypothesised (Nora, 1968) and remained as a mainstream theory for a long time along with the concept that most CHD cases were isolated. While only around 20% of cases are related to genetic syndromes (Eskedal *et al*, 2004), an increased incidence of non-syndromic cases in subsequent pregnancies of 2 – 10% suggests a genetic component is relevant in the pathogenesis of CHD as well (reviewed by Wessels and Willems, 2010). New technology allowed powerful analysis identifying genetic causes and inheritance patterns linked to cardiovascular malformation whereas epidemiological studies have identified separate modifiable risk factors that could potentially lead to cardiac teratogenicity. *Table 1.1* shows genetic factors related to CHD aetiology.

Genetic cause	Mechanism/Outcome
Chromosome disorders	Down Syndrome, DiGeorge Syndrome
Gene deletions	Williams Beurer Syndrome
Single gene disorders	Alagille Syndrome, Marfan Syndrome, Noonan Syndrome
Non-syndromic single gene disorders	NKX2.5 gene: ASD, tricuspid valve abnormalities GATA4 gene: ASD, VSD
Copy number variation	Isolated CHD or associated to developmental delay
Single nucleotide polymorphisms	VEGF gene link to certain isolated cases of TOF
RNA abnormalities: micro RNA dysfunction	VSD link
Epigenetics: remodelling complexes and growth regulators dysfunction	Altered morphogenesis, hypertrophy

Table 1.1. Genetic aetiology of CHD. Abbreviations: ASD: atrial septal defect; CHD: congenital heart disease; GATA4: GATA binding protein 4; NKX2.5: NK2 homeobox 5; RNA: ribonucleic acid; TOF: Tetralogy of Fallot; VEGF: vascular endothelial growth factor; VSD: ventricular septal defect. Original table based on information by Pierpont *et al*, 2007; Huang *et al*, 2010; Richards and Garg, 2010; Gelb and Chung, 2014.

Identification of genes and pathways involved in anomalous heart development has been possible in syndromic and familial CHD cases; however the small percentage of monogenic genotypes as well as the large amount of susceptibility genes involved presenting low penetrance mutations make it a challenge to reveal clear pathogenesis pathways (reviewed by Wessels and Willems, 2010). In addition to this, investigation of single nucleotide mutations through high throughput sequencing techniques in sporadic CHD cases has produced data suggesting that several different pathways can produce a single specific defect (Smith *et al*, 2009).

Well known environmental risk factors during cardiac development in pregnancy include maternal illness, exposure to environmental agents and therapeutic use of certain drugs (Jenkins *et al*, 2007; Sibiude *et al*, 2014); while others such as alcohol consumption are believed to affect heart formation, however a clear correlation has failed to be established (Sun *et al*, 2015). Factors which can be modified in order to prevent CHD are shown in *Table 1.2*.

Environmental factor	Example
Maternal illness and conditions	Phenylketonuria, diabetes, rubella, febrile illnesses, influenza, epilepsy
Maternal therapeutic drug exposure	Thalidomide, vitamin A cogeners/retinoids, antibiotics, antiviral/antiretroviral agents: zidovudine, anticonvulsants, sedatives/hypnotics
Maternal non-therapeutic drug exposure	Heavy alcohol consumption?
Maternal environmental exposure	Organic solvents, herbicides, pesticides, and rodenticides, groundwater contamination

Table 1.2. Environmental factors contributing to the aetiology of CHD. Original table based on information by Jenkins *et al*, 2007; Huang *et al*, 2010.

1.2.3 Types of Congenital Heart Disease

Congenital heart disease can be classified into cyanotic or acyanotic taking into account their pathophysiologic basis and clinical presentation where right to left shunts cause decreased pulmonary blood flow and passing of non-oxygenated blood into the left ventricle which pumps mixed blood with low oxygen levels into the general circulation (reviewed by Adelman, 2011). Cyanotic status however may not always follow this classification parameters, the direction of shunting may gradually change as pulmonary vascular resistance varies causing acyanotic lesions such as ventricular septal defects (VSD) to evolve into cyanosis in certain cases. In addition to this, tetralogy of Fallot (TOF) cases can be defined as cyanotic or acyanotic referring only to the patient's condition at one specific point in time rather than the defect itself (reviewed by Jacobs, 2013).

Table 1.3 presents the classification and relative incidence of CHD according to cyanotic and acyanotic presentation.

Acyanotic	Cyanotic
Left-to-right shunts Ventricular septal defect (VSD) (20%) Atrial septal defect (ASD) (10%) Patent ductus arteriosus (PDA) (10%) Atrioventricular septal defect (AVSD) (2–5%) Aortopulmonary window (rare) **Left-sided obstructive lesions** Coarctation of the aorta (CoA) (10%) Congenital aortic stenosis (10%) Interrupted aortic arch (1%) Mitral stenosis (rare)	**Right-to-left shunts** TOF (10%) Pulmonary stenosis (10%) Pulmonary atresia: (5%) with intact ventricular septum with ventricular septal defect Tricuspid atresia (3%) Ebstein anomaly (0.5%) **Complex mixing defects** Transposition of the great arteries (TGA) (5–8%) Total anomalous pulmonary venous connection (2%) Truncus arteriosus (3%) Hypoplastic left heart syndrome (2%)

Table 1.3. Pathophysiological classification of lesions in congenital heart disease and approximate incidence. Table modified from Jacobs, 2013, with permission from John Wiley and Sons.

Classification according to segmental division of the cardiac anatomy provides an alternative comprehensive tool for practice. *Table 1.4* summarizes the segmental system of CHD classification including significant lesions in each category.

I. Great veins		**IV. Ventricles**	
Systemic veins	Systemic venous anomaly, superior or inferior vena cava	Right ventricle	TOF Double-chamber right ventricle
Pulmonary veins	Anomalous pulmonary venous connection	Ventricular septum	VSD
II. Atria		Left ventricle	Single ventricle, Hypoplastic left heart syndrome
Right atrium	ASD	**V. Ventriculoarterial junction**	
Atrial septum		Right ventriculoarterial valve	Pulmonary stenosis, insufficiency, atresia
Left atrium		Common ventriculoarterial valve	Truncus arteriosus
III. Atrioventricular junction		Left ventriculoarterial valve	Aortic stenosis, insufficiency, atresia
Right atrioventricular valve	Tricuspid stenosis or regurgitation Ebstein anomaly	Both ventriculoarterial valves	Transposition of the great arteries Double-outlet right or left ventricles
Common atrioventricular valve	AVSD	**VI. Great arteries**	
Left atrioventricular valve	Mitral stenosis or regurgitation	Pulmonary artery	Pulmonary arterial stenosis, sling
		Aorta	Subaortic stenosis,AoC Interrupted aortic arch
		Both great arteries	PDA
		Coronary arteries	Anomalous origin of coronary artery

Table 1.4. Segmental Classification of CHD. Abbreviations: AoC: aortic coarctation; ASD: atrial septal defect; AVSD: atrial-ventricular septal defect; PDA: patent ductus arteriosus; TOF: Tetralogy of Fallot; VSD: ventricular septal defect. Table modified from Jacobs, 2013, with permission from John Wiley and Sons.

Septal Defects

Septal defects occur as a communication between the right and left heart and may be present between the atria (ASD), the ventricles (VSD) or both (AVSD) occurring as a result of failure to close these communications during foetal life and birth (reviewed by Backer and Mavroudis, 2013; Mavroudis *et al*, 2013).

VSDs are the most common CHD with a prevalence of 2.64 per 1000 live births (van der Linde *et al*, 2011), representing one fifth of CHD when isolated and over half of all CHD cases when associated with other major defects. VSD manifestations depend on the size of the defect, ranging from asymptomatic undiagnosed cases to severe presentations, in addition pulmonary vascular resistance determines the degree of left to right shunt where oxygenated blood from the LV passes to the RV, if shunting is excessive clinical manifestations involving congestive heart failure will take place (reviewed by Mavroudis *et al*, 2013).

Management of septal defects depends on the size and manifestations of the defect, if surgical repair is required cardiac internal anatomy is usually accessed through RA sectioning and the defect is corrected placing a patch (Backer and Mavroudis, 2013; Mavroudis *et al*, 2013).

Tetralogy of Fallot

The pathological foundation of TOF was described as early as 1671 by Niels Stenson, however it was not until 1888 that Arthur Louis Etienne Fallot described the tetralogy that is still the basis to the contemporary description of TOF characterised by narrowing or atresia of the pulmonary outflow, VSD, overriding aorta and right ventricular hypertrophy (reviewed by Stewart *et al*, 2013).

TOF is the most common cyanotic CHD presenting a worldwide incidence of 0.34 per 1000 livebirths showing a higher prevalence in Asia (van der Linde *et al*, 2011). Variants of TOF may include pulmonary stenosis, absent pulmonary valve, atrio-ventricular canal (AVSD), double outlet right ventricle (DORV), pulmonary atresia-VSD and pulmonary atresia-VSD with multiple aortopulmonary collateral arteries (MAPCAs) (Jacobs, 2000).

The restricted outflow of the pulmonary artery causes inadequate blood oxygenation, in addition to this the communication between ventricles is large and non-restrictive in most cases causing the pressure to be equal in both ventricles; the flow direction through the VSD depends on the severity of the pulmonary outflow restriction, if the obstruction is severe a large right to left shunt would occur causing deoxygenated blood from the RV to enter the LV, resulting in poorly oxygenated blood reaching the systemic circulation causing patients to be exposed to hypoxia in a chronic manner (Apitz *et al*, 2009). RV hypertrophy is attributed to the high pressure resulting from pulmonary stenosis.

TOF is frequently diagnosed prenatally; however most affected children present the condition after birth and are diagnosed after clinical suspicion is confirmed with transthoracic echocardiography (reviewed by Apitz *et al*, 2009). Management for the condition is based on surgical correction of the defect which involves accessing the internal cardiac anatomy through an incision in the right atrium, excision of myocardial tissue obstructing the right ventricle outflow tract and VSD closure (Stewart *et al*, 2013).

Hypoplastic Left Heart Syndrome

Hypoplastic left heart syndrome (HLHS) is a term used to describe a group of CHDs presenting with severely underdeveloped left heart structures, including hypoplasia of the ascending aorta and the left ventricle which is unable to sustain systemic circulation becoming dependant on the right ventricle through a patent ductus arteriosus involving mixing of oxygenated and un-oxygenated blood (reviewed by Hirsch *et al*, 2013).

HLHS has a prevalence of 0.26 per 1000 livebirths (Ferencz *et al*, 1985) and 28% of HLHS cases are diagnosed through prenatal ultrasounds which are highly sensitive for major CHD (Montaña *et al*, 1996).

Surgical correction of HLHS aims to create separate systemic and pulmonary circulations supported by a single right ventricle; it is performed in a staged approach starting with a first stage or Norwood procedure which is usually performed during the first 30 days of the patient's life and must obtain systemic blood flow from the right ventricle to the aorta and coronary arteries without obstruction, relieve any obstruction to pulmonary venous return and limit pulmonary blood flow through an appropriate systemic to pulmonary artery shunt (reviewed by Hirsch *et al*, 2013; Shillingford *et al*, 2013).

Stage two involves a bidirectional Glenn or hemi-Fontan procedure, it is usually performed between 4 to 6 months of age (Lamberti *et al*, 1990) and aims to preserve right ventricular function providing adequate pulmonary blood flow while decreasing volume overload at the right ventricle until the patient can go through Fontan completion. The bidirectional Glenn procedure consists of performing an anastomosis between the superior vena cava and the pulmonary artery (reviewed by Shillingford *et al*, 2013) while the hemi-Fontan involves a connection of the superior vena cava and the right atrial junction to the pulmonary arteries, augmentation of the branch pulmonary arteries, and placement of a temporary patch between the pulmonary arteries and the right atrium (reviewed by Hirsch *et al*, 2013).

Finally, stage three or completion Fontan is done from 18 to 24 months of age and involves connecting the inferior vena cava to the pulmonary arteries using a lateral tunnel or extra cardiac method. In the lateral tunnel method, a tunnel shaped patch is placed inside the atrium and connects it to the pulmonary artery directing blood from the inferior vena cava straight to the pulmonary artery while the extra cardiac method deviates blood from the inferior vena cava outside the heart through a synthetic tube leading to the pulmonary artery (reviewed by Hirsch *et al*, 2013).

1.3 Cardiac Surgery and Cardiopulmonary Bypass

Children presenting with CHD have different degrees of complexity and the treatment for patients requiring surgical correction according to the diagnosis and individual circumstances entails a multidisciplinary effort that involves cardiologists, surgeons, anaesthetists and intensive care among other subspecialties. During a proportion of interventions, the work of haemodynamists is crucial as the use of a bypass system makes these procedures possible.

1.3.1 Cardiopulmonary Bypass

CPB is a system designed to replace the functions of heart and lungs by diverting circulation to monitored devices in order to support the patient during cardiac procedures. The development of this technique drastically broadened the spectrum of corrective procedures that are possible to perform nowadays.

Numerous attempts of devices managing the concept of extracorporeal circulation were made before the 1950s (Dale and Schuster, 1928; DeBakey, 1934), however it was John Gibbon who developed and applied CPB successfully to an animal, and later used CPB managing to complete the repair of an atrial septal defect with a surviving patient (Gibbon, 1954). During following decades CPB methods were refined to efficaciously reproduce circulatory processes occurring in normal physiology in an effective and secure way to the patient.

The CPB circuit starts with a cannula usually inserted on the right atrium or in the superior and inferior vena cava from where blood is drained into an extracorporeal system and directed to a venous reservoir; blood is then pumped through a heat exchanger and an oxygenator where gas interchange takes place. Oxygenated blood goes through a filter and bubble trap which removes micro-aggregates and air particles and finally returns to the ascending aorta through a cannula from where oxygenated blood circulates to tissues, gas exchange takes place and the blood returns to the right atrium starting the process once again. Blood obtained from suction and vents during surgery is stored in a separate cardiotomy reservoir and is microfiltered before reaching the venous reservoir (reviewed by Hessel and Edmunds, 2003; Kouchoukos *et al*, 2003; Lawson *et al*, 2013).

Clamps on arterial and venous lines can be used to control flow; the system additionally includes safety devices, sensors for pressure, temperature, blood gases, pH and oxygen saturation as well as sites for obtaining blood samples; cardioplaegic solutions are administered and controlled through a separate circuit within the system while on occasions a haemoconcentrator is added in order to remove water and small molecules (reviewed by Hessel and Edmunds, 2003) (*Figure 1.2*).

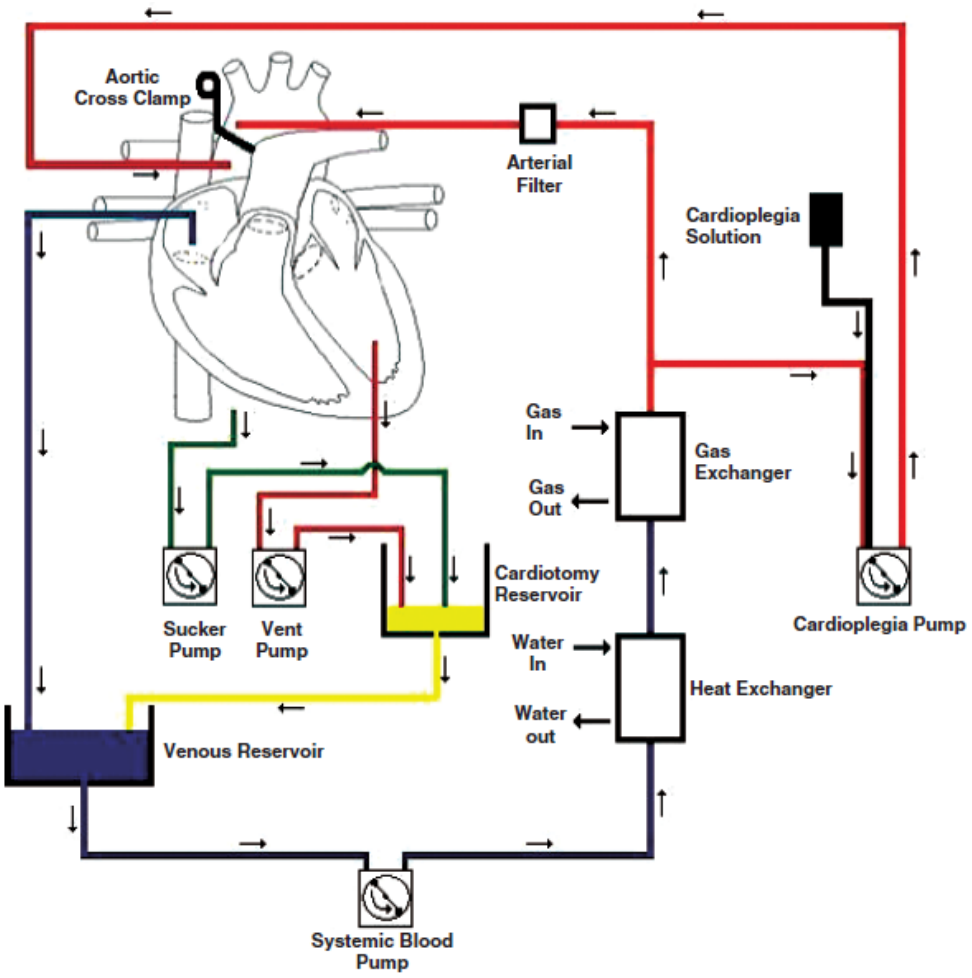

Figure 1.2. Schematic representation of the CPB circuit. Image from Machin and Allsager, 2006, reproduced with permission from Oxford University Press.

The bypass process has several stages of high technical complexity, *Table 1.5* shows a simplified account of the main events taking place during CPB. Heparin is administered for anticoagulation before the system is established, initially the heart is cannulated and connected to the bypass machine which reduces the work load of the heart while pumping highly oxygenated blood (100% O_2). In a second stage the aorta is clamped stopping blood flow to the heart, starting a period of ischaemia protected cardioplegia; in the next phase the heart is reperfused with 100% O_2 blood in order to protect the brain, and beating is re-

established; finally the bypass system is removed and the heart workload is restored. Protamine is administered in order to reverse the anticoagulation effects of heparin once the surgical results are satisfactory and the need for restarting CPB is extremely unlikely (reviewed by Lawson *et al*, 2013; Whiting *et al*, 2015).

Stage	Procedure	Heart
I	Heart is cannulated and connected to the bypass machine	Reduced work load High O_2
II	Aortic cross-clamp	No myocardial blood flow Ischaemia protected cardioplegia
III	Aortic cross clamp removal	Reperfusion High PO_2 Heart beating Reduced work load
IV	Bypass system removal	Re-establishment of circulation and work load

Table 1.5 Cardiopulmonary bypass stages. Original table.

1.3.2 Preventive measures

From the implementation of CPB, improvement of the system, technology and materials used have been a constant priority, however the unavoidable insults involving cardiac surgery and CPB have required the utilisation of additional protective measures aimed to minimise the risks to the patient.

Hypothermia

Reduction of the patient´s temperature is one of the main protective strategies used during cardiac surgery for myocardial and systemic protection. It reduces the metabolic rate and hence oxygen consumption; at a temperature of 32°C oxygen consumption in the whole body is reduced by 45% (reviewed by Chai, 2013). Hypothermia has been proven to lower the occurrence of brain ischaemia (Bernard *et al*, 2002). Metabolic rate in the brain is reduced exponentially as the temperature drops causing minimal psychomotor adverse effects with circulatory arrest for up to 41 minutes at 18°C (Wypij *et al*, 2003). Deep (<20°C) and moderate (>25°C) hypothermia have been showed to inhibit inflammatory responses and to delay the onset of inflammation after cardiac surgery (Kimura *et al*, 2002; Drapalova *et al*, 2014).

Cardioplegia

Cardioplegia is achieved through the use of solutions causing depolarization or hyperpolarization of the membrane and mechanical arrest of the heart in order to stop all electrical and contractile activity. The composition of solutions varies according to institutions as there is no consensus regarding the most appropriate method, likewise there is no clear conclusion on the use of hypothermia in favour of cardioplegia or both (reviewed by Chai, 2013).

Cardioplegia can be induced by using blood or crystalloid solutions; blood cardioplegia would present certain advantages such as the ability to transport oxygen, a similar osmotic and electrolyte composition and the capacity to reduce oxidative stress by scavenging free radicals with a relative benefit of blood over crystalloids (Zeng *et al*, 2014; reviewed by Chai, 2013).

A paediatric study found that cold blood cardioplegia followed by warm blood reperfusion preserved adenosine triphosphate (ATP) in cyanotic patients (Modi *et al*, 2004), however there is no definite conclusion on the advantages of one approach and the decision is made by particular institutions and surgical teams.

High O_2 administration

High concentration of oxygen reaching 100% is routinely delivered during and after CPB in order to prevent cellular hypoxia as oxygen delivery may be altered by several factors including changes in microcirculation, hypothermia, fluid shift, blood loss and myocardial dysfunction. This preventive measure, although necessary, is not harmless as it can increase systemic oxidative stress and has an impact in the metabolic processes taking place within the heart after hypoxia and reperfusion (reviewed by Spoelstra-de Man *et al*, 2015).

Further preventive measures involve adequate management during and after CPB including haemofiltration, leukocyte filtration, the use of heparin coated circuits, use of steroids, filters to avoid air and emboli, and maintainance of pressures, antigoagulation, pH levels, haematocrit and glycaemia control (reviewed by Berkowitz and Gaynor, 2013).

1.4 Ischaemic Reperfusion Injury

Organs can be safely exposed to ischaemia for an established period of time which can be extended by reducing metabolic needs; cardioplegia and hypothermia are routinely used as protective measures during cardiac surgeries in order to allow the exposure of the heart to ischaemia while limiting irreversible damage (Hearse *et al*, 1976; Dapralova *et al*, 2014).

Early reperfusion has the potential to limit the injury and improve postoperative outcome, nevertheless reperfusion itself triggers processes including oxidative stress that result in a further insult to the susceptible cardiac tissue increasing the injury already caused by ischaemia in a process known as ischaemic reperfusion injury (IRI) first described in a dog model more than fifty years ago (Jennings *et al*, 1960); therefore the final injury area results of multiple events taking place during ischaemia and reperfusion including microvascular and contractile dysfunction, arrhythmia and myocyte death (reviewed by Hausenloy and Yellon, 2013).

After the myocyte membrane loses integrity, enzymes such as lactate dehydrogenase, creatine-kinase (CK), creatine-kinase cardiac isoenzyme (CK-MB) and cardiac troponin (cTn) are released; these enzymes are used as markers for the diagnosis and establishment of the extent of the final cardiac injury (reviewed by Halestrap and Pasdois, 2009; Fathil *et al*, 2015).

In addition to this, systemic reactions take place in response to extracorporeal circulation; the contact of blood with foreign surfaces triggers inflammatory and immunologic responses throughout the body which may cause a systemic inflammatory response syndrome (SIRS) that could result in severe end organ damage, leading to neurological injury, lung and renal dysfunction (reviewed by McGuinness *et al*, 2008; Pągowska-Klimek *et al*, 2015).

1.4.1 Ischaemia

After the aorta is clamped during the second stage of CPB, the heart is rendered ischaemic. Low levels of oxygen and ATP are received by the myocardium forcing it to go into an anaerobic metabolism through glycolisis causing lactic acid to accumulate and the potential of hydrogen (pH) to drop (reviewed by Tapuria *et al*, 2008; Kharbanda *et al*, 2010). Most of the ATP is obtained by glycolysis and is consumed by the mitochondria in order to maintain its membrane potential (reviewed by Murphy and Steenbergen, 2008).

Compensatory mechanisms are triggered; excessive positively charged protons activate the sodium/hydrogen (Na^+/H^+) pump exchanger (NHE), introducing an overload of Na^+. As there is a lack of ATP the sodium/potassium (Na^+/K^+) ATPase, usual compensatory mechanism and ATP dependant, is not able to compensate for the excess of Na^+ triggering the NA^+/Ca^{2+} pump which balances Na levels at the cost of mitochondrial and cytosolic Ca^{2+} surplus leading to a state of cardiomyocite hypercontractility (Tani and Neely, 1989; reviewed by Hausenloy and Yellon, 2013). Ca^{2+} overload is further enhanced due to low transport of cytosolic Ca^{2+} into the sarcoplasmic reticulum (Krause and Hess, 1984; Kaplan *et al*, 1992).

As a response to low ATP, adenylate kinase acts rising adenosine monophosphate (AMP) levels, some AMP is converted into adenosine which enters a purine degradation pathway; these nucleosides drip out of the cell, producing vasodilation and an eventual depletion of adenine nucleotides (reviewed by Halestrap and Pasdois, 2009).

1.4.2 Reperfusion

After the circulation is restored once the aorta is de-clamped, the heart receives a sudden supply of blood containing high oxygen concentration (100%) which is used in order to protect the brain during surgery. The oxygen received is excessive for the adapted cardiac tissue resulting in significant harm (Hearse *et al*, 1973) whilst starting the production of reactive oxygen species (ROS) along with macrophage activation while the pH levels go rapidly back to normal (reviewed by Tapuria *et al*, 2008). Restored pH levels lead to a loss of cellular viability in a 'pH paradox' (Bond *et al*, 1991); this in combination with the very specific conditions resulting from ischaemia: oxidative stress, adenine nucleotide depletion and high Ca^{2+} levels which are not compensated fast enough, results in the opening of the mitochondrial permeability transition pore (MPTP), a protein complex which acts as a nonselective channel of the inner mitochondrial membrane (reviewed by Halestrap and Pasdois, 2009). This causes the mitochondrial membrane potential to collapse resulting in ATP exhaustion, leakage of apoptotic factors and further rise of ROS leading to cell death by apoptosis and necrosis (reviewed by Murphy and Steenbergen, 2008). Intercellular junctions disseminate these effects to neighbouring cells increasing the final cardiac injury (reviewed by Kharbanda, 2010).

1.4.3 Mechanisms

Hypercontrature of cardiomyocites / Ca^{2+} overload

High Ca^{2+} concentration results after cellular transporters attempt to balance conditions of reperfusion and ischaemia; in addition sarcolemmal L-type Ca^{2+} channels and a poor import of cytosolic Ca^{2+} into the sarcoplasmic reticulum contribute to excessive concentration of Ca^{2+} (reviewed by Turer and Hill, 2010).

Excessive cytosolic Ca^{2+} induces contraction in an uncontrolled manner once ATP production recovers causing hypercontractility (reviewed by Rodríguez-Sinovas *et al*, 2007); in addition to this, rigor-induced cellular shortening takes place as a response to a slow metabolic recovery while cytosolic ATP is sustained at minimal levels as would occur in severely harmed mitochondria after reperfusion (Ventura-Clapier and Veksler, 1994).

Electrical coupling between cells is an important process that links cardiomyocytes through gap junctions in specialised areas allowing coordinated contraction, Ca^{2+} overload disrupts this process leading to arrhythmias (reviewed by Ruiz-Meana *et al*, 2008). Connexin 43, a gap junction protein, has been linked to the spread of IRI as prolonged ischaemia causes the protein to dephosphorylate increasing permeability between cells (Schulz *et al*, 2003).

Animal studies have shown that avoiding Ca^{2+} overload by the use of intracoronary Ca^{2+} channel antagonists or by inhibiting the Na^+/H^+ pump exchanger limits the resulting injury (Klein *et al*, 1989; Gumina *et al*, 1999). Large clinical trials however have not reproduced these positive results in humans where reducing Ca^{2+} overload by using eniporide, the same inhibitor of the Na^+/H^+ antiporter used in the mentioned animal study, did not have a beneficial effect over the final injury in the clinical setting (Zeymer *et al*, 2001). Similarly, the use of caldaret, a compound that inhibits Na^+/Ca^{2+} exchange and potentiates Ca^{2+} intake to the sarcoplasmic reticulum, failed to show benefit (Bär *et al*, 2006). It has been suggested that different timing of drug administration between animal and human studies which due to clinical circumstances in myocardial infarct patients was later than animal protocols, could be linked to the conflicting results as Ca^{2+} overload would be expected to have happened already (reviewed by Ibanez *et al*, 2011).

pH restoration

The rapid normalization of pH to physiologic levels plays an important role in the mechanisms of injury; the effect of acidosis in myocardial viability after exposure to ischaemia has been assessed in rat culture models showing that a normal pH with or without re-oxygenation was linked to a 90% loss of viability while maintaining acidosis resulted in survival of all cultured cells, linking a fast return to a normal pH to cardiac injury independently of other factors that may contribute to the injury as well (Bond *et al*, 1991).

Controlled acidotic media proved to prevent hyper-contractility and spiking of cytosolic Ca^{2+} (Ladilov *et al*, 1995); in addition low pH re-oxygenation prevented MPTP opening and depolarization (Kim *et al*, 2006). It has also been suggested that bicarbonate ($HCO3^-$) transport plays an important role in pH recovery during reperfusion, hence inhibiting the Na+/HCO3- symporter (NBS) along with the NHE prevent hypercontracture (Schäfer *et al*, 2000).

Mitochondrial permeability transition pore (MPTP)

The mitochondrial membrane is formed by an outer membrane (OM) and an internal membrane (IM) which during physiologic conditions is extremely selective or virtually impermeable, however permeability may occur under specific circumstances leading to apoptosis and necrosis; permeability is the consequence of the organization of the proteins adenine nucleotide translocator from the IM and voltage-dependent anion channel from the OM into the MPTP which is formed between the two membranes (reviewed by Kroemer and Reed, 2000; Bernardi *et al*, 2015).

MPTP opens in response to Ca^{2+} overload, a reaction further promoted by the presence of ROS, inorganic phosphate and a low IM potential, as this happens the IM is depolarised and the matrix swells causing the OM to breach, as a consequence proteins from the intermembrane space reach the cytosol triggering caspases leading to cellular death (reviewed by Heusch *et al*, 2010; Bernardi *et al*, 2015).

Preventing MPTP opening has been shown to reduce IRI *in vivo* and *ex vivo* models (Ong *et al*, 2014); inhibition of MPTP opening using cyclosporine-A, an immunosuppressive drug

that acts blocking the action of cyclophiline-D with a pore component, has been proven to prevent IRI in several *ex-vivo* studies and is linked to Ca^{2+} control (Nazareth *et al*, 1991; Griffiths *et al*, 2000). Similar findings were obtained after a pilot clinical trial showed that administration of cyclosporine-A in patients during reperfusion treatment of myocardial infarction resulted in a smaller infarct when compared to placebo (Piot *et al*, 2008).

Oxidative stress and endothelial dysfunction

Endothelial dysfunction was first shown in coronary arteries of dogs exposed to ischaemia and reperfusion which failed to relax in response to vasodilators such as thrombin (Ku, 1982) and acetylcholine (VanBenthuysen *et al*, 1987); this effect was strongly present after reperfusion, not so following ishcaemia only, and persisted in a chronic manner due to abnormally regenerated endothelial cells (Kaeffer *et al*, 1996), it has been suggested that it is established before necrosis takes place (Tsao *et al*, 1990).

Free radical increase has shown to be involved in this process as relaxation was preserved when superoxide scavengers such as SOD were used (Mehta *et al*, 1989); free radicals act through an inhibitory effect in the production of NO which would fail to inhibit neutrophil activation while free radicals potentiate neutrophil adhesion to the endothelium making it possible for an amplified endothelial injury to take place (reviewed by Laude *et al*, 2001). Endothelial injury leads to a rise in vascular permeability and signalling for inflammatory cells (reviewed by Turer *et al*, 2010). Furthermore, NO has a well-known role inhibiting platelet aggregation (Radomski *et al*, 1987), hence the lack of NO activity could lead to vasospasm as well as thrombosis.

During reperfusion significant oxidative stress is generated starting with a rise in free radicals, which are highly reactive, unstable and have a short life; free radicals and oxidants such as ROS are part of physiologic metabolism and signalling while kept under control by homeostatic scavenging systems, however their increase in stress situations can cause significant damage to cellular components through membrane lipid peroxidation, protein denaturation and deoxyribonucleic acid (DNA) breakage (reviewed by Zweier and Talukder, 2006; Zhou *et al*, 2015).

Myocardial injury has been linked to oxidative stress in animal and human studies (Zweier *et al*, 1989; Ferrari *et al*, 1990). ROS production and build up, in addition to antioxidant consumption have been associated to injury and cardiac contractile dysfunction (reviewed by Ibanez *et al*, 2011). Evidence suggests a cardioprotective effect of free radical scavengers such as antioxidants, superoxide dismutase (SOD) and glutathione peroxidase (GSHpx) including influence of the expression of genes linked to antioxidant processes to the outcome after ischaemia (reviewed by Marczin *et al*, 2003).

NO is a relatively stable free radical and a gaseous signalling molecule with particular physiologic and pathologic functions; according to its concentration and external conditions it can provide protection or additional damage. It is typically accepted that low concentrations of NO have a protective effect involving cyclic guanosine monophosphate (cGMP) signalling and an inhibition of mitochondrial Ca^{2+} accumulation while having the opposed ability to increase superoxide production and its detrimental consequences (reviewed by Halestrap and Pasdois, 2009; Zhao *et al*, 2015).

NO is synthesised from L-arginine by the enzyme nitric oxide synthase (NOS) (Moncada *et al*, 1989); two of its isoforms are constitutive: endothelial NOS (eNOS) and neuronal (nNOS), while inducible NOS (iNOS) is expressed as a response to inflammatory signalling and can produce higher levels of NO (reviewed by Ibanez *et al*, 2011; Zhao *et al*, 2015).

Low NO availability has been recognised as a possible IRI mechanism, therefore investigation of NO donors as potential preventive measures has attracted interest. Nicorandil, a mitochondrial ATP sensitive potassium (mitoK_{ATP}) channel opener and NO donor proved beneficial when administered as a single dose to infarct patients before reperfusion in an initial considerably large clinical trial (Ishii *et al*, 2005), however conflicting results were obtained in an even larger clinical trial which found no difference on infarct size after administering nicorandil in a continuous dose, it should be noted that further oral nicorandil was linked to higher ejection fraction during patient recovery (Kitakaze *et al*, 2007).

Hydrogen sulfide (H_2S) is a different gas messenger involved in vascular relaxation, angiogenesis and IRI; it has been linked to cardioprotection, NO activity, mitoK_{ATP} channels, kinase pathways and inhibition of oxidative stress; it has been shown that neonatal rat cardiomyocytes treated with a H_2S donor stimulated enzymatic activity reducing levels of

ROS (Sun *et al*, 2012). IRI studies have shown H_2S cardiac protection effects including apoptosis reduction and smaller infarct size (Li *et al*, 2015).

Enzymatic activity of super oxide dismutase (SOD) has a role controlling the levels of ROS, there are three active forms in humans including soluble, copper-zinc SOD (CuZnSOD, SOD1), Mitochondrial manganese SOD (MnSOD, SOD2) and extracellular SOD (ecSOD, SOD3) (reviewed by Bresciani *et al*, 2015).

Stimulation of SOD has proven protective in several animal models of IRI (Chen *et al*, 1998; Wang *et al*, 1998). These results were not always reproduced and two small initial clinical trials failed to show a protective benefit from the administration of human recombinant SOD during post-ischaemic procedures (Murohara *et al*, 1991; Flaherty *et al*, 1994).

Since then further investigation has shown that increased activity of CuZn SOD and MnSOD has a protective effect during IRI circumstances (Sun *et al*, 2012); a study performed in transgenic (Tg) mice presenting with overexpression of cardiomyocyte specific extracellular SOD (ecSOD) resulted in increased availability of NO and a directly measured attenuation of ROS related to a smaller injury implying a possible therapeutic pathway (Obal *et al*, 2012); ecSOD was also assessed in an intra-myocardial transplantation model of gene modified bone marrow cells obtaining smaller infarct size after ischaemia and reperfusion involving the control of apoptotic factors (Pan *et al*, 2014).

Apoptosis

Evidence suggests that during ischaemia necrosis accounts for cardiomyocyte death while apoptosis is initiated during ischaemia but does not take place until reperfusion (Gottlieb *et al*, 1994; Freude *et al*, 2000); an acceleration of apoptosis has also been observed during reperfusion in animal models and a human trial (Fliss *et al*, 1996; Veinot *et al*, 1997).

Apoptosis is one of the consequences of several processes taking place during ischaemia and reperfusion including gene activation, stimulation of caspases, mitochondrial activity including MPTP opening, loss of membrane potential and cytochrome C release, nuclear and cellular shrinkage and fragmentation, selective modification of the cellular membrane and DNA fragmentation by the activation of endonucleases (reviewed by Buja, 2005).

Preventing apoptosis mechanisms using caspase-3 inhibitors has successfully reduced injury size with the protective effect taking place only when accurately timing the intervention before reperfusion (Mocanu *et al*, 2000; Zhao *et al*, 2003; Lee *et al*, 2011).

Autophagy

Autophagy is a physiologic intracellular process that degrades and recycles long lived organelles and proteins, this process can be further triggered by stress and it has been recognised that autophagy activity is raised in the myocardium after ischaemia (Yan *et al*, 2005).

Reduction of autophagy by the use of the inhibitor urocortin during IRI in a neonate and adult rat *in vitro* model resulted in the inhibition of cellular death (Valentim *et al*, 2006); conversely a second study performed in atrial derived cultured cells proved that enhanced autophagic activity had protective properties against IRI (Hamacher-Brady *et al*, 2006). An analysis of different pathways leading to autophagy has brought to light that its mechanisms during ischaemia are different to those during ischaemia/reperfusion along with evidence that autophagy would be protective during ischaemia while playing a damaging role during reperfusion (Matsui *et al*, 2007).

Inflammation and Immunity

Humoral Response

Complement

The complement cascade is part of the innate immune system consisting of more than 30 proteins that interact in different pathways in order to act against bacteria, link innate to adaptive immunity, and remove immune complexes and products of inflammatory injury (reviewed by Walport, 2001).

Complement can be triggered by ischaemia and is exacerbated during reperfusion; ischaemia triggers the complement cascade as during necrosis mitochondrial components including cardiolipin are released and bind to early factors of the complement cascade such as C1, C2,

C3 and C4 and in addition remain as binding sites for later components including C5a and C5b (Rossen *et al*, 1994).

It has been suggested that late complement mediators such as C5 have a clear role in the physiopathology of IRI while early components participation including C3a has not been elucidated but seems to be minor (Busche and Stahl, 2010).

Active complement mediators such as C5b-9 alter the production of ROS and Ca^{2+} flux, contributing to IRI (Kim *et al*, 1987; Berger *et al*, 1993). Interaction between endothelial cells and leukocytes altering vascular homeostasis is mediated by the complement; C5a stimulates vasodilation and promote neutrophil chemotaxis, aggregation and production of ROS but also stimulates vasoconstriction through the production of thromboxane from the interaction of platelets with polymorphonuclear cells. C5b-9 influences endothelial response modifying its interaction with neutrophils by an increase in the production of von Willebrand factor while leukocyte adhesion molecule is upregulated through the action of nuclear factor kappa B (NF-κB), it alters endothelium relaxation and stimulates leukocyte activation and migration while causing neutrophils to produce ROS, proteolytic enzymes and arachidonic acid metabolites (reviewed by Stahl *et al*, 2012).

Inhibition of C5 has proven to protect against cardiac IRI in a rat model (Vakeva *et al*, 1998), clinical studies assessing the efficacy of the C5 inhibitor pexelizumab have shown inconclusive results with initial promising evidence showing cardioprotection during percutaneous interventions after myocardial infarction (Granger *et al*, 2003) and coronary artery bypass graft (CABG) procedures using CPB (Shernan *et al*, 2004; Verrier *et al*, 2004). However, larger trials showed only a non-significant positive effect when applied to similar groups of patients (APEX AMI investigators *et al*, 2007; Smith *et al*, 2006).

Further investigation has been done on C5a receptors, whose lack has proven to be protective in a murine model involving a reduced recruitment of inflammatory cells to the injury site presenting a lower endothelial transmigration (Mueller *et al*, 2013).

Paediatric surgeries using CPB have been proven to activate the complement lectin pathway which has a link to the development of SIRS (Pągowska-Klimek *et al*, 2015); furthermore patients presenting insufficiency of lectin binding factors had a lower risk of developing SIRS after cardiac surgery (Pągowska-Klimek *et al*, 2016).

ROS

ROS are involved in the physiopathology of IRI as mentioned before, in addition to its oxidative stress effects they have a role as inflammatory triggers inducing the action of cytokines and leukocyte chemotaxis (reviewed by Frangogiannis *et al*, 2002).

Cytokines

Several cytokines play a role on the mediation and establishment of IRI, factors involved in the process include cellular adhesion molecules such as selectins as well as interleukins. Mast cells localised at the injury site degranulate and release tumour necrosis factor alpha (TNF-α) which starts a cytokine cascade by stimulating the activity of IL-6 in leukocytes leading to the induction of intercellular adhesion molecule-1 (ICAM-1) which is responsible for neutrophil response (Frangogiannis *et al*, 1998).

TNF-α and IL-1 in combination with ROS are also responsible for triggering the activity of NF-κB which is an important transcription factor controlling the up and down regulation of several inflammatory, adhesion and growth mediators making it a key control point for stress response, immune function and oncogenesis (reviewed by Tornatore *et al*, 2012).

Cellular Response

Leukocyte activity and migration after injury follow an established model mediated by cellular adhesion molecules (CAMs) starting with thetering which is facilitated by selectins where transient adhesion induces cellular rolling; triggering follows through the mediation of chemokines causing integrin activation to then continue with strong adhesion, mediated by integrins, and migration as leukocytes travel into the tissue influenced by promigratory factors (Adams and Shaw, 1994; reviewed by Cook-Mills *et al*, 2011).

After an ischaemic episode neutrophils are trapped in site and adhere to the capillary endothelium blocking reperfusion; chemotactic signalling changes the shape of neutrophils making them less malleable, they accumulate and initiate the action of adhesion molecules (reviewed by Frangogiannis *et al*, 2002).

Neutrophils rarely interact with the vessel wall, they roll instead in a process mediated by the CAM selectin family which include L-selectin (CD65L), E-selectin (CD26E), and P-selectin (CD62P); L-selectin is constitutively expressed by leukocytes while E-selectin follows *de novo* synthesis only and P-selectin is constitutively found in platelets and endothelium, however all three molecules are involved in leukocyte migration into tissues (Hahne *et al*, 1993). Different patterns of P-selectin expression have been shown in a rat IRI model (Chukwuemeka *et al*, 2005), while blocking E and L-selectin resulted in attenuated neutrophil migration in sheep (Carter *et al*, 2000).

Strong adhesion of neutrophils to the endothelium requires the activity of CD11/CD18 (β2) integrins over ligands in the endothelium such as ICAM-1, activation of CD11/CD18 takes place after the neutrophil is exposed to chemotactic molecules including IL-8 and platelet activating factor (PAF); transmigration finally occurs as a result of chemotactic stimulus and activity of platelet-endothelial cell adhesion molecule-1 (PECAM-1) (reviewed by Albelda *et al*, 1994; Lyck and Enzmann, 2015).

Interaction between ICAM-1 and the CD11b component may be crucial for triggering respiratory dysfunction in the neutrophil leading to oxidative injury in the myocyte (Entman *et al*, 1992). Blocking PECAM-1 has effectively limited IRI in rat and cat models through inhibiting transendothelial migration of neutrophils (Gumina *et al*, 1996; Murohara *et al*, 1996).

1.5 Conditioning of the heart

Ischaemic cardiac conditioning consists of the application of several brief periods of ischaemia and reperfusion before (pre-conditioning), during (per-conditioning) or after ischaemia (post-conditioning). This model of controlled ischaemia triggers endogenous cardioprotective mechanisms which act after the stimulus has been withdrawn in order to limit IRI (Zhao *et al*, 2003; Cheung *et al*, 2006; Bøtker *et al*, 2010).

1.5.1 Pre-conditioning

During planned periods of heart ischaemia such as in elective cardiac surgeries, there is the potential to apply upfront preventive strategies. Several established techniques previously reviewed in this chapter are routinely applied during surgery to limit myocardial injury allowing the safe conduct of the procedure. Despite this, myocardial injury occurs particularly with extended periods of cardiopulmonary bypass and ischaemia.

Pre-conditioning has emerged as a potential strategy which may provide additional protection to the myocardium. Conditioning prior to cardiac surgery involving CPB can be either pharmacological or mechanical; the research in this thesis will focus on mechanical pre-conditioning only.

1.5.2 Direct ischaemic pre-conditioning

Direct ischaemic preconditioning involves the exposure of the heart to brief periods of ischaemia through aortic clamping previous to the ischaemic phase of CPB in order to generate protection against ischaemia and IRI during CPB through the activation of protective pathways involving endothelial function, blood flow and a reduction of inflammatory cell activity (reviewed by Tapuria *et al*, 2008).

This process, first tested in a canine model thirty years ago (Murry *et al*, 1986) and subsequently in human patients (Yellon *et al*, 1993) has been evaluated in numerous trials producing beneficial results regarding its systemic effects and over different organs including the heart by conferring protection against IRI (Codispoti *et al*, 2006; Reutzel-Selke

et al, 2008; Franchello *et al*, 2009) while producing important changes including metabolic adaptation and facilitating cell survival (Nadtochiy *et al*, 2015).

Unfortunately the invasive nature of the intervention is a major drawback. Aortic clamping has been linked to pulmonary complications, heart failure and spinal cord injuries secondary to ischaemia (reviewed by Gelman, 1995). Although clamping is a necessary measure during CPB, additional insults could cause harm to the aorta and the already vulnerable myocardium.

1.5.3 Remote ischaemic preconditioning

Remote ischaemic preconditioning (RIPC) has emerged as a non-invasive alternative to direct preconditioning. It is based on the principle that inducing non-lethal ischaemic stimulus to a tissue bed remote from the heart could potentially trigger the same protective mechanisms as direct conditioning, providing local and systemic resistance to subsequent ischaemic exposure and IRI (Kharbanda *et al*, 2002).

The RIPC concept, was initially realised in a dog model when a myocardial region was protected from IRI by inducing temporary ischaemia by coronary occlusion subtending a different myocardial region (Przyklenk *et al*, 1993). Later, using a pig model it was found that stimulus in non-myocardial tissue could protect the heart as limb preconditioning was proven to reduce the extent of myocardial infarction (Kharbanda *et al*, 2002).

RIPC works through local and systemic pathways including neural, humoral and subcellular responses which act through two identified phases of protection, an early phase or first window of protection and a late phase or second window of protection.

1.5.4 Early phase of protection

During the early protective phase acute fast responses take place through post-translational modification of existing proteins, with protective effects starting immediately after the preconditioning stimulus. It has been shown that inhibition of protein synthesis does not alter the RIPC response (Thornton *et al*, 1990); thus the process is not dependant on gene

activation and protein synthesis but on the regulation of metabolic pathways which decay with time, resulting in protection loss after 3 to 4 hours (reviewed by Tapuria *et al*, 2008).

Mechanisms acting during the acute phase of protection are transmitted through humoral and neural pathways (Lim *et al*, 2010), inter-organ communication through extracellular vesicles has been recently suggested (Giricz *et al*, 2014) as well as a role for non-neuronal cardiac cholinergic communication (Oikawa *et al*, 2015).

Neural Pathway

The neural pathway starts with the activation of afferent fibres by substances produced locally at the stimulus area such as bradykinin, adenosine and calcitonin gene-related peptide (CGRP) which act on their efferent counterparts leading to cardiac effects (reviewed by Aimo *et al*, 2015; Gill *et al*, 2015).

This hypothesis has been supported by loss of protection after nerve sectioning in the stimulus area and after the use of receptor antagonists (Lim *et al*, 2010; Loukogeorgakis *et al*, 2005). Furthermore, neurotransmitters such as CGRP have been linked to protection activation (Wolfrum *et al*, 2005). The neural component of RIPC has also been proven by obtaining cardiac protection after the stimulation of skin nociceptors alone, which resulted in sympathetic activity and cardiac sensory responses producing heart protection related to trauma in animal models (Jones *et al*, 2009).

Humoral Pathway

Humoral RIPC activity depends on the local production of substances that would reach the myocardium through the bloodstream as the full effect of RIPC does not seem to be present unless circulation is restored (reviewed by Aimo *et al*, 2015).

The humoral theory is supported by experimental evidence showing that transfusion of blood from a conditioned animal resulted in cardiac protection in a second recipient animal (Dickson *et al*, 1999). Furthermore it has been demonstrated that human blood dialysate following RIPC provided protection in rabbit hearts, suggesting cross species effects

(Shimizu *et al*, 2009). In addition, models of heart transplant showed that when RIPC was induced in the recipient animal, the donor heart presented a reduced infarct size after transplantation; as no neural connection was present, the effects can be attributed to humoral activity (Konstantinov *et al*, 2005).

Early phase of protection pathway

Subcellular mechanisms start after the release of adenosine, bradykinin and opioids, all agonist ligands to G protein coupled receptors (GPCR), which transmit intracellular signals initially through the phosphoinositide-3-kinase (PI3K) – protein kinase B (Akt) pathway involving anti apoptotic survival kinases (reviewed by Murphy and Steenbergen, 2008). This stimulates the activity of the eNOS signaling pathway which includes interactions with nitric oxide (NO) and plays a critical role for cardioprotection as shown by loss of the protective effects after inhibition of eNOS activity (Yang *et al*, 2013).

This process leads to the reduction of anaerobic glycolysis, producing lower levels of lactate and less acidosis, a factor that contributes to reduce intracellular Ca. Furthermore, during ischemia ATP is consumed at a lower rate in the conditioned tissue, preventing ATP depletion (Murry *et al*, 1990; Finegan *et al*, 1995).

In addition, GPCR agonists start a cascade via the activation of protein kinase C (PKC), which controls mitochondrial ATP sensitive potassium (mitoK$_{ATP}$) channels which regulate mitochondrial matrix volume; after PKC activation mitoK$_{ATP}$ channels open resulting in a reduction of the mitochondrial membrane potential, decreasing Ca uptake and its concentration during reperfusion thus preserving mitochondrial integrity (reviewed by Murphy and Steenbergen, 2008; Costa, 2013; Simkhovich *et al*, 2013).

Preservation of mitochondrial enzyme activities through post-translational mechanisms have been shown to attenuate tissue hyperoxygenation, reducing the formation of ROS during ischaemia (Zhu *et al*, 2007).

PKC has also been linked to inhibition of apoptosis as it interacts with the MPTP directly, preventing it from opening and thus stopping the collapse of the mitochondrial membrane potential (Baines *et al*, 2003). *Figure 1.3* shows the RIPC cascade.

Numerous mediators act during this process, recently the small protein stromal cell-derived factor-1 (SDF-1α) has been hypothesised to be involved, SDF-1α is hypoxia induced and has the ability to promote cardioprotection acutely through its receptor CXCR4 which controls responses through the reperfusion injury salvage kinase (RISK) pathway; increased levels of SDF-1α in plasma were linked to infarct size reduction, presenting a loss of the effects after experimental CXCR4 receptor block (Davidson *et al*, 2013).

In addition certain miRNAs that influence signalling pathways such as P-Akt and MAPK have been identified as early RIPC mediators (Li *et al*, 2014).

Early onset of increased coronary blood flow and a higher diastolic volume have been reported which seem to prevent reperfusion induced arrhythmias and to limit infarct size while preserving left ventricular function (reviewed by Costa *et al*, 2013).

1.5.5 Late phase of protection

Mediated by a high rate synthesis of new cardioprotective proteins, the mechanisms involving the second window of protection respond to gene activation, producing a phenotypic modification in the cardiac tissue related to inflammation and oxidative stress. Protective effects are seen 12 to 24 hours after the stimulus and remain active for 3 to 4 days (Kuzuya *et al*, 1993; Tang *et al*, 1996; Rizvi *et al*, 1999; reviewed by Costa *et al*, 2013).

After the RIPC stimulus signalling molecules acting as triggers are released causing the activation of kinases which stimulate the action of transcription factors, causing up-regulation or inhibition of target genes coding for proteins which work as late phase mediators influencing inflammation, MPTP opening and endothelial dysfunction (*Figure 1.3*) (reviewed by Tapuria *et al*, 2008; Costa *et al*, 2013).

Certain mechanisms such as the opening of mitoK_{ATP} channels, which allows the conservation of the mitochondrial membrane potential are involved in both early and late phases of protection (Xu *et al*, 2001).

Attenuation of tissue hyperoxygenation is achieved during late RIPC by improving oxygen consumption in the mitochondria through the induction of synthesis of mitochondrial proteins consequently maintaining its enzymatic activity (Li *et al*, 2011). It has been

established that mitochondrial and death receptor pathways of apoptosis are also regulated during late RIPC (Stein *et al*, 2007). An involvement of protein kinase A (PKA), PI3K and serine threonine kinase Akt through the stimulation of NO synthesis has been found in animal endothelial cells (Bellis *et al*, 2009).

Candidate genes coding for transcription factors, antioxidants, glycolysis enzymes and inflammatory genes have been linked to preconditioning (reviewed by Simkhovich *et al*, 2003; Li *et al*, 2011). Certain genes such as *NF-κB*, and hypoxia-inducible factor 1 (*HIF-1*) have been identified as transcription factors that play an important role in late RIPC (reviewed by Tranter *et al*, 2010; Ong and Hausenloy, 2012).

Although *NF-κB* mediates inflammation during IRI it triggers an opposite effect on the heart after 24 hours of the RIPC stimulus as its increased levels cause its inhibitor IκB to act resulting in a reduction of NF-κB after reperfusion, followed by iNOS enhanced expression and NO production which would have anti-inflammatory effects, with the overall process reflecting in a reduction of infarct size and improved ventricular performance (reviewed by Costa *et al*, 2013).

During the second window of protection even more enhanced anti-inflammatory reactions take place as genes involved in apoptosis, chemotaxis, exocytosis, migration, cellular adhesion and innate immunity are supressed. Protective genes such as heat shock proteins and genes acting against oxidative stress have been found to be upregulated while the expression of pro-inflammatory mediators was reduced in a human expression profile study (Konstantinov *et al*, 2004).

The wide variety of agents acting during the late phase of protection may involve humoral or neural activity, however they are not referred in this thesis as acting in the humoral or neural pathways that mediate the initial steps of the process but as final effectors.

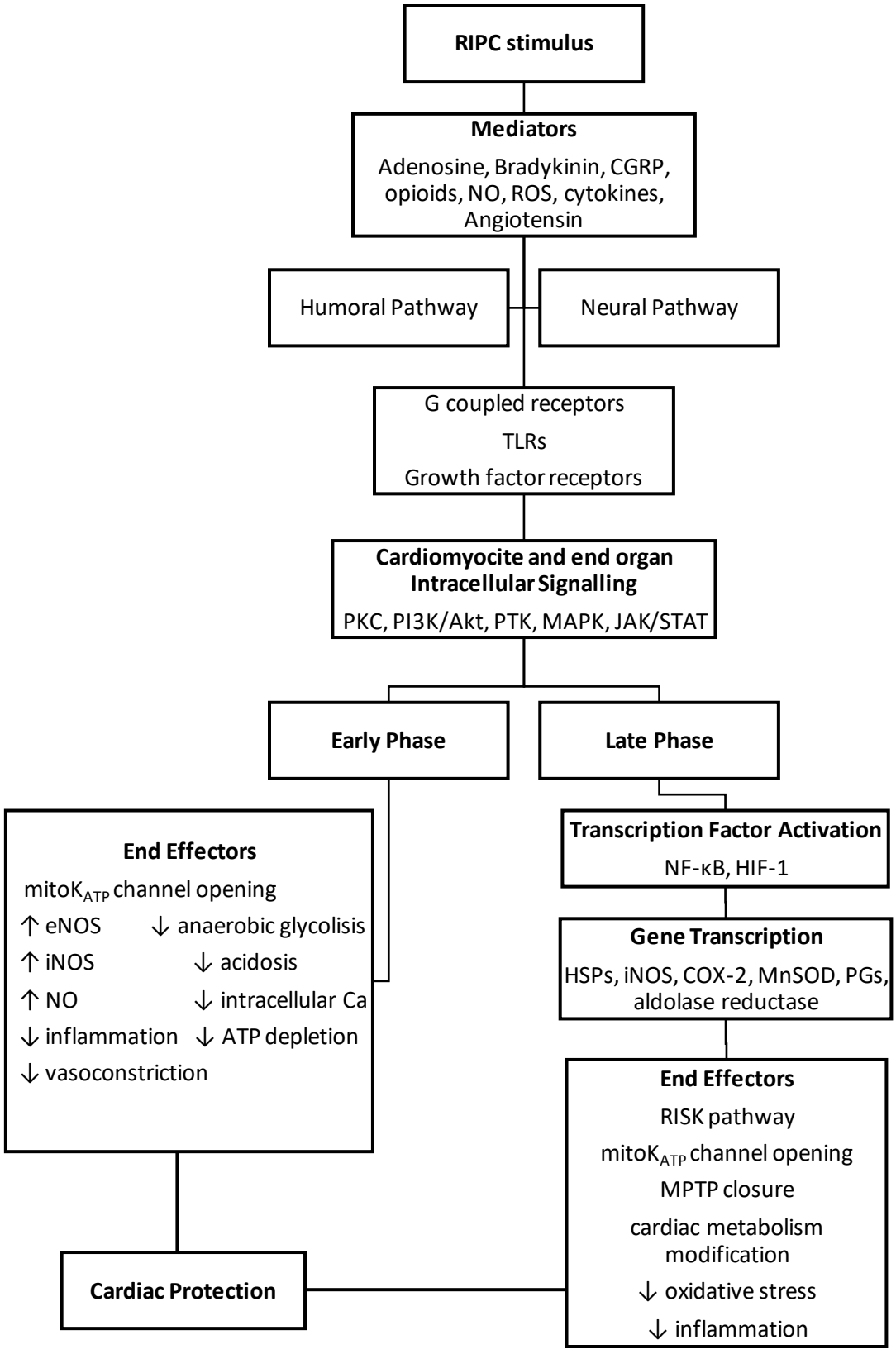

Figure 1.3. Mechanisms of early and late phases of RIPC protection. After RIPC stimulus in an organ such as a limb, initial mediators are rapidly upregulated and reach the cardiac and end organ tissues through humoral and neural pathways while starting a systemic

response, intracellular signalling leads to the activity of end effectors after post-translational protein modification during the early phase of protection. At the same time signalling molecules activate transcription factors leading to stimulating the expression of key genes which then act on late phase end effectors. These modifications prevent the metabolic and toxic effects taking place during IRI providing cardiac protection. Abbreviations: CGRP: calcitonin gene related peptide; COX-2: cyclooxygenase 2; eNOS: endogenous nitric oxide synthase; HIF-1: hypoxia inducible factor 1; HSPs: heat shock proteins; iNOS: inducible nitric oxide synthase; JAK/STAT: janus kinase/signal transducers and activators of transcription; MAPK: mitogen-activated protein kinase; mitoKATP: mitochondrial potassium ATP dependent; MnSOD: mitochondrial antioxidant manganese superoxide dismutase; MPTP: mitochondrial permeability transition pore; NF-κB: nuclear factor kappa B; NO: nitric oxide; PI3K/Akt: phosphoinositide 3-kinase/protein kinase B; PGs: prostaglandins; PKC: protein kinase C; PTK: protein tyrosine kinase; RISK: reperfusion injury salvage kinase; RIPC: remote ischaemic preconditioning; ROS: reactive oxygen species; TLRs: toll like receptors. Original figure based on information by Aimo *et al*, 2015; Costa *et al*, 2013; Crowley and McIntyre, 2013; Endre 2011; Gill *et al*, 2015; Hausenloy and Yellon, 2008; Lehotský *et al*, 2009; Martin-Puig *et al*, 2015; Yellon and Downey, 2003.

Inflammatory response

RIPC has been shown to have an effect on inflammatory responses which starts after the initial stimulus and is considerably heightened during the late phase of protection, causing a suppressed proinflammatory transcription in circulating leukocytes with effects on chemotaxis, innate immunity, signalling, and apoptosis (Konstantinov *et al*, 2004). Reduced adhesion and modified expression patterns have also been found in neutrophils affecting cytokine production, exocytosis and phagocytosis in humans (Shimizu *et al*, 2010).

An initial pattern of higher inflammatory activity after RIPC has been shown in cardiac surgical patients which would be expected to trigger latter protective reactions; high levels of cytokines such as IL-1β, IL-8 and TNF-α, as well as elevated HIF-1α and procaspase-3 levels and high myeloperoxidase activity by the time of bypass were shown combined with a cardioprotective effect after surgery (Albrecht *et al*, 2013).

Table 1.6 presents animal studies supporting the numerous mechanisms involved in the RIPC process for both phases of protection. While most studies have produced positive results reflected in infarct size reduction among other parameters, more recent publications have found detrimental results linked to RIPC, specially linked to neonatal myocardium (Schmidt *et al*, 2014b).

Study	Species	RIPC phase	Mechanism	Mechanism/ Effector	Outcome
Gho *et al*, 1996	Rat	Early	Neural pathway	-	Infarct size reduction, protection lost by ganglion block.
Tang *et al*, 1999	Rabbit	Early and late	Neural pathway	CGRP	Infarct size and CK reduction.
Schoemaker and van Heijningen, 2000	Rat	Early	Neural pathway*[1]	Bradykinin	High bradykinin reduced infarct size.
Ding *et al*, 2001	Rabbit	Early	Neural pathway	Adenosine	Infarct size reduction, effect reduced by adenosine antagonists and abolished by nerve section.
Wang *et al*, 2001	Rat	Late	-	iNOS	Infarct size and neutrophil infiltration reduction, effects lost by iNOS inhibition.
Xiao *et al*, 2001	Rat	Late	Neural activity (CGRP)	iNOS, CGRP	Infarct size and CK reduction, effects lost by CGRP and iNOS inhibition.
Hu *et al*, 2002	Rat	Late	Neural activity	CGRP	Infarct size and CK reduction, increased CGRP expression.
Liem *et al*, 2002	Rat	Early	Neural pathway	Adenosine	Reduced infarct size, effect abolished by ganglion block.
Patel *et al*, 2002	Rat	Early	Humoral pathway	Opioids	Infarct size reduction, effect attenuated by Naloxone (opioid receptor antagonist).
Weinbrenner *et al*, 2002	Rat	Early	Humoral pathway	PKC	Reduced infarct size by PKC up-regulation, ganglion block did not interfere
Li *et al*, 2004	Mouse	Late	-	iNOS, NF-κB	Infarct size reduction, increased NF-κB and iNOS. Effects lost in animals with iNOS or NF-κB deletions
Schulte *et al*, 2004	Mouse	Late	Neural or humoral activity	Adenosine A1 receptors	Infarct reduction, effect lost in A1 receptor knockout mice.
Wolfrum *et al*, 2005	Rat	Early	Neural and humoral pathways	Bradykinin, PKC	Infarct reduction, effects abolished by prevention of PKC activation either by bradykinin antagonist or by autonomic ganglion block.

Konstantinov *et al*, 2005	Pig	Early	Humoral pathway	mitoK$_{ATP}$ channel activation	Infarct size reduction in preconditioned recipients of cardiac transplant after donor heart denervation.
Zhang *et al*, 2006	Rat	Early	-	κ-opioid receptors	Infarct size reduction, similar effect produced by a κ-opioid receptor agonist and abolished by its antagonist.
Heidbreder *et al*, 2008	Rat	Early	Humoral pathway	MAPK pathways	Infarct size reduction, abolished effects by various MAPK inhibitors.
Jones *et al*, 2009	Mouse	Early	Neural pathway	Bradykinin, PKC, mitoK$_{ATP}$ channels	Infarct size and apoptosis reduction after skin nociceptors stimulation.*[2]
Wu *et al*, 2011	Rat	Late phase	-	MnSOD, mitoK$_{ATP}$	Infarct size and ventricular arrhythmia reduction, increased MnSOD. Effects lost after mitoK$_{ATP}$ channel blocker use.
Cai *et al*, 2013	Mouse	Late phase	Humoral activity	HIF-1, IL-10	Infarct size reduction linked to IL-10 activated by HIF-1, effect lost after HIF-1 inhibition.
Davidson *et al*, 2013	Rat	Early phase	Humoral pathway	SDF-1α, CXCR4 receptor	Infarct size reduction, SDF-1α increased in plasma. Effects lost after CXCR4 receptor block.
Rassaf *et al*, 2014	Mouse	Early phase	Humoral pathway	Nitrite	Infarct size, mitochondrial respiration and ROS production reduced, linked to higher nitrite levels.
Brandenburger *et al*, 2014	Rat	Early phase	-	miRNA miR-1	miR-1 differential expression.
Li *et al*, 2014	Mouse	Early and late phases	Humoral	miR-144	miR-144 was increased by RIPC in blood and myocardium and decreased by IRI.
Giricz *et al*, 2014	Rat	Early phase	Inter-organ communication	Extracellular vesicles	RIPC increased extracellular vesicles. Perfusates from preconditioned donors mediated a reduction in recipients' infarct size.
Hibert *et al*, 2014	Rat	Early phase	Humoral pathway	-	RIPC regulated proteins involved in oxidation, inflammation and metabolism.

Schmidt *et al*, 2014a	Pig	Early phase	-	-	RIPC combined with metabolic support with glucose and insulin reduced infarct size while RIPC alone had a tendency for infarct size increase (non significant).
Schmidt *et al*, 2014b	Rabbit	Early phase	Humoral pathway	-	Reduced infarct size and better LV function in adult hearts. Increased infarct size and poorer LV function in neonatal hearts after RIPC.
Oikawa *et al*, 2015	Mouse	Early phase	Non-neuronal cardiac cholinergic system	Acetylcholine	RIPC increased ATP cardiac content.

Table 1.6. Animal studies on the mechanisms of RIPC. Abbreviations: CGRP: calcitonin gene-related peptide; CK: creatin kinase; CXCR4: Chemokine (C-X-C Motif) Receptor 4; iNOS: inducible nitric oxide synthase; miR: microRNA; mitoK_{ATP} channel: mitochondrial ATP-sensitive potassium channel; MnSOD: mitochondrial antioxidant manganese superoxide dismutase; MPKA: mitogen-activated protein kinase; NF-κB: nuclear factor kappa B; PKC: protein kinase C; ROS: reactive oxygen species; SDF-1α: stromal-derived-factor-1α. Original Table.

*[1] Although the focus of the study was on neural or humoral pathways specifically, overlaps may have been possible.

*[2] Preconditioning stimulus was not ischaemic, used in order to support neural pathway.

1.6 RIPC human experience

Increasing interest in the potential benefits of RIPC has led to an increase in human trials over recent years. Patients undergoing planned elective cardiac surgery and CPB present the ideal conditions for the application of preconditioning and its potential benefits. RIPC adult evidence is more ample, however adult RIPC outcomes may not be applicable in children due to age related differences in physiology and immature responses.

1.6.1 RIPC adult evidence

Numerous RIPC clinical trials have been performed in recent years, a literature search resulted in 23 adult human randomised controlled trials (RCT) applying RIPC to patients undergoing cardiac surgery and CPB presenting an analysis of cardiac injury and other parameters. The majority of studies recruited patients presenting coronary disease who required CABG procedures (14), four studies analysed the intervention before valve surgery, four trials included any type of cardiac surgery, and one trial recruited high risk cardiac procedures only. *Table 1.7* shows a summary of the relevant adult RIPC trials to date.

Study protocols induced ischaemia through the inflation of a blood pressure cuff in an upper or lower limb during 3 or 4 ischaemic periods lasting 4, 5 or 10 minutes each intercalated with equal periods of reperfusion. Twenty one trials performed the intervention after anaesthesia induction and before the surgery started. One study applied RIPC prior to anaesthesia induction (Slagsvold *et al*, 2014), one started the intervention at the moment of the first surgical incision (Young *et al*, 2002), while only one trial applied the intervention 18 hours before surgery (Wagner *et al*, 2010).

Outcome measures for all trials included markers of cardiac injury such as cardiac troponin I (cTnI), cardiac troponin T (cTnT), high sensitivity cardiac troponin T (hscTNT), CK-MB, B-type natriuretic peptide (BNP), N-terminal pro-brain natriuretic peptide (NTproBNP), cardiac function parameters or cardiac tissue analysis. In addition certain studies included haemodynamic criteria, inflammatory markers, assessment of renal function with acute kidney injury (AKI) criteria and creatinine measurements, as well as neurocognitive dysfunction and brain injury parameters such as S100 (See *Table 1.7*).

The data obtained by these trials presents promising results for the application of RIPC, with 16 studies presenting positive outcomes, six studies finding absolutely no benefit from the intervention and two trials showing detrimental effects (*Table 1.7*). One trial reported higher troponin levels and longer need of adrenaline support in the RIPC group (Young *et al*, 2012) while a second study found a higher incidence of a composite endpoint of myocardial infarction and new arrhythmias in the RIPC group with no difference in cardiovascular outcome after six months in procedures using isoflurane as the anaesthetic (Lucchinetti *et al*, 2012).

Four studies looked at myocardial tissue expression of specific genes, finding higher expression of HIF-1α and procaspase 3 before CPB as well as higher myeloperoxidase and IL-1β after CPB (Albrecht *et al*, 2013), increased phosphorylation of signal transducers and activators of transcription 5 (STAT5) during reperfusion (Heusch *et al*, 2012). However no difference in iNOS or eNOS expression was demonstrated after RIPC (Wagner *et al*, 2010). Finally microRNA array analysis failed to identify differential expression due to RIPC (Slagsvold *et al*, 2014).

Tissue analysis showed that RIPC stimulus resulted in increased mitochondrial respiration, Akt presented a significantly higher activation before aortic clamp and higher phosphorylation of its substrates during reperfusion (Slagsvold *et al*, 2014). In addition, the activity of metalloproteinases 2 and 9 (MMP-2/9), which are involved in tissue remodelling following myocardial damage, was attenuated after RIPC and correlated to lower cTnT levels (Zitta *et al*, 2014).

Study	Type of trial	Patients	Surgery	RIPC Protocol	Outcome measures	Results
*Hausenloy *et al*, 2007	Double blinded RCT	57 adults C: 30 RIPC: 27	CABG	3 x 5 min cycles in right upper limb by BPC inflation to 200 mmHg after AI	cTnT	↓ cTnT at 6, 12, 24, and 48 h (p=0.005)
Venugopal *et al*, 2009	Single blinded RCT	45 adults C: 22 RIPC: 23	CABG	3 x 5 min cycles in right upper arm by BPC inflation to 200 mmHg after AI	cTnT	↓ cTnT at 72 h (p=0.017)
Ali *et al*, 2010	Single blinded RCT	100 adults C: 50 RIPC: 50	CABG	3 x 5 min cycles in upper arm by BPC inflation to 200 mmHg after AI	CK-MB	↓ CK-MB at 8, 16, 24 and 48 h (p=0.026)
Li *et al*, 2010	Double blinded RCT	81 adults C: 27 RIPC: 26 Percondition: 28	Valve replacement	3 x 4 min cycles in right lower limb BPC inflation to 600 mmHg after AI	cTnI and haemodynamic parameters	No significant difference for RIPC. Per-conditionig showed benefit.
Rahman *et al*, 2010	Double blinded RCT	162 adults C: 82 RIPC: 80	CABG	3 x 5 min cycles in left upper limb after AI	cTnT and haemodynamic parameters	No significant difference
Thielmann *et al*, 2010	Single blinded RCT	53 adults C: 27 RIPC: 26	CABG	3 x 5 min cycles in left upper arm by BPC inflation to 200 mmHg after AI	cTnI, renal function, mortality, cardiac and cerebro-vascular events	↓ cTnI at 6, 12, 24, and 48 h (P<0.0001)
Wagner *et al*, 2010	Single blinded RCT	101 patients C: 35 RIPC: 33 Tramadol: 33	CABG	3 x 5 min cycles upper limb ischaemia by BPC inflation to 40 mmHg above BP 18 hours before surgery	cTnI at 8, 16 and 24 hours after surgery, iNOS and eNOS expression in myocardium	↓ cTnI at 8 hours after surgery in the RIPC group (p=0.043) ↑ cTnI at all time intervals after surgery in the tramadol group (p<0.018; p<0.001;p<0.03)

Study	Type of trial	Patients	Surgery	RIPC Protocol	Outcome measures	Results
Choi *et al*, 2011	RCT blinded clinicians	n: 76 C: 38 RIPC: 38	Valve surgery	3 x 10 min cycles in thigh by BPC inflation to 250 mmHg after AI	Renal injury markers, AKI, CK-MB, clinical parameters	↓ CK-MB at 24 h (p=0.017) and shorter ICU stay (p=0.013) after RIPC
Karuppasamy *et al*, 2011	RCT blinded patients and surgeons	n: 54 C: 27 RIPC: 27 M:45 F:9	CABG	3 x 5 min cycles in left upper arm by BPC inflation to 200 mmHg after AI	cTnI, CK-MB, BNP, IGF-1, myostatin and cytokines in blood, haemodynamic parameters	No significant difference
Wu *et al*, 2011	RCT no blinding information	n: 75 C:25 RIPC I: 25 RIPC II: 25 M:26 F:49	Mitral valve replacement	I: 3 × 5 min cycles in right upper arm by BPC inflation to 200 mmHg II: I + 2 × 10 min cycles in right thigh by BPC inflation to 450 mmHg All protocols after AI	cTnI, clinical parameters	↓ cTnI in RIPC II group (no statistic information provided)
Heusch *et al*, 2012	Single blinded RCT	24 C: 12 RIPC: 12	CABG	3 x 5 min cycles in left upper arm by BPC inflation to ≥200 mmHg after AI	cTnI, myocardial expression of cardioprotective proteins	↓ cTnI 72 hours after surgery (p=0.023) ↑ phosphorylation of STAT5 during reperfusion in myocardium
Kottenberg *et al*, 2012	Single blinded RCT	n: 72 M: 60, F: 12 RIPC + IF:20 RIPC + PF: 14 C +IF: 19 C + PF: 19	CABG	3 x 5 min cycles in left upper arm by BPC inflation to 200 mmHg after AI	cTnI, creatinine, inotropic support	RIPC + IF: ↓ cTnI AUC (p=0.004), peak (p=0.004) and serial (p<0.041) compared to IF alone. RIPC + PF showed no significant difference compared to PF alone

Study	Type of trial	Patients	Surgery	RIPC Protocol	Outcome measures	Results
Lomivorotov *et al*, 2012	RCT	80 patients C: 40 RIPC: 40	CABG	3 x 5 min cycles in right arm by BPC inflation to 200 mmHg after AI	cTnI, CK-MB and haemodynamic parameters	↑ cardiac index immediately after RIPC in the treatment group ($p<0.05$)
Lucchinetti *et al*, 2012	Double blinded RCT	57 patients C:28 RIPC:27	CABG	4 x 5 min cycles in right thigh by BPC inflation to 300 mmHg after AI	hscTnT peak and AUC, NTproBNP, hsCRP, S100, clinical outcome	↑ incidence of new arrhythmias and myocardial infarctions in RIPC group ($p=0.036$).
Xie *et al*, 2012	Double blinded RCT	73 patients C: 35 RIPC: 38	Valve replacement	3 x 5 min cycles in right upper limb by BPC inflation to 200 mmHg after AI	cTnI, NYHA class, echo assessment 3 months after surgery	↓ cTnI 6, 12, 24, 48 and 72 hours after surgery ($P<0.0001$), higher NYHA functional class ($p=0.0298$) Improved EF ($p=0.0049$)
Young *et al*, 2012*	Double blinded RCT	96 patients C: 48 RIPC: 48	High risk cardiac procedures	3 x 5 min cycles in upper limb by BPC inflation to 200 mmHg at first surgical incision	hscTnT, noradrenaline use, AKI category, haemodynamic parameters	↑ hsTNT in the RIPC group after 12 hours of surgery ($p=0.05$), longer postoperative adrenaline support ($p=0.04$)
Albrecht *et al*, 2013	Double blinded RCT	61 patients C: 29 RIPC: 32	Cardiac surgery with CPB	4 x 5 min cycles in upper limb by BPC inflation to 200 mmHg after AI	cTnT, cardiac tissue protein expression of HIF-1α, procaspases and IL-1β, myeloperoxidase activity, serum IL profile before and after CPB	↓ cTnT in the first 48 h after surgery ($p<0.05$), ↑ HIF-1α ($p<0.05$), procaspase 3 ($p<0.05$) in tissue, ↑ IL-1β, IL-8 and TNF-α ($p<0.05$) in serum before CPB. ↑ myeloperoxidase ($p<0.05$) and concentration of IL-1β ($p<0.05$) in tissue after CPB.

Study	Type of trial	Patients	Surgery	RIPC Protocol	Outcome measures	Results
Meybohm *et al*, 2013	Double blinded RCT	n:180 C: 90 RIPC: 90	Cardiac surgery with CPB	4 x 5 min cycles in upper limb by BPC inflation to 200 mmHg, 15 mmHg above SBP after AI	Neurocognitive dysfunction, cTnT, renal dysfunction, clinical parameters	No significant difference
Thielmann *et al*, 2013 *	Double blinded RCT	329 patients C: 167 RIPC: 162	CABG	3 x 5 min cycles in left upper limb by BPC inflation to 200 mmHg after AI	cTnI AUC in the first 72 h after surgery, mortality	↓ cTnI AUC lower in RIPC (p=0·022). ↓ mortality at 1.54 (SD 1.22) years (p=0·046).
Slagsvold *et al*, 2014	Double blinded RCT	60 patients C:30 RIPC: 30	CABG	3 x 5 min cycles in upper limb by BPC inflation to 200 mmHg before AI	Mitochondrial respiration in cardiac tissue, Akt activation.	↓ mitochondrial respiration in controls (p=0.02). ↑ Akt activation before aortic clamp (p=0.04) and ↑ phosphorylation of Akt substrates at reperfusion (p<0.01) in RIPC group
Zitta *et al*, 2014	Double blinded RCT	35 patients C: 17 RIPC: 18	Cardiac surgery with CPB	4 x 5 min cycles in upper limb by BPC inflation to 200 mmHg after AI.	MMP-2/9 activity in cardiac tissue, cTnT	Before CPB MMP-2/9 were attenuated in the RIPC group MMP-2 ($p < 0.05$). MMP-9 ($p < 0.01$). MMP activity was correlated with cTnT serum levels postoperatively.
Hausenloy *et al*, 2015	Multi-centre double blinded RCT	1612 patients C: 811 RIPC: 801	CABG high risk	4 x 5 min cycles in upper arm by BPC inflation to 200 mmHg after AI	Mortality at 12 months, hscTnT AUC, inotrope score, AKI, ICU stay.	No significant difference

Study	Type of trial	Patients	Surgery	RIPC Protocol	Outcome measures	Results
Meybohm *et al*, 2015	Multi-centre double blinded RCT	1385 patients C: 693 RIPC: 692	CV surgery with CPB	4 x 5 min cycles in upper limb by BPC inflation to ≥200 mmHg, at least 15 mmHg higher than SBP after AI	Composite of MI, stroke, AKI by discharge. Any component by day 90.	No significant difference

Table 1.7. RIPC adult clinical trials investigating cardiac response after cardiac surgery and CPB. Only significant results are reported. Abbreviations: AI: anaesthesia inductionAKI: acute kidney injury; Akt: protein kinase B; AUC: area under the curve; BNP: B-type natriuretic peptide; BP: blood pressure; BPC: blood pressure cuff; CABG: coronary artery bypass graft; CK-MB: creatin kinase cardiac isoenzyme; CPB: cardiopulmonary bypass; cTnI: cardiac troponin I; cTnT: cardiac troponin T; CV: cardiovascular; EF: ejection fraction; eNOS: endothelial nitric oxide synthase; F: female; hsCRP: high-sensitivity C-reactive protein; hscTnT: high sensitivity troponin T; IF: isoflurane; IGF-1: insulin-like growth factor 1; iNOS: inducible nitric oxide synthase; M: male; MI: myocardial infarction; MMP-2/9: metalloproteinases 2 and 9; NTproBNP: N-terminal pro-brain natriuretic peptide; NYHA: New York heart association; PF: propofol; RCT: randomised controlled trial; RIPC: remote ischaemic preconditioning. Original Table.
*isofluorane

Recently five meta-analysis have gathered the information from RCTs assessing the efficacy of RIPC in adult cardiac surgeries, four of them have found beneficial effects from the intervention, in spite of this they have not been able to demonstrate clinical benefit.

A meta-analysis looking into CABG surgery only and RIPC incorporated 9 studies resulting in a lower cTnI and cTnT after RIPC, this difference persisted after excluding an off-pump study (no CPB performed) and trials using a flurane for anaesthesia, however the benefit was not translated to clinical effects (D'Ascenzo *et al*, 2012).

A second meta-analysis of 15 adult clinical trials including 1155 patients showed an overall protective cardiac effect of RIPC, finding a possible influence on the outcome by the type of surgery, and an undermining impact caused by the use of volatile anaesthetics and β-blockers on the final effect of RIPC (Zhou *et al*, 2013).

A meta-analysis including 25 studies of cardiac, pulmonary and renal outcomes found after analysis of 16 cardiac focused adult and paediatric RCTs that RIPC resulted in a significant reduction of cardiac injury which persisted after subgroup analysis for all types of surgical procedures, yet again this did not translate into better clinical outcomes (Haji Mohd Yasin *et al*, 2014).

A meta-analysis including 19 trials and 1235 patients analysed cTnI values after several types of cardiac surgery, including CABG, valve procedures and paediatric CHD repair concluding that RIPC reduced cTnI values significantly finding no difference in morbidity or mortality (Yang *et al*, 2014).

Finally, the largest meta-analysis including 2200 patients from 23 cardiac trials did not find a significant outcome on any of the clinical end-points when the information was pooled together, including mortality, myocardial infarction, troponin release, stroke, renal failure, ICU and hospital length of stay (Healy *et al*, 2014).

All these meta-analysis presented overlap in the studies used, it is to be noted that they presented a high proportion of male participants as would be expected from the higher prevalence of coronary artery disease in males and their need for CABG surgery, these analysis also included one off-pump study and trials focused in outcome measures other than cardiac injury such as renal, pulmonary and brain responses.

1.6.2 RIPC paediatric evidence

Nine trials analysed RIPC response in children going through correction of CHD using CPB, *Table 1.8* presents a summary of the relevant studies. The intervention involved 3 or 4 periods of ischaemia in a lower or upper limb induced by the inflation of a blood pressure cuff, intercalated with periods of reperfusion; seven trials investigated the early phase of protection performing the intervention during or after anaesthesia induction or 5 to 10 minutes before CPB, one focused on the second window of protection carrying out RIPC 24 hours before surgery (Pavione *et al*, 2012), while only one trial applied a dual protocol combining the effects of early and late responses applying the intervention 24 hours and 1 hour before surgery (Zhou *et al*, 2010).

Outcome measures included cTnI, CK, CK-MB, NTproBNP, inflammatory mediators, haemodynamic and clinical parameters, as well as renal and brain injury markers.

Within the paediatric setting four trials found benefit regarding lower cardiac injury markers and inflammatory mediators while one trial reported a lower likelihood of arrhythmia linked to RIPC. Three studies concluded there was no significant difference for any of the outcome measures analysed between treatment groups.

One of the studies finding no benefit from the intervention recruited TOF patients only and reported its results in two different publications after analysing RV protein expression and leukocyte mitochondrial respiration; patients showed an overall high proportion of phosphorylated proteins which could be due to the chronic cyanosis caused by the pathology (Pepe *et al*, 2013) and an upregulated plasma expression of proteins involved in metabolism, haemostasis, immunity and inflammation after RIPC (Hepponstall *et al*, 2015).

Only one additional study explored myocardial protein expression finding upregulation of HSP-70 in right atrial myocardium linked to cardiac benefits after RIPC (Zhou *et al*, 2010).

Author, year	Type of trial	Patients	Surgery	Protocol	Outcome measures	Results
Cheung *et al*, 2006	Double blinded RCT	37 children C: 20 RIPC: 17	CHD repair	4 x 5 min cycles in lower limb by BPC inflation to 15 mmHg above SBP 5 to 10 min before CBP	Haemodynamic state, Inflammatory mediators, cTnI	↓cTn in IPC group (p=0.04), better haemodynamic condition (p=0.03) ↓ IL-10 at 3 h (p= 0.03) ↓TNF-α at 6 h (p=0.0001)
Zhou *et al*, 2010	Double blinded RCT	60 infants C: 30 RIPC: 30	CHD repair	3 x 5 min cycles in left upper limb by BPC inflation to 240 mmHg 24 h and 1 h before surgery	Haemodynamic state, cTnI, CK, CK-MB, IL-6, IL-8, IL-10, TNF-α, protein HSP-70 expression in RA tissue	Better haemodynamic state (p=0.04), ↓cTnI at 2 (p=0.007) and 4 h after surgery (p=0.000) ↓CK (p=0.03), ↓CK-MB (p=0.049), ↓ IL-6, IL-8 and TNF-α, ↑ IL-10. Higher HSP-70 in RIPC group (p=0.006)
Luo *et al*, 2011	Double blinded RCT	60 children 1-5 y/o C: 20 RIPC:20 Post: 20	VSD repair	3 x 5 min cycles in thigh by BPC inflation to 200-300 mmHg after AI	Haemodynamic state, cTnI, CK-MB	↓ CK-MB in pre (p=0.021) and post-conditioning groups (p=0.024). ↓ cTnI in pre (p=0.013) and post-conditioning groups (p=0.022).
Lee *et al*, 2012	RCT	55 infants with PHT	VSD repair	4 x 5 min cycles in thigh by BPC inflation to 30 mmHg above SBP after AI	Haemodynamic state, cTnI	No significant difference
Pavione *et al*, 2012	Single blinded RCT	22 children 1 month-2 y/o C:10 RIPC:12	CHD repair	4 x 5 min cycles in lower limb by BPC inflation to 15 mmHg above SBP 24 h before surgery	cTnI, NTproBNP, NF-κB, IL8 and IL10	↓ NTproBNP in RIPC group (p=0.02)

Author, year	Type of trial	Patients	Surgery	Protocol	Outcome measures	Results
Jones *et al*, 2013	Blinded clinicians and data analysis RCT	Cyanotic neonates n: 39 C: 19 RIPC: 20	TGA switch or HLHS Norwood procedures	4 x 5 min cycles in lower limb by BPC inflation to 15 mmHg above SBP after AI	cTnI, renal (NGAL) and brain injury (S100) markers	No significant difference.
Pepe *et al*, 2013 *	Double blinded RCT	Children 1 month and older n: 40 C: 20 RIPC: 20	TOF repair	4 x 5 min cycles in lower limb by BPC inflation to 20 mmHg above SBP after AI	Protein expression in RV biopsy, leukocyte mitochondrial respiration, cTnI	No significant difference. A high proportion of total proteins were phosphorylated in both groups.
McCrindle *et al*, 2014	Double blinded RCT	Neonates to 17 y/o n: 299 C: 151 RIPC: 148	primary surgical repair with CPB	4 x 5 min cycles in left thigh by BPC inflation to 15 mmHg above SBP during AI	Postoperative stay, serial clinical and laboratory measurements	↓ likelihood of arrhythmia (p=0.05), no other significant finding.
Hepponstall *et al*, 2015*	Double blinded RCT	n: 40 C: 20 RIPC: 20	Primary TOF repair	4 x 5 min cycles in lower limb by BPC inflation to 20 mmHg above SBP after AI	Protein expression analysis in plasma	Upregulation of proteins involved in metabolism, haemostasis, immunity and inflammation in RIPC group 6 h after CPB.

Table 1.8. Paediatric RIPC cardiac trials. *different publications based on analysis performed in the same clinical trial. Abbreviations: AI: AI; BPC: blood pressure cuff; C: control; CHD: congenital heart disease; CK: creatin kinase; CK-MB: creatin kinase cardiac isoenzyme; CPB: cardiopulmonary bypass; cTnI: cardiac troponin I; HLHS: hypoplastic left heart síndrome; NGAL: neutrophil gelatinase-associated lipocalin; NTproBNP: N-terminal pro-B-type natriuretic peptide; RCT: randomised controlled trial; PHT: pulmonary hypertension; RA: right atrium; RIPC: remote ischaemic preconditioning; RV: right ventricle; SBP: systolic blood pressure; TOF: Tetralogy of Fallot; TGA: transposition of the great arteries; VSD: ventricular septal defect. Original Table

Evidence is conflicting regarding the benefits of RIPC in paediatric subjects. A meta-analysis including 359 paediatric patients from 7 RCTs identified no benefit with regard to cTnI levels, inotropic score, duration of mechanical ventilation and hospital length of stay, however concluded that ICU length of stay was significantly reduced after RIPC (Tie *et al*, 2014). However a further meta-analysis by the same authors incorporated two additional RCTs including those analysed in *Table 1.8* and an additional trial focused on post-perative kidney injury which reported a lack of efficacy (Pedersen *et al*, 2012), concluded none of the parameters considered before including ICU stay were altered by RIPC. The publication emphasised the heterogeneity of the trials and the need for further investigation (Tie *et al*, 2015).

The research in this thesis is focused on cardiac injury indicators, end organ damage and clinical response after cardiac surgery in paediatric patients as well as basic science analysis of expression patterns and behaviour of several elements mediating the process of IRI and RIPC.

Chapter 2. Materials and Methods

2.1 Research Objectives

Principle research objective

To assess whether RIPC provides protection against IRI through the expression of protective genes in cardiac tissue of children presenting congenital heart defects undergoing surgical correction and CPB.

Secondary research objective

To assess whether RIPC is effective against end organ damage and systemic inflammatory responses.

2.2 Study Design

General design: Randomised clinical trial

Primary outcome measures

- Measurement of final cardiac injury after CPB assessed by markers of cardiac injury and echocardiography.
- Protein and mRNA expression in the cardiac tissue related to the RIPC process.

Secondary outcome measures

- End organ damage assessment by measurement of markers relevant to lung and kidney function and evaluate a possible benefit from RIPC.
- Systemic immune response assessment by measurement of inflammatory mediators such as cytokines and nitric oxide metabolites.

Quality and assessment of research

Scientific quality was assessed by Professor Fiona Lyall –academic supervisor (AS) and by Mr Mark Danton – chief investigator (CI). Statistical aspects of the research were assessed by a statistician within the CI's institution, Dr David Young.

Statistical Analysis

Previous echocardiography data analysed by this research group in 50 paediatric patients showed a marked mean decline of postoperative right ventricle velocity of 4.705 cm/s compared to preoperative values with a standard deviation of 2.798.

We hypothesised a 50% decrease (2.35 cm/s) of this decline with the trial intervention. Supposing that in the treated group the reduction was 2.35 cm/s with the same standard deviation then a sample size of 24 patients in each group would be required for 80% power to detect a significant difference between the two groups.

2-Sample t Test
Testing mean 1 = mean 2 (versus not =)
Calculating power for mean 1 = mean 2 + difference
Alpha = 0.05 Assumed standard deviation = 2.798

Sample Difference	Target Size	Power	Actual Power
2.35	24	0.8	0.812798

Using a sample of 31 patients in each of the control and treated groups would allow for 90% power to detect a significant difference between the two groups (2-sample t test).

2-Sample t Test
Testing mean 1 = mean 2 (versus not =)
Calculating power for mean 1 = mean 2 + difference
Alpha = 0.05 Assumed standard deviation = 2.798

Sample Difference	Target Size	Power	Actual Power
2.35	31	0.9	0.902046

Using a sample of size 40 in each group would allow a difference of 1.77 cm/s to be detected between the change in the control group vs. the change in the treated group

2-Sample t Test
Testing mean 1 = mean 2 (versus not =)
Calculating power for mean 1 = mean 2 + difference
Alpha = 0.05 Assumed standard deviation = 2.798

Sample Size	Power	Difference
40	0.8	1.77477

i.e. If the change in velocity in the control group is 4.7 cm/s, then as long as the change in the treated group is no more than about 3.0 there is 80% power to detect a significant difference between the changes in the two groups.

2.3 Ethical approval

Ethical approval was granted after a lengthy and meticulous application process in July 2012 by the West of Scotland Research Ethics Service, reference number: 12/WS/0137 and Research and Development (R&D) GN12KH253.

An amendment to the protocol was later performed with the relevant ethics authorisation obtained in October 2013. All approval documents can be seen on *Appendix 1*.

A research passport was obtained in order for the principle investigator (PI) Maribel Verdesoto to be allowed to have contact with patients and their guardians during the process of informed consent.

2.4 Subject Enrolment

This single centre randomised controlled trial started recruitment in January 2013 at the Scottish National Paediatric Cardiac Service based at the Royal Hospital for Sick Children - Yorkhill, Glasgow.

Identification

Children who underwent elective cardiac surgery for the correction of CHD were identified as potential participants by review of the cardiac surgical waiting list and clinical presentations at the cardiac team weekly meeting held on Fridays. This involved the health care team and Mr Mark Danton (CI) took responsibility to coordinate the clinical team process and identify potential patients. The PI attended the meetings as a strategy to improve patient recruitment from 2014.

Patients were assessed according to the following criteria:

Inclusion Criteria:

- Children undergoing cardiac surgery for correction of congenital heart defects utilizing cardiopulmonary bypass and cardioplegia.
- Children whose parents understand the child's condition, the purpose of the study and are willing to participate.

Exclusion Criteria:

- Children whose parents either are unwilling or do not have sufficient understanding of the study.
- Emergency operations, where there is insufficient time to establish the study protocol.
- Premature children presenting a corrected gestational age under 35 weeks.
- Patients with known viral blood infections (e.g. HIV, Hepatitis B) or severe congenital infection.
- Patients with severe preoperative brain injury.

Approach

Parents and patients were approached at the outpatient clinic when possible. The consultant surgeon in charge met the parents and child and introduced the study.

Detailed information on every aspect of the trial was provided by the PI at patients' admission, usually 24 – 48 hours before surgery, allowing for any questions or comments from the parents, guardians or children to be made. A comprehensive information sheet, previously approved by ethics services, was provided according to the age of the participants (see *Appendix 2*). Sufficient time to read it and carefully consider their participation in the study was allowed according to the patients and parents preferences.

Recruitment

If the patient, parent or legal guardian were happy to participate in the study, a consent form was signed by the PI as well as the patient's parent or legal guardian; children between 7 to 11 years old, in addition, signed an assent form to establish their acceptance to take part; children from 12 to 15 years old signed the consent form with their parents and 16 year olds signed a young person consent form as they are able to provide full consent independently. Participants held the right to withdraw from the trial at any point without requiring to provide an explanation.

All consent forms were filed in the clinical notes of the patient and a copy was kept in the study site file.

2.5 Randomisation

After informed consent was obtained patients were classified according to their diagnosis into a "tissue" group when the surgical procedure to be performed required cardiac tissue excision and into a "non-tissue" group when it did not. Trial numbers were assigned preceded by the code "M" for the "tissue" group and "B" for the "non-tissue" group. Randomisation was done within each group into a "RIPC" treatment or a "Control" group. Randomisation was performed using open access online software for the generation of true random numbers available online (www.random.org). *Figure 2.1* shows the structure of the trial.

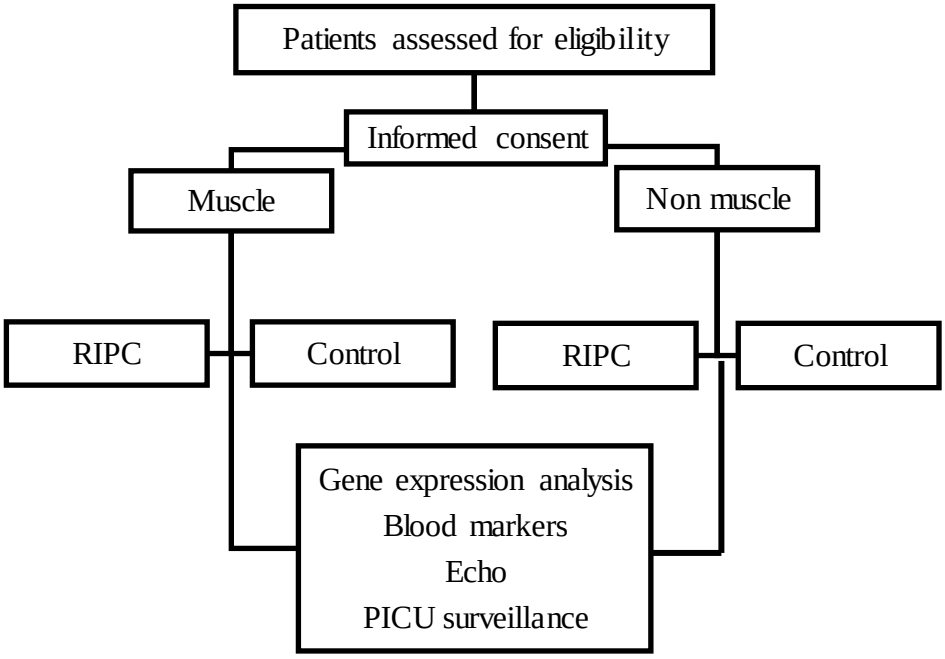

Figure 2.1. RIPC clinical trial structure. Abbreviations: PICU: paediatric intensive care unit; RIPC: remote ischaemic preconditioning.

2.6 Intervention

Patients assigned to the treatment group had a blood pressure cuff placed on their leg when their weight was under 10 kg, or arm if their weight was above 10 kg. RIPC was performed by 3 cycles of 5 minute ischaemia induced by inflation of the cuff to 40 mmHg above the patient's systolic blood pressure. This protocol was performed at two phases: 15 to 20 hours before surgery and during anaesthesia immediately prior to surgery in order to achieve early and late RIPC protection phases as described by Zhou *et al*, 2010. This process was performed by Mr Mark Danton or the liaison nursing team at the cardiac ward and by Ms Isobel McLeod or Mr Mark Danton at the operating theatre; it remained blinded for the PI.

2.7 Blood sampling

Blood samples were obtained from all patients at six different time points as shown in *Table 2.1*; Baseline samples were taken after anaesthesia induction before surgery. Pre-bypass samples were obtained from the right atrium after heparin infusion and post-bypass samples were drawn before protamine administration, both drugs of standard use during the bypass process. An early postoperative sample was drawn at PICU arrival of the patient (PO1) followed by an early morning sample on the following day (day 2 – PO2) and the day after (day 3 – PO3). Samples were spun within 4 hours of extraction in a centrifuge for 10 minutes at 2000 g to obtain plasma which was aliquoted and stored at -70°C.

	Baseline	**Pre CPB**	**Post CPB**	**PO1**	**PO2**	**PO3**	**Total**
Blood amount	2.6 ml	1.2 ml	1.2 ml	3.8 ml	3.6 ml	3.6 ml	16 ml
Site of extraction	IV line	Right atrium	Right atrium	IV line	IV line	IV line	
Place of extraction	Cardiac ward	Operating theatre	Operating theatre	PICU	PICU	PICU	

Table 2.1. Blood sampling schedule during RIPC trial. Amount of blood drawn for each sample is shown in ml. Abbreviations: CPB: cardiopulmonary bypass; IV: intra-venous; PICU: paediatric intensive care unit; PO: post-operative.

2.8 ELISA analysis of blood markers

Enzyme-linked immunosorbent assay (ELISA) DuoSet® assays from R&D Systems were used to measure blood sample markers according to the manufacturer´s protocol. *Table 2.2* shows the kit used for each analyte.

These immunoassays use a sandwich format which detects a target antigen present in the sample, the antigen is trapped between a capture and detector antibodies, the detection antibody is labelled with biotin and the sandwich complex is detected by enzyme-conjugated streptavidin (reviewed by Stanker and Hnasko, 2015). Tetramethyl benzidine (TMB) is then used as a substrate solution which forms a water soluble blue reaction after oxidation, the reaction is then stopped using a sulfuric acid solution in order to prevent change in the optical density (OD), this final reaction changes the colouration to yellow (reviewed by Crowther, 2009).

Analyte	Kit	Catalogue number
IL-6	Duoset Human IL-6	DY206-05
IL-8	DuoSet Human CSCL8/IL-8	DY208
IL-10	Duoset Human IL-10	DY217B
TNF-α	Duoset Human TNF-α	DY210-05
ICAM-1	DuoSet Human ICAM-1/CD54	DY720-05
VCAM-1	DuoSet Human VCAM-1/CD106	DY809
Cystatin-C	DuoSet Human Cystatin C	DY1196

Table 2.2 ELISA kits used for analysis. Abbreviations: ICAM-1: intercellular adhesion molecule 1; IL: interleukin; TNF-α: tumour necrosis factor α; VCAM-1: vascular cell adhesion molecule 1.

Reagent diluent (0.15 bovine serum albumin (BSA), 0.05% Tween 20, 20 mM Trizma base, 150 mM NaCl) was used for all IL-8 and IL-10; all other analysis were performed using Reagent diluent (1% BSA in phosphate-buffered saline (PBS), pH 7.2-7.4, 0.2 μm filtered, R&D Systems, catalogue number DY995), as it was thought it would produce less background interference.

The capture antibody was diluted to the working concentration in PBS (*Table 2.3*) without carrier protein. A 96 well Cornig microplate was immediately coated with 100 μl of capture antibody per well using a multichannel pipette. The microplate was sealed with an adhesive strip and incubated overnight at room temperature.

Analyte	**Concentration**
IL-6	2 μg/ml
IL-8	4 μg/ml
IL-10	2.0 μg/ml
TNF-α	4.0 μg/ml
ICAM-1	4.0 μg/ml
VCAM-1	2.0 μg/ml
Cystatin-C	4.0 μg/ml

Table 2.3. Capture antibody working concentration for ELISA analysis. Abbreviations: ICAM-1: intercellular adhesion molecule 1; IL: interleukin; TNF-α: tumour necrosis factor α; VCAM-1: vascular cell adhesion molecule 1.

The next day the capture antibody was removed from the wells. Washing buffer (10% PBS 10x, 0.05% Tween 20) was then used with a squirt bottle in order to wash each well for a total of three times ensuring complete removal of all content between washes. After the last wash the microplate was blotted against clean paper towels in order to remove remaining washing buffer.

Plates were blocked using 200 µl reagent diluent (RD) per well, they were covered and incubated for a minimum of one hour at room temperature followed by washing and blotting as described above.

100 µl of neat or diluted plasma samples (*Table 2.4*) or standards representing the appropriate concentrations to generate a sigmoid curve (*Table 2.5*) were added to each well; nothing was added to blank assigned wells. Plates were covered and incubated for at least one hour at room temperature followed by washing and blotting.

Analyte	**Dilution factor**
IL-6	Neat
IL-8	Neat
IL-10	Neat
TNF-α	Neat
ICAM-1	1:200
VCAM-1	1:3000
Cystatin C	1:1500

Table 2.4. Dilution factor used for plasma sample analysis. Neat samples were not diluted. Abbreviations: ICAM-1: intercellular adhesion molecule 1; IL: interleukin; TNF-α: tumour necrosis factor α; VCAM-1: vascular cell adhesion molecule 1.

Analyte	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**
IL-6	600	300	150	75	37.5	18.75	9.375
IL-8	2000	1000	500	250	125	62.5	31.250
IL-10	2000	1000	500	250	125	62.5	31.250
TNF-α	1000	500	250	125	62.5	31.25	15.625
ICAM-1	2000	1000	500	250	125	62.5	31.250
VCAM-1	1000	500	250	125	62.5	31.25	15.625
CystatinC	2000	1000	500	250	125	62.5	31.250

Table 2.5. Standard concentration used. The table shows seven different standard concentrations for each analyte. Concentration is shown in pg/ml. Abbreviations: ICAM-1: intercellular adhesion molecule 1; IL: interleukin; S: standard; TNF-α: tumour necrosis factor α; VCAM-1: vascular cell adhesion molecule 1.

100 µl of detection antibody diluted in RD to the appropriate concentration (*Table 2.6*) were added to each well, plates were covered and incubated at room temperature for at least one hour to then be washed and blotted.

Analyte	Concentration
IL-6	50 ng/ml
IL-8	20 ng/ml
IL-10	75 ng/ml
TNF-α	400 ng/ml
ICAM-1	100 ng/ml
VCAM-1	200 ng/ml
Cystatin-C	250 ng/ml

Table 2.6. Detection antibody working concentration for ELISA analysis. Abbreviations: ICAM-1: intercellular adhesion molecule 1; IL: interleukin; TNF-α: tumour necrosis factor α; VCAM-1: vascular cell adhesion molecule 1.

100 µl of Streptavidin-HRP working dilution were added to each well. Plates were covered and incubated for 20 minutes at room temperature and away from direct light. Washing and blotting was repeated.

100 µl of substrate solution were added to each well, plates were placed away from direct light and monitored for colour change until the higher standards reached an adequate blue intensity. 50 µl of Stop solution (sulfuric acid solution - H_2SO_4 2N) were then added changing the blue colouration to yellow.

The optical density (OD) of each well was determined immediately using a microplate reader and Dynex technologies revelation 4.25 software using a test filter of 450 nm and wavelength correction of 550 nm.

The reader generates OD measurements after subtracting blank values which reflect background reading. The software uses a 4 Parameter Logistic regression model producing a sigmoid curve with mean OD values for duplicate standard measurements which represent the known concentrations of the analyte (*Figure 2.2*). A coefficient of determination value (r^2) is produced as a measure of how well the regression model fits the data, ranging from 0, where there is no fit, to 1 representing an exact fit of the regression linking a relationship between the dependent variable and a linear regression of the independent variable (reviewed by Randolph and Myers, 2013; Lee, 2014). The software then places the unknown samples´ OD values within the curve, producing comparative concentration values.

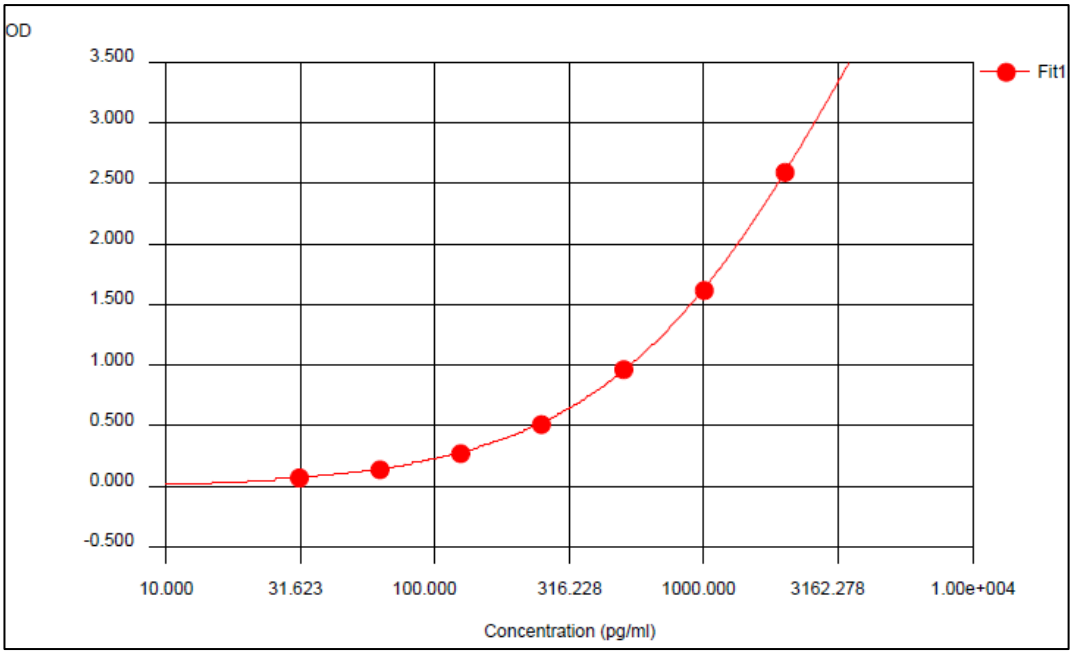

Figure 2.2. Standard curve produced from mean OD values of standards for I-CAM analysis.

Concentration values outwith the standard curve limits were accepted up to 1.5 times the highest standard value and 0.5 times the lowest standard, eg. If a standard curve had 1000 pg/ml and 15.625 pg/ml as the highest and lowest standard values respectively, 1500 pg/ml would be the highest acceptable value and 7.8125 pg/ml would be the lowest acceptable value with readings exceeding or under this limit being assigned the highest or lowest accepted value as this range is reliable while anything beyond this limit is not a credible measure and lies in a zone of unknown value.

Every sample was analysed in duplicate, after obtaining concentration values these were averaged. Values for diluted samples were then multiplied by the dilution factor.

2.9 Tissue sampling and incubation

"Tissue" group

In preparation for obtaining tissue specimens culture medium was supplemented with foetal bovine serum (FBS) and antibiotic/antimycotic solution and placed into an incubator which was set to high oxygen flow (60%) at least three hours before the specimen was expected.

Tissue specimens were obtained from the operating theatre and immediately divided into two sections; one of them was placed into RNAlater® (Sigma), a solution designed to stabilize and protect RNA with ribonuclease (RNase) inactivation, while the second section was placed into the enriched culture medium and was promptly brought into the laboratory to be incubated at 37°C, 60% O_2 and 5% CO_2 flow for four hours in order to mimic surgical conditions as patients receive high O_2 concentration during the reoxygenation process. After incubation the tissue was placed into RNAlater® solution and stored at -4°C for at least 24 hours before processing.

"Non tissue" group

Samples from the "non-tissue" group did not go through the incubation process due to the reduced amount of tissue available; specimens were placed into RNAlater® and stored at -4°C until RNA extraction.

Tissue was resected at times according to surgical protocols and due to particular circumstances regarding each case, variability is possible based on the time the tissue was obtained relative to CPB and cross clamping, implying longer exposure to hypoxia and inflammatory responses for some specimes.

2.10 RNA extraction

Myocardial tissue was stored at least overnight in RNAlater® solution (Sigma-Aldrich, catalogue number R0901) at -4°C in order to stabilize the RNA, this solution allows unfrozen storage maintaining the quality and quantity of RNA. Before extraction the tissue was removed from the solution and carefully weighed.

The RNeasy® Fibrous Tissue Midi Kit (Qiagen, catalogue number 75742) was used for the first 15 specimens obtained until the manufacturer discontinued the kit, from then on the RNeasy® Fibrous Tissue Mini Kit (Qiagen, catalogue number 74704) was used.

The method used is designed for treating fibrous tissue such as skeletal muscle or myocardium which are rich in collagen, connective tissue and contractile proteins. The tissue is initially lysed in buffers and diluted before adding Proteinase K, a serine protease used in order to ensure complete removal of proteins including histones, which could interfere with the accurate quantitation and hybridization reactions and later inhibit reverse transcription and amplification by PCR (reviewed by Farrell, 2010).

The difference between the two kits used consist only on the volume of tissue that is used while processing as a single sample, the Midi Kit allows samples up to 250 mg to be processed while the Mini Kit has a limit of 30 mg; as a result samples exceeding this limit were split when using the RNeasy® Fibrous Tissue Mini Kit. The process for each kit was performed as follows:

RNeasy® Fibrous Tissue Midi Kit

Tissue ≤250 mg was added to 2 ml of Buffer RLT, provided with the kit and previously supplemented with 10 µl beta-mercaptoethanol (ß-ME) per 1 ml buffer RLT in order to be disrupted and homogenised using a Polytron PT1600E homogeniser (Kinematica) at full speed until complete homogenization was achieved; 4 ml RNase-free water and 65 µl of proteinase K (>600 mAU/ml, solution) were then added, mixed and incubated at 55°C for 20 minutes. Samples were then spun at 4000 x g at 25°C for 5 minutes and transferred to a new sterile Falcon tube (Sigma-Aldrich, cat. no: Z617849), 0.5 volumes of 100% ethanol were added and mixed, supernatant volume was usually around 6 ml, requiring 3 ml of ethanol to be added.

A volume of 3 ml was transferred to a RNeasy Midi column in a 15 ml collection tube to be centrifuged at 4000 x g at 25°C for 5 minutes to then discard the flow-through, this step was repeated until the complete lysate was used.

2 ml of Buffer RW1 were added to the column and spun at 4000 x g at 25°C for 5 minutes, flow-through was discarded.

Deoxyribonuclease (DNase) stock solution was previously prepared by dissolving lyophilised DNase I in RNase-free water and 20 µl of this solution was mixed with 140 µl Buffer RDD, the resulting 160 µl were added to the membrane in the spin column and incubated for 15 minutes at room temperature in order to ensure the absence of genomic DNA contamination.

After the incubation was completed, 2 ml of Buffer RW1 were added to the column which was centrifuged at 4000 x g at 25°C for 5 minutes, flow-through was discarded, 2.5 ml of Buffer RPE were added and spun for 2 minutes at 4000 x g at 25°C, followed by the addition of further 2.5 ml of RPE Buffer and centrifugation at 4000 x g at 25°C for 5 minutes.

The column was placed in a new tube and 150 µl of RNase-free water were added and spun at 4000 x g at 25°C for 3 minutes. For some samples the final elution step was repeated with another volume of water.

RNeasy® Fibrous Tissue Mini Kit

Tissue ≤30 mg was added to 300 µl of Buffer RLT, the tissue was disrupted and homogenised and then 590 µl RNase-free water and 10 µl of proteinase K were added and incubated at 55°C for 10 minutes. Tubes were spun at 10000 x g at 25°C for 3 minutes and the supernatant was transferred to a new tube and 0.5 volumes of 100% ethanol were added and mixed.

The supernatant volume was usually around 1 ml, requiring 0.5 ml of ethanol to be added.

A volume of 700 µl was transferred to a RNeasy Mini column in a 2 ml collection tube which was spun at 80000 x g at 25°C for 15 seconds; the flow-through was discarded, this step was repeated until the complete lysate was used.

350 µl of Buffer RW1 were added to the column and spun at 8000 x g at 25°C for 15 seconds, the flow-through was discarded.

Previously prepared and aliquoted 10 µl DNase stock solution was mixed with 70 µl Buffer RDD and added to the membrane in the spin column and incubated for 15 minutes at room temperature.

After incubation was completed, 350 µl of Buffer RW1 were added to the column which was then spun at 8000 x g at 25°C for 15 seconds, the flow-through was discarded and 500 µl Buffer RPE were added and spun for 15 seconds at 8000 x g at 25°C, buffer RPE was added for a second time and tubes were spun for 2 minutes at 8000 x g and 25°C.

The column was placed in a new tube and 50 µl of RNase-free water were added and spun at 4000 x g at 25°C for 3 minutes. For some samples the final elution step was repeated with another volume of water.

RNA concentration

RNA concentration was determined using a NanoDrop® instrument (Thermo Scientific) which not only produces results of RNA concentration quantification in ng/µl but provides information on the quality of the RNA. Purity is determined by a ratio of absorbance at 260 nm and 280 nm, a value between 1.8 and 2.1 shows optimal purity (*Figure 2.3*). RNA was aliquoted at 50 µl and stored at -70°C.

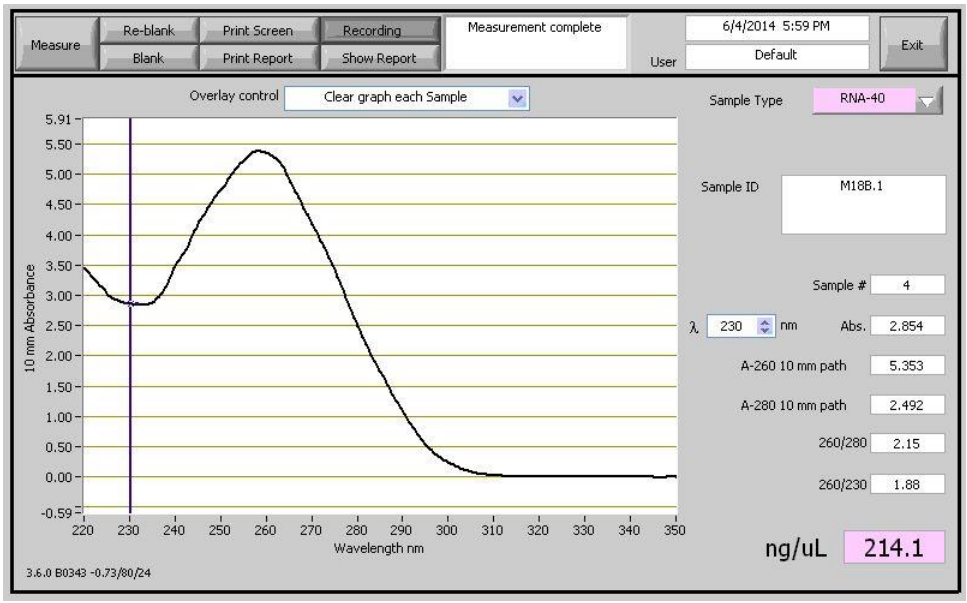

Figure 2.3. RNA concentration measurement using Nanodrop instrument. Original image.

2.11 Reverse Transcription PCR

A reverse transcription PCR (RT-PCR) reaction was performed using extracted RNA from myocardium in order to obtain complimentary DNA (cDNA). The reaction was done using the QuantiTect® Reverse Transcription Kit (Qiagen, cat. no. 205310) and GoScript™ Reverse Transcriptase (Promega, cat. no. A5003).

GoScript™ reverse transcriptase was used instead of the QuantiTect® kit enzyme as previous optimization showed that it produced cDNA of superior quality.

The reaction was performed using 100ng RNA which were calculated according to the RNA concentration for each sample.

A genomic DNA elimination reaction was first done in a MicroAmp® Fast Reaction Tube (Life technologies cat no 4358297) using the Quantitect RT kit with the following setup:

Wipeout buffer	2µl
Template RNA (100ng)	?µl
RNase free water	?µl
Total reaction volume	14µl

The reaction was run on a PCR StepOnePlus® instrument (applied Biosystems); 42°C for 3 minutes followed by the addition of GoScript reverse transcriptase, RT buffer and RT primer mix to each tube as follows:

	1 sample
GoScript reverse transcriptase	1 µl
RT buffer	4 µl
RT primer mix	1 µl
Total	6 µl

This master mix was added to each tube reaching a total volume of 20 µl which were then run on the PCR StepOnePlus® instrument (applied Biosystems) for 30 minutes at 42°C followed by 5 minutes at 95°C. The obtained cDNA was then stored at -20°C.

2.12 Real Time (Quantitative) PCR

Real time PCR was performed using TaqMan gene expression analysis assays (Applied Byosistems), which use the 5´ nuclease activity of *Taq* DNA polymerase. The assays used are shown in *Table 2*.7.

	Gene name	**Gene aliases**	**Gene symbol**	**Assay Design**	**Amplicon length**	**Assay ID**
Actin B	actin, beta	BRWS1, PS1TP5 BP1	ACTB	Amplicon spans exons and probe does not span exons	171	Hs999999 03_m1
HSP-20	heat shock protein, alpha-crystallin-related, B6	Hsp20	HSPB6	Probe spans exons	96	Hs003289 33_m1
HSP-22	heat shock 22kDa protein 8	HSP22, CMT2L, DHMN2	HSPB8	Probe spans exons	66	Hs002050 56_m1
HSPB7	heat shock 27kDa protein family, member 7 - cardiovascular	RP11-5P18.6, cvHSP	HSPB7	Probe spans exons	79	Hs002052 96_m1
HO-1	heme oxygenase (decycling) 1	HMOX1 D, HO-1, HSP32	HMOX 1	Probe spans exons	82	Hs011102 50_m1
HSP-60	heat shock 60kDa protein 1 (chaperonin)	CPN60, GROEL, HSP60	HSPD1	Probe spans exons	81	Hs010367 53_g1
HSP-70	heat shock 70kDa protein 1A	HSP70-HSP72, HSPA1	HSPA1 A	Both primers and probe map within a single exon	124	Hs003591 63_s1
HSP-90	heat shock protein 90kDa alpha, cytosolic, class A member 1	EL52, HSP86, HSP89A, HSP90A, HSPC1, HSPCA	HSP90 AA1	Both primers and probe map within a single exon	133	Hs007437 67_sH

	Gene name	Gene aliases	Gene symbol	Assay Design	Amplicon length	Assay ID
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	EBP-1, KBF1, NF-kB1, NFKB-p105, NFKB-p50, NFkappaB	NFKB1	Probe spans exons	66	Hs00765730_m1
HIF-1α	hypoxia inducible factor 1, alpha subunit	HIF-1A, HIF-1alpha, HIF1, HIF1-ALPHA, MOP1	HIF1A	Probe spans exons	76	Hs00153153_m1
TLR-2	toll-like receptor 2	CD282, TIL4	TLR2	Probe spans exons	80	Hs00610101_m1
TLR-4	toll-like receptor 4	ARMD10, CD284, TLR-4, TOLL	TLR4	Probe spans exons	89	Hs00152939_m1
MYD-88	myeloid differentiation primary response 88	MYD-88D	MYD-88	Probe spans exons	104	Hs00182082_m1
CBS	cystathionine-beta-synthase	HIP4	CBS	Probe spans exons	59	Hs00163925_m1
CTH	cystathionase (cystathionine gamma-lyase)	-	CTH	Probe spans exons	110	Hs00542284_m1
Apelin	Apelin	APEL, UNQ471, XNPEP2	APLN	Probe spans exons	79	Hs00175572_m1
Apelin receptor	apelin receptor	AGTRL1, APJ, APJR, HG11	APLNR	Both primers and probe map within a single exon	98	Hs00270873_s1

Table 2.7. Genes analysed and assay information.

Heart positive control cDNA (Primer Design) and all cDNA samples were prepared in a 1:4 dilution using RNAse free water. A master mix for each of the primers was prepared using TaqMan Gene Expression Master Mix (Applied Byosistems) and RNAse free water as follows:

	1 reaction	4 reactions + 1
Sample	1 µl	5 µl
H_2O	4 µl	20 µl
Total	5 µl	25 µl

β-actin was used as endogenous control in order to normalise levels of relative expression within different RNA samples.

5 µl of water (negative control), samples, or positive control were added to the respective wells using a sterile 96 well plate MicroAmp® Fast Optical Reaction Plate (Applied Biosystems) followed by the addition of 15 µl master mix to the relevant wells. The plate was secured with a plastic cover and placed into the PCR StepOnePlus® instrument (Applied Biosystems) using a comparative threshold cycle (Ct) method in order to perform PCR amplification involving an initial holding stage at 50°C for two minutes and 95°C for ten minutes followed by 50 cycles including a denaturation stage at 95°C for 15 seconds and an annealing/extending stage at 60°C for one minute.

Quantitation is based on the point in which amplification of the gene of interest reaches a threshold cycle (Ct) which reflects a significant increase over the gene´s baseline measure, this threshold discriminates between background signal and real amplification.

StepOne software then produces a relative quantitation value (RQ) after several comparisons including the gene of interest, the endogenous control (β-actin), and the positive control sample using the following formulas:

$$\Delta Ct = Ct \text{ gene of interest} - Ct \text{ endogenous control}$$

$$\Delta\Delta Ct = \Delta Ct \text{ (unknown sample)} - \Delta Ct \text{ (positive control)}$$

$$RQ = 2^{-\Delta\Delta Ct}$$

2.13 PICU surveillance

Study monitoring at PICU included duration of intubation and PICU stay, paired blood gases analysis, respiratory/ventilatory parameters and inotrope support every six hours until post-operative day 2. In addition the use of inhaled nitric oxide, extracorporeal membrane oxygenation (ECMO) support and events such as cardiac arrest were noted. Laboratory analysis performed daily as per routine at the unit including urea & electrolytes, liver function tests, full blood count and coagulation parameters were registered as well. *Table 2.8* shows the different parameters and timing at which they were recorded.

Section	**Parameters**	**Timing**
Laboratory analysis	Urea & electrolytes Liver function tests Full blood count Coagulation parameters	Arrival, second day morning, third day morning
Paired blood gases	Arterial and venous acid/base Oxygen status Electrolytes Metabolites	Every six hours for 48 hours
Ventilator parameters	M-FiO_2 M-Pmean Airway type	Every six hours for 48 hours
Inotrope support	Inotrope dosis and score	Every six hours for 48 hours
Inhaled NO	Use and dosis	Every six hours for 48 hours
ECMO	Use and duration	First 48 hours PICU stay
Events	Cardiac arrest	First 48 hours PICU stay

Table 2.8. PICU surveillance parameters. Abbreviations: ECMO: extracorporeal membrane oxygenation; M-FiO_2: mean fraction of inspired oxygen; M-Pmean: mean airway pressure; NO: nitric oxide; PICU: paediatric intensive care unit.

Duration of PICU stay depends on clinical decisions by intensive care specialists who assess in detail all aspects of individual patient recovery and particular support requirements. Guidelines have established discharge criteria based on the resolution of the conditions requiring intensive care support and on the consideration that complex care is no longer needed for paediatric patients.

These criteria include evidence of stable hemodynamic and respiratory parameters with minimal oxygen requirements, no further need of drugs such as antiarrhythmics, inotropics or vasodilators, or assurance that low doses can be safely managed in other care units; appropriate control of cardiac dysrhythmias, neurologic stability, no further need for intracranial pressure monitoring equipment or hemodynamic monitoring catheters.

Patients in need of chronic support such as mechanical ventilation or routine dialysis whose critical illness has been resolved and who are stable may also be discharged. Finally, if the health care team and the patient's family consider there would be no benefit from further support or treatment, discharge is advised (American Academy of Pediatrics – Society of Critical Care Medicine, 1999).

2.14 Statistical Analysis

Data distribution was assessed through histograms and the Shapiro Wilk test. Data is presented as mean and SD for normally distributed variables, or median and interquartile range for non-normal distribution.

Demographic data

Demographic data did not present normal distribution, hence nonparametric analysis was undertaken. Mann-Whitney tests were carried out in order to determine differences in numerical values between groups (2 tailed), while chi-square test was used for categorical data (gender and cyanosis status). Correlation between demographic variables were tested using the nonparametric Spearman´s rank correlation test (r_s).

Independent sample t-test or Mann-Whitney tests were carried out in order to determine differences between groups according to group allocation for surveillance data according to each time point.

Plasma markers

Correlation of each marker to demographic data including age, weight, gender, cyanosis status, CPB and aortic clamp duration were tested using the Pearson test (r) for normally distributed data or Spearman´s rank correlation test (r_s) for non-parametric data. Correlation of markers to cTnI levels was also explored.

Differences between time points were analysed using repeated measures ANOVA in normally distributed data applying post hoc tests using the Bonferroni correction., while repeated samples Friedman´s analysis of variance followed by post-hoc comparisons between time points using Wilcoxon signed rank tests with Bonferroni correction for multiple testing was applied for non-parametric data.

The association of cTnI PO1 measurements with independent variables was explored using a linear regression analysis for independent variables. Significant variables from univariate analyses were used in a multivariate model followed by backwards selection in order to establish predictor relationships.

Independent samples t-tests or Mann-Whitney tests were carried out in order to determine differences between RIPC or control groups (2 tailed).

Gene expression analysis

Correlation to gender, age, weight, cyanosis status, and post-operative cTnI levels were explored using Pearson analysis (r) in normally distributed data and Spearman analysis in non-parametric data (r_s).

Expression differences according to atrial or ventricular tissue type were analysed using independent sample t-tests or Mann Whitney tests.
Paired samples t-test or the non-parametric Wilcoxon signed-rank test for paired measures were used to explore differences before and after tissue incubation.
Mann Whitney tests or independent samples t-test were used as appropriate in order to determine differences according to RIPC or control allocation for each time point at baseline and after incubation.

All analyses were performed using SPSS Software version 23 at a significance level of 5%.

Statistical analysis for this study was started under the guidance of Dr Charles McSharry (Institute of Infection, Immunity and Inflammation – University of Glasgow) who provided tools for the analysis of demographic data and RIPC vs control comparisons. Further advice was provided by Dr David Young. Finally, all statistic work was discussed with Miss Nicola Greenlaw and Dr Bernard Francq (Robertson Centre for Biostatistics – University of Glasgow) close to the completion of this study in order to assure the appropriate tests were being used.

Chapter 3. General results

Recruitment took place from January 2013 to March 2015. Information on patients who were approached for considering their participation in the study was not stored from the start of the trial, however based on data collected during the last recruitment year approximately 71 patients were approached in total, with 15 refusing participation.

56 patients were recruited for the trial, 50 patients completed follow-up and 48 patients were included in RIPC analysis; *Figure 3.1* shows the patient flow during enrolment, allocation and analysis of the trial.

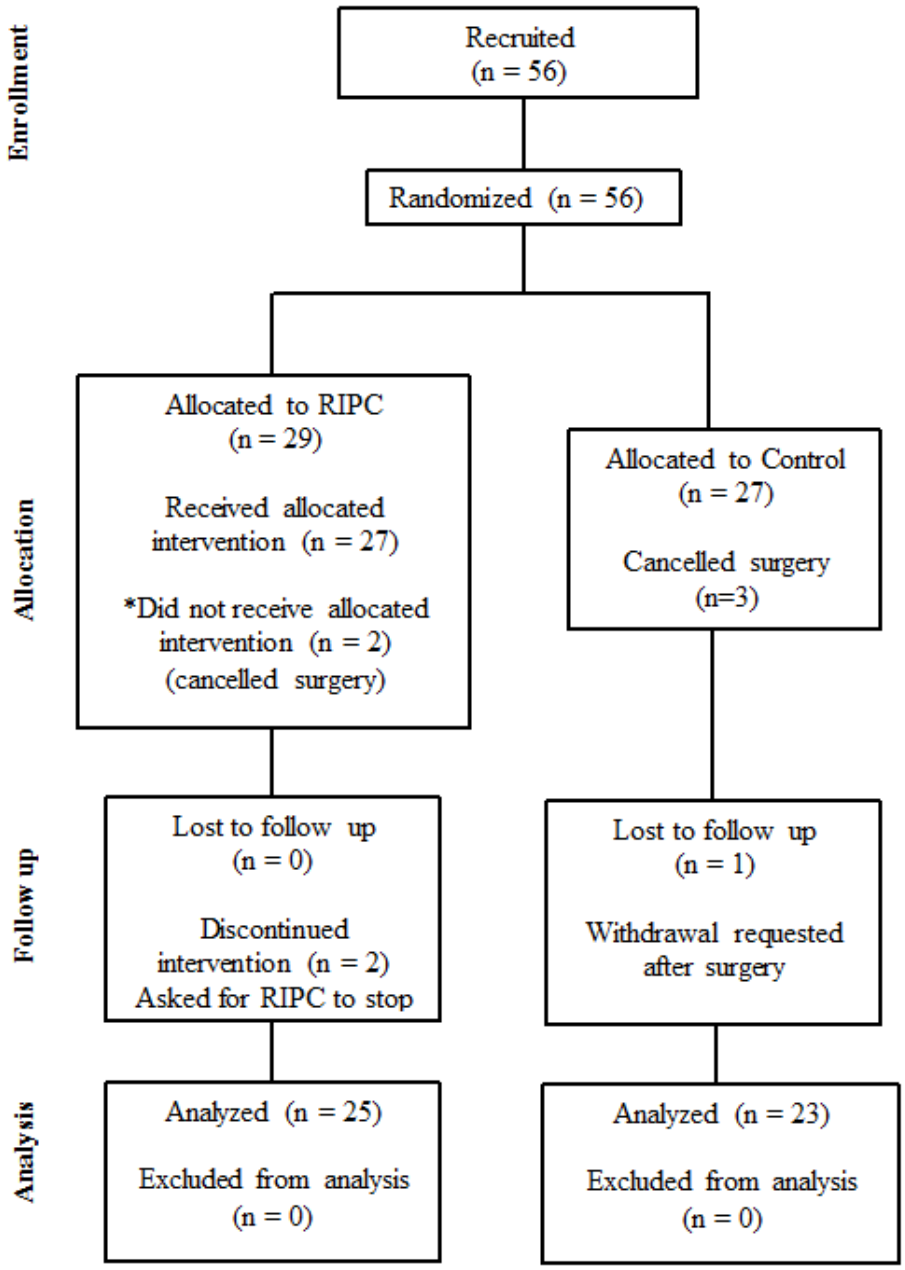

Figure 3.1. Patient flow from recruitment to analysis. CONSORT based diagram. *patients received first stage of intervention the day before surgery. Abbreviations: CONSORT: consolidated standards of reporting trials; RIPC: remote ischaemic preconditioning.

3.1 Demographics

Demographic data for all participants is presented in *Table 3.1*.

Patient	Gender	Age (months)	Weight (kg)	Diagnosis	Procedure
B01	female	19	10,25	Aortic regurgitation	Aortic valve repair
B02	female	4	5	AVSD	AVSD repair
B03	female	3	4,2	AVSD	AVSD repair
B05	Male	104	22	CoA, VSD, RV hypoplasia	TCPC
B06	Male	0,6	3,08	TGA, VSD	Switch, VSD closure
B07	female	2,36	4,58	AVSD, PDA	AVSD repair, PDA closure
B08	Male	177	55,05	Aortic regurgitation	Valve replacement
B09	female	12	6,93	VSD, PDA	Closure of PDA, VSD
B10	Male	5	6,73	HLHS	Norwood (Glenn)
B11	female	7	7,15	LPA absent	Reconstruction of LPA
B12	female	5	5,9	VSD	atrial septectomy
B13	male	10	9,15	Tricuspid atresia, VSD	Bi-directional Glenn
B14	female	16	9,45	Aortic stenosis	Aortic valve repair
B15	male	11	10	Aortic stenosis	Open AV repair + Annuloplasty
B16	male	58	16,2	Tricuspid atresia, TGA	Completion of TCPC/Fontan
B17	female	79	17,8	Aortic stenosis	aortic valve repair
B18	male	50	16,6	Tricuspid atresia	Fontan
B19	male	56	18,8	Tricuspid atresia, VSD	TCPC
B20	male	56	17,4	Double inlet RV, double outlet RV	TCPC
B21	male	4	15	HLHS	TCPC
B22	male	39	17,8	Sinous venosus, ASD	Repair of pulmonary venous connections
B23	male	118	22,3	TGA, AVSD, situs inversus	TCPC
B28	male	20	10	AVSD, TGA, DORV	Bilateral Glenn
B29	female	11	7,18	AVSD, TOF	TOF repair
B31	female	59	17,14	VSD, aortic cuspid prolapse	VSD closure, aortic valve repair
M01	male	104	24,25	Sub aortic stenosis	resection of subaortic membrane, myectomy

Patient	Gender	Age (months)	Weight (kg)	Diagnosis	Procedure
M02	male	17	9,45	Pulmonary atresia, VSD	VSD closure, RV-PA conduct
M03	male	35	12,22	PV atresia, VSD	PA reconstruction, VSD closure
M04	female	7	5,88	TOF	VSD and PDA closure
M05	female	18	12,2	Subaortic stenosis, CoA	Relief of subaortic stenosis, Ao valvotomy
M06	male	56	19,6	VSD, CoA, subaortic stenosis	Relief of sub aortic stenosis
M07	female	94	28	TOF, Subpulmonary stenosis	Tissue resection, pacemaker replacement
M09	female	6	6,64	TOF	TOF repair
M10	female	124	30,45	Sub aortic obstruction	LVOT relief of sub valve obstruction
M11	female	11	8,25	TOF	TOF repair
M12	female	60	21,05	Truncus arteriosus	RV-PA conduit
M13	female	12	8,99	TOF	TOF repair
M14	female	2	3,65	Pulmonary atresia, VSD (TOF type)	Fallot type procedure
M15	male	8	8,11	TOF	TOF repair
M16	female	6	7,7	Pulmonary atresia, VSD (TOF type)	Pulmonary atresia repair,VSD closure
M17	male	37	13,55	Pulmonary atresia, VSD, MAPCAs	VSD closure, RV to PA conduct
M18	female	9	8,86	TOF	TOF repair
M20	male	2	4,42	Pulmonary atresia	RV-PA plasty
M21	female	8	6,3	TOF, disconnected LPA	TOF repair, LPA to MPA anastomosis
M22	female	6	5,83	TOF	TOF repair
M23	male	116	34,9	Double outlet RV (TOF type)	Pulmonary valve replacement
M24	male	3	5,19	TOF	TOF repair
M26	male	3	6,82	TOF, PDA, Co LPA	TOF repair, LPA plasty
M27	male	163	42,5	RVOT obstruction, VSD	RVOT tissue resection, VSD closure
M28	female	4	5,96	TOF	TOF repair

Table 3.1. Demographic characteristics of recruited patients. Abbreviations: ASD: atrial septal defect; AV: aortic valve; AVSD: atrio-ventricular septal defect; CoA: aortic coarctation; DORV: double outlet right ventricle; HLHS: hypoplastic left heart syndrome; LPA: left pulmonary artery; LVOT: left ventricle outlet tract; MAPCAs: major aorto pulmonary collateral arteries; MPA: main pulmonary artery; PA: pulmonary artery; PDA: persistent ductus arteriosus; PV: pulmonary valve: RV: right ventricle; RVOT: right ventricle outlet tract; TCPC: total cavopulmonary connection; TGA: transposition of the great arteries; TOF: tetralogy of Fallot; VSD: ventricular septal defect.

Cyanosis status in this study was defined according to the clinical diagnosis of the patient and degree of repair, as detailed in *Table 3.2*. Oxygen saturation was not considered pre-operatively in order to determine cyanosis in each patient.

Cyanotic	**Non-cyanotic**
DORV	Aortic stenosis
HLHS	ASD
Pulmonary atresia	AVSD
RV hypoplasia	CoA
TOF	Mitral regurgitation
Tricuspid atresia	Previously corrected TOF and DORV
	Sub aortic stenosis
	TGA
	VSD

Table 3.2. Diagnosis classification according to cyanotic presentation. Abbreviations: ASD: atrial septal defect; AVSD: atrio-ventricular septal defect; CoA: aortic coarctation; DORV: double outlet right ventricle; HLHS: hypoplastic left heart syndrome; TGA: transposition of the great arteries; TOF: tetralogy of Fallot; VSD: ventricular septal defect.

Correlation between demographic characteristics including gender, age, weight, cyanosis status, anaesthesia duration, CPB duration and aortic cross clamp duration were analysed for all patients (n=50). As expected, weight correlated to age (r_s=0.944, p<0.0001) and CPB duration correlated to aortic cross clamp duration (r_s=0.716, p<0.0001).

In addition gender presented a correlation to weight (r_s=0.356, p=0.011). Variation of weight according to gender was significant with f: 7.18(5.90-10.25) vs m: 15(9.15-19.60), p=0.013. Aortic cross clamp duration tended to be longer in female patients, however the difference did not reach statistical significance with f: 111(47-133) vs m: 49(0-128), p=0.054 (*Figure 3.2*).

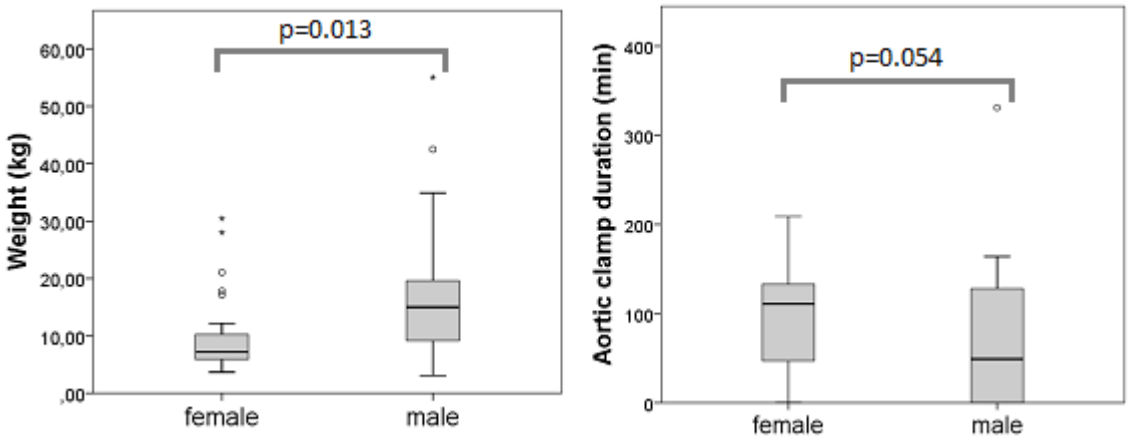

Figure 3.2. Variation on weight and aortic clamp duration according to gender.

3.2 RIPC analysis

3.2.1 Demographic and baseline characteristics

Table 3.3 shows baseline characteristics of the patients, there was no significant difference in gender, age, weight, cyanosis status, anaesthesia, CPB or aortic clamp duration between RIPC and control groups.

	RIPC	**Control**	**P value**
Gender f:m	12:13	12:11	0.773
Age (months)	19 (6-58)	9 (5-56)	0.463
Weight (Kg)	12.22 (6.82-17.40)	8.11 (6.30-17.80)	0.502
Cyanosis status (cyanotic : non cyanotic)	14 cyanotic 11 non-cyanotic	13 cyanotic 10 non-cyanotic	0.971
Anaesthesia duration (min)	400 (320-470)	380 (330-490)	0.695
CPB duration (min)	142 (104-202)	151 (101-213)	0.861
Aortic cross clamp duration (min)	69 (41-133)	86 (23-131)	0.934

Table 3.3. Baseline characteristics of participants according to group allocation. Analysis included 48 patients who were analysed according to the study intervention. Data is presented as ratios or median – IQ range as appropriate. Abbreviations: CPB; cardiopulmonary bypass; f: female; IQ: interquartile range; m: male; min: minutes; RIPC: remote ischaemic preconditioning.

3.2.2 Length of stay and intubation

Length of intubation in days did not differ significantly according to group allocation with RIPC: 0.36(0.10-3.44) vs Control: 2.79(0.46-4.83), p=0.150. Length of stay in PICU was significantly different with RIPC: 1.79(0.94-3.88) vs Control: 4.93(1.63-7.20), p=0.029 (*Figure 3.3*).

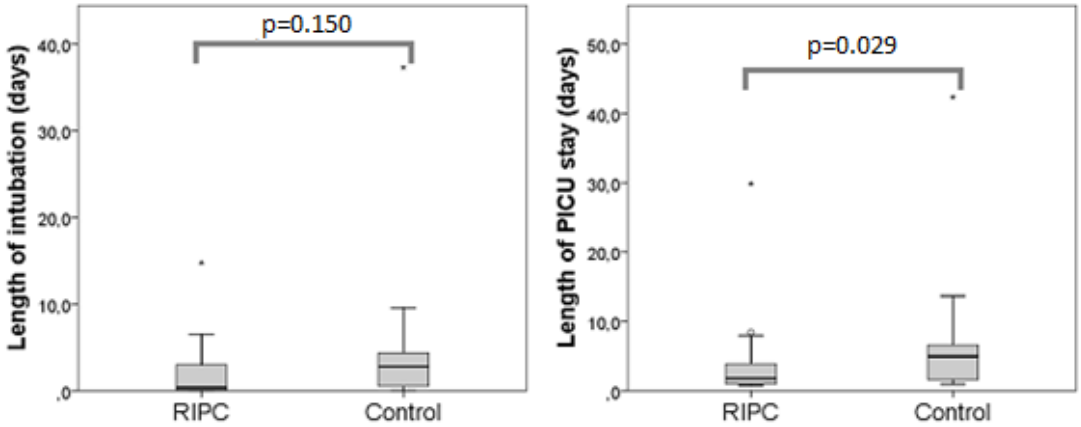

Figure 3.3. Length of intubation and PICU stay according to group allocation. Abbreviations: PICU: paediatric intensive care unit; RIPC: remote ischaemic preconditioning.

3.2.2 Inotrope score

Table 3.4 summarises descriptive data for inotrope score according to group allocation, inotrope score was not significantly different in RIPC compared to control for any of the time points analysed (*Figure 3.4*). AUC analysis revealed no difference between groups at RIPC 112.50 (20.10-373.50) vs Control 307.50(18.00-526.50), p=0.463.

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	25	5.0(0.3-7.5)	23	5.0(2.0-12.5)	0.540
6 h	25	6.0(1.3-11.5)	23	9.5(2.0-12.5)	0.583
12 h	25	3.0(0.0-11.5)	23	8.0(0.0-14.5)	0.433
18 h	25	0.0(0.0-10.5)	23	8.0(0.0-16.5)	0.170
24 h	25	0.0(0.0-10.5)	23	7.5(0.0-13.5)	0.225
30 h	25	0.0(0.0-10.5)	23	5.0(0.0-13.5)	0.355
36 h	25	0.0(0.0-5.3)	23	3.8(0.0-13.5)	0.133
42 h	25	0.0(0.0-0.6)	23	5.0(0.0-11.5)	0.141

Table 3.4. Descriptive data for inotrope score according to group allocation for each time point.

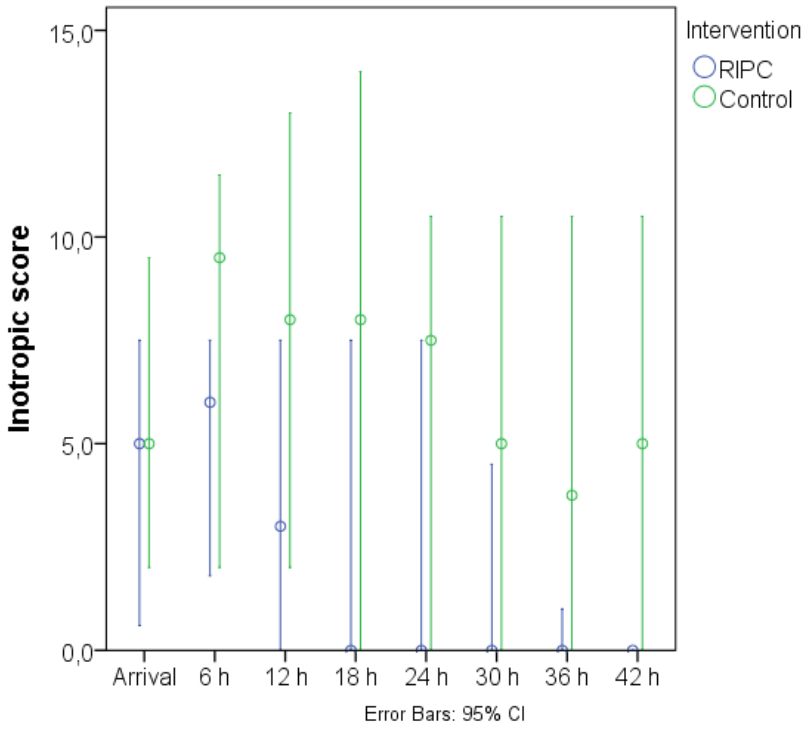

Figure 3.4. Inotrope score according to RIPC or control allocation across time points. Values are expressed in medians. Abbreviations: h: hours; RIPC: remote ischaemic preconditioning.

3.2.2 Ventilation parameters

Mean fraction of inspired oxygen (M-FiO_2) descriptive data is shown in *Table 3.5;* M-FiO_2 was significantly lower in the RIPC group at PICU arrival compared to control (*Figure 3.5*); however AUC comparison between both groups was not significant with RIPC 2351.29±539.37 vs Control 2211.32±731.48, p=0.646.

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	21	49.7(40.0-60.0)	21	55.0(50.0-85.0)	0.029
6 h	19	50.0(34.0-60.0)	19	52.0(40.0-90.0)	0.402
12 h	17	39.7(30.0-54.0)	18	54.0(35.0-71.0)	0.184
18 h	13	40.0(30.0-50.0)	18	53.5(41.0-70.0)	0.062
24 h	10	40.0(40.0-60.0)	14	55.5(35.2-81.0)	0.285
30 h	9	50.0(40.5-59.0)	14	47.5(40.0-71.0)	1.00
36 h	10	47.0(40.0-50.0)	14	45.0(40.0-70.0)	0.977
42 h	10	50.0(40.2-80.0)	15	41.0(30.3-72.0)	0.311

Table 3.5. Descriptive data for M-FiO_2 according to group allocation for each time point.

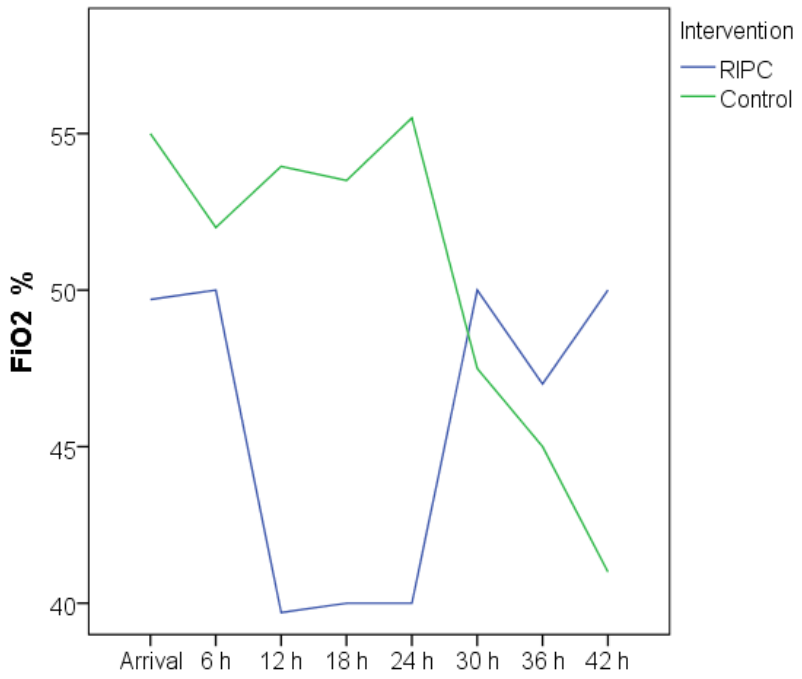

Figure 3.5. M-FiO_2 variation according to RIPC or control allocation across time points. Values expressed in medians – IQ range. Abbreviations: M-FiO_2: mean fraction of inspired oxygen; h: hours; RIPC: remote ischaemic preconditioning.

Analysis of PaO_2–FiO_2 ratio, a common indicator of oxygenation did not result in any significant differences between treatment and control groups (*Table 3.6*, *Figure 3.6*).

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	21	38.5 (21.5-60.2)	20	26.8(16.2-45.8)	0.273
6 h	15	31.0(18.3-55.6)	17	26.3(18.2-54.0)	0.390
12 h	12	33.5(18.5-50.8)	16	25.3(14.8-35.8)	0.302
18 h	8	28.9(22.4-33.4)	15	29.1(19.8-48.0)	0.728
24 h	5	24.22 ± 2.77	11	23.55 ± 12.39	0.909
30 h	6	26.24 ± 9.59	11	24.22 ± 12.37	0.735
36 h	8	26.71 ±11.55	9	25.25 ± 11.88	0.800
42 h	7	20.99 ±13.38	10	22.35 ± 12.31	0.830

Table 3.6. Descriptive data for PaO_2–FiO_2 ratio according to group allocation for each time point. Abbreviations: PaO_2: partial pressure of oxygen; FiO_2: fraction of inspired oxygen; h: hours; RIPC: remote ischaemic preconditioning.

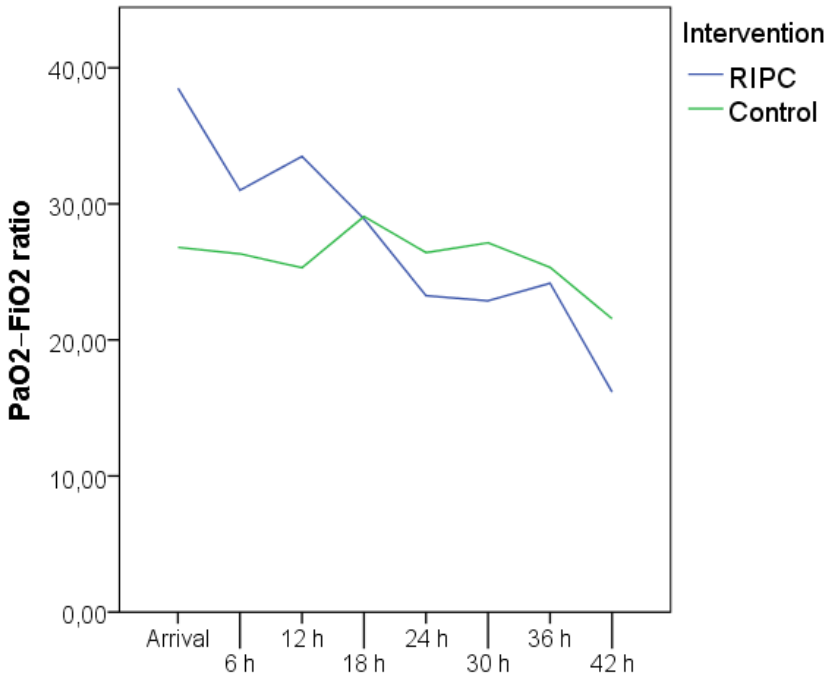

Figure 3.6. PaO_2–FiO_2 ratio variation according to RIPC or control allocation across time points. Values expressed in medians. Abbreviations: PaO_2: partial pressure of oxygen; FiO_2: fraction of inspired oxygen; h: hours; RIPC: remote ischaemic preconditioning.

M-Pmean descriptive data is shown in *Table 3.7*. Values did not vary according to group allocation for any time point (*Figure 3.7*). AUC comparison between both groups was not significant with RIPC: 418.11±43.24 vs Control: 427.67±56.68, p=0.705.

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	19	8.0(7.0-10.1)	21	10.0(8.0-11.0)	0.069
6 h	17	9.0(8.0-11.0)	18	10.1(9.0-14.0)	0.195
12 h	11	11.0(8.9-12.0)	17	10.0(9.5-12.0)	0.781
18 h	10	10.0(8.0-11.0)	16	10.0(9.0-11.0)	0.737
24 h	8	10.5(9.25-13.0)	14	10.0(9.0-12.0)	0.868
30 h	8	11.0(9.5-12.0)	13	10.0(9.0-12.0)	0.972
36 h	7	11.0(8.0-12.0)	14	9.5(7.0-12.0)	0.799
42 h	8	11.35(9.0-12.0)	14	9.0(7.0-12.0)	0.482

Table 3.7. Descriptive data for M-Pmean according to group allocation for each time point. Abbreviations: M-Pmean: mean airway pressure; h: hours; RIPC: remote ischaemic preconditioning.

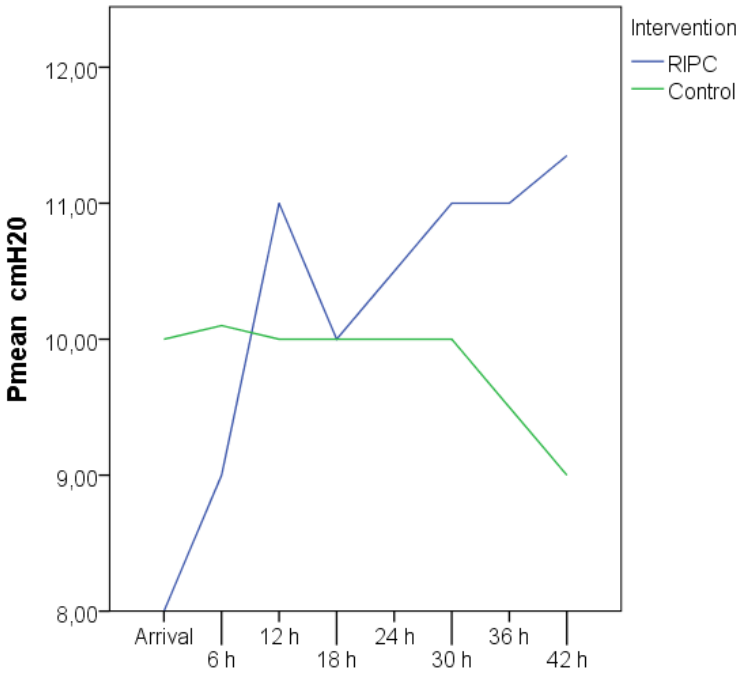

Figure 3.7. M-Pmean variation according to RIPC or control allocation across time points. Values expressed in medians. Abbreviations: M-Pmean: mean airway pressure; h: hours; RIPC: remote ischaemic preconditioning.

3.2.3 Arterial blood gases

Descriptive information on the partial pressure of carbon dioxide (PCO_2) is shown on *Table 3.8*. PCO_2 was significantly higher in the control group 42 hours after surgery (*Figure 3.8*).

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	25	6.26±0.97	22	6.16±1.18	0.739
6 h	21	5.56±0.76	21	5.73±0.94	0.519
12 h	23	5.38±0.61	21	5.48±0.69	0.616
18 h	16	5.62±0.66	20	5.32±0.92	0.284
24 h	10	5.55±0.71	15	5.56±0.83	0.975
30 h	9	5.39±1.01	14	5.54±0.62	0.670
36 h	12	5.71±0.96	13	5.81±0.52	0.747
42 h	8	5.2(5.0-5.7)	12	5.8(5.4-6.4)	**0.047**

Table 3.8. Descriptive data for PCO_2 according to group allocation for each time point. Abbreviations: PCO_2: partial pressure of carbon dioxide; h: hours; RIPC: remote ischaemic preconditioning.

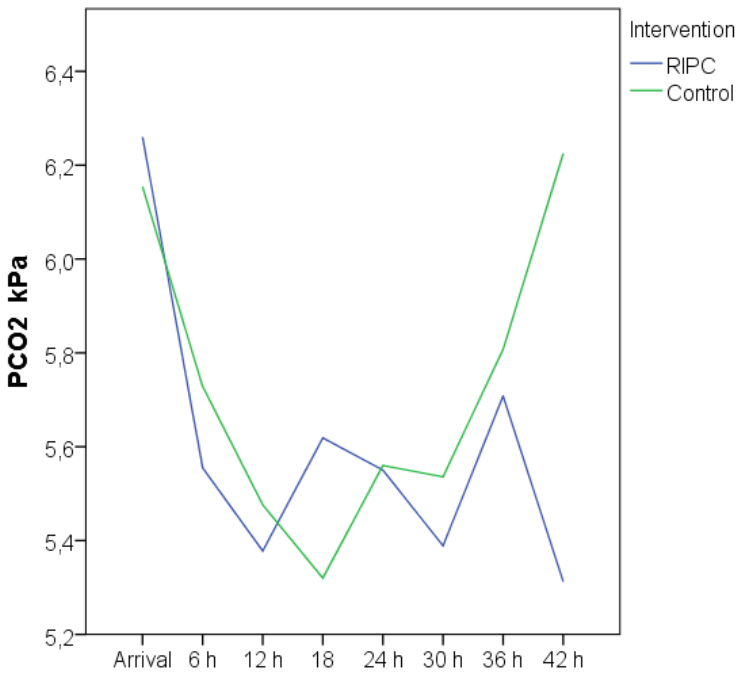

Figure 3.8. PCO_2 variation according to RIPC or control allocation across time points. Values expressed in means. Abbreviations: PCO_2: partial pressure of carbon dioxide; h: hours; RIPC: remote ischaemic preconditioning.

Variation was not significantly different for concentration of hydrogen (CH) (*Table 3.9; Figure 3.9*), PO_2 (*Table 3.10; Figure 3.10*), HCO_3 (*Table 3.11; Figure 3.11*), O_2sat (*Table 3.12; Figure 3.12*), glucose (*Table 3.13; Figure 3.13*) and lactate (*Table 3.14; Figure 3.14*) for any time point.

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	25	52.0(47.8-57.6)	22	49.0(47.6-61.8)	0.949
6 h	20	48.10±5.85	21	50.72±5.24	0.138
12 h	23	48.73±7.42	21	48.54±4.57	0.921
18 h	16	47.3(45.2-51.3)	20	48.2(42.5-51.1)	0.718
24 h	10	47.9(45.0-49.7)	15	48.7(42.8-50.1)	0.935
30 h	9	45.3(38.9-46.5)	14	44.0(38.1-47.5)	0.734
36 h	12	42.7(38.7-46.4)	13	43.0(41.0-45.4)	0.689
42 h	8	42.7(38.7-46.4)	12	43.0(41.0-45.4)	0.270

Table 3.9. Descriptive data for CH according to group allocation for each time point. Abbreviations: CH: concentration of hydrogen; h: hours; RIPC: remote ischaemic preconditioning.

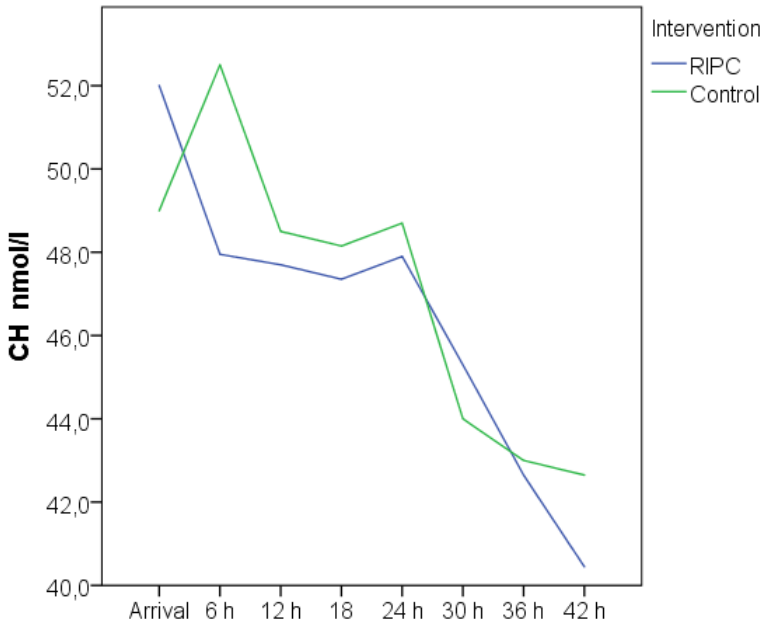

Figure 3.9. M-Pmean variation according to RIPC or control allocation across time points. Values expressed in medians. Abbreviations: CH: concentration of hydrogen; h: hours; RIPC: remote ischaemic preconditioning.

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	25	15.4(10.8-23.9)	22	13.1(10.8-23.5)	0.515
6 h	20	13.1(10.9-20.0)	21	15.1(11.5-19.8)	0.896
12 h	23	13.1(9.0-16.4)	21	11.7(10.0-18.0)	0.707
18 h	16	10.8(9.4-14.2)	20	11.6(10.1-19.7)	0.290
24 h	10	11.1(9.3-13.1)	15	11.0(5.7-16.7)	0.765
30 h	9	10.6(9.5-13.5)	14	11.7(8.6-14.9)	0.926
36 h	12	10.1(8.9-11.9)	12	10.8(9.0-11.4)	1.00
42 h	8	10.20±4.27	12	9.58±2.94	0.702

Table 3.10. Descriptive data for PO_2 according to group allocation for each time point¡Error! Marcador no definido.**.** Abbreviations: PO_2: partial pressure of oxygen; h: hours; RIPC: remote ischaemic preconditioning.

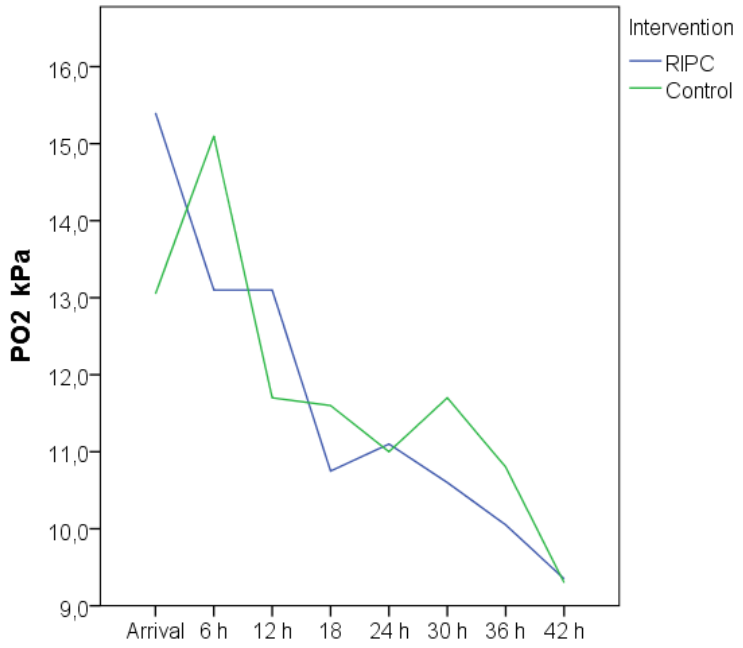

Figure 3.10. PO_2 variation according to RIPC or control allocation across time points. Values expressed in medians. Abbreviations: PO_2: partial pressure of oxygen; h: hours; RIPC: remote ischaemic preconditioning.

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	25	22.19±1.996	22	21.67±2.34	0.420
6 h	20	21.46±2.41	21	20.83±2.59	0.428
12 h	23	20.77±2.99	21	20.97±2.83	0.828
18 h	16	21.38±2.96	20	21.23±4.08	0.903
24 h	10	20.97±3.05	15	21.95±3.73	0.496
30 h	9	22.20±2.20	14	23.27±3.89	0.462
36 h	12	-0.51±2.97	13	-1.35±4.87	0.766
42 h	8	23.71±3.55	12	24.60±4.46	0.647

Table 3.11. Descriptive data for HCO_3 according to group allocation for each time point. Abbreviations: HCO_3: bicarbonate; h: hours; RIPC: remote ischaemic preconditioning.

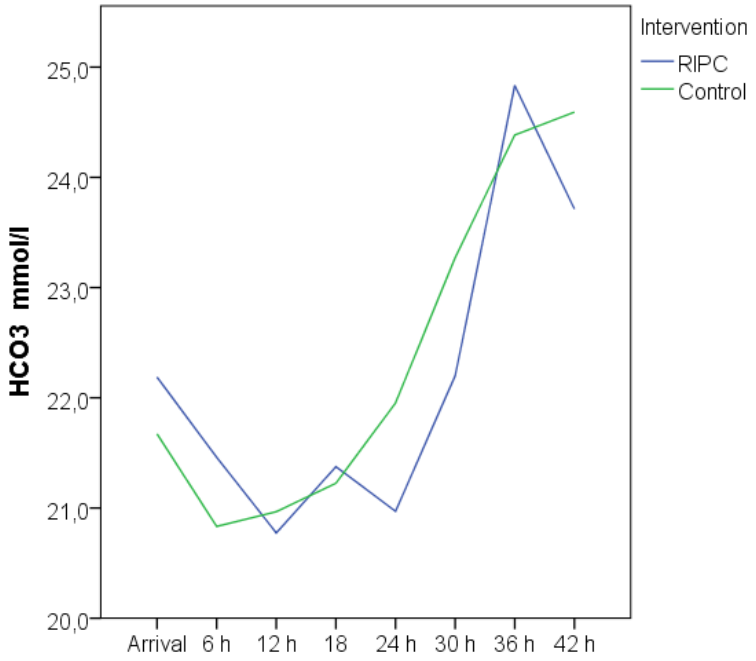

Figure 3.11. HCO_3 variation according to RIPC or control allocation across time points. Values expressed in means. Abbreviations: HCO_3: bicarbonate; h: hours; RIPC: remote ischaemic preconditioning.

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	25	99(97-100)	22	99(97-100)	0.810
6 h	20	99(97-99)	21	99(98-100)	0.570
12 h	23	99(95-99)	21	98(96-100)	0.933
18 h	16	97(96-99)	20	99(97-100)	0.336
24 h	10	97(95-98)	15	98(94-99)	0.978
30 h	9	98(96-98)	14	98(94-99)	0.829
36 h	12	97(95-99)	13	97(96-98)	0.810
42 h	8	96(93-99)	12	96(90-98)	0.851

Table 3.12. Descriptive data for O_2 saturation according to group allocation for each time point. Abbreviations: O_2: oxygen; h: hours; RIPC: remote ischaemic preconditioning.

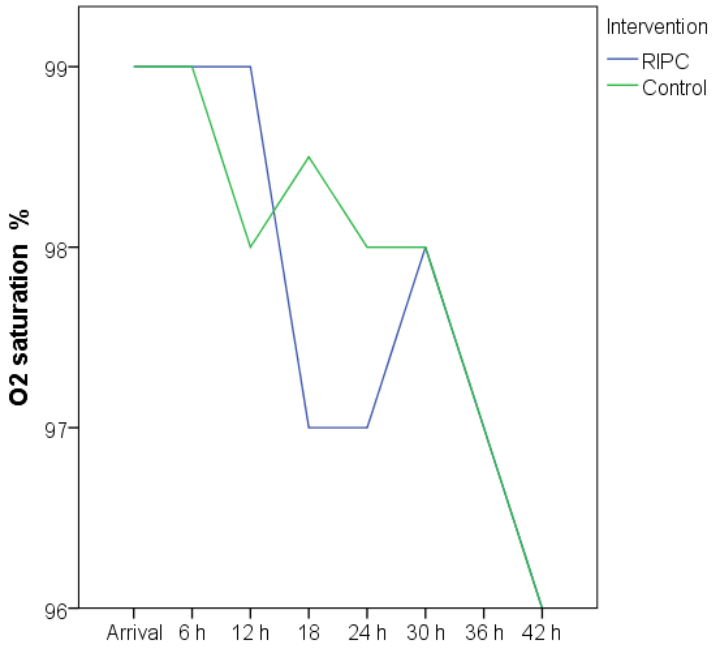

Figure 3.12. O_2 saturation variation according to RIPC or control allocation across time points. Values expressed in medians. Abbreviations: O_2: oxygen; h: hours; RIPC: remote ischaemic preconditioning.

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	25	6(4-7)	22	6(5-7)	0.609
6 h	20	6(6-8)	21	7(5-8)	0.497
12 h	23	6.03±1.27	21	6.57±1.39	0.187
18 h	16	6.19±1.27	20	6.23±1.46	0.919
24 h	10	6.00±1.10	15	6.93±1.66	0.132
30 h	9	6(6-7)	14	6(6-8)	0.643
36 h	12	6.93±1.92	13	7.47±2.31	0.537
42 h	8	6(6-7)	12	7(6-7)	0.384

Table 3.13. Descriptive data for glucose levels according to group allocation for each time point. Abbreviations: h: hours; RIPC: remote ischaemic preconditioning.

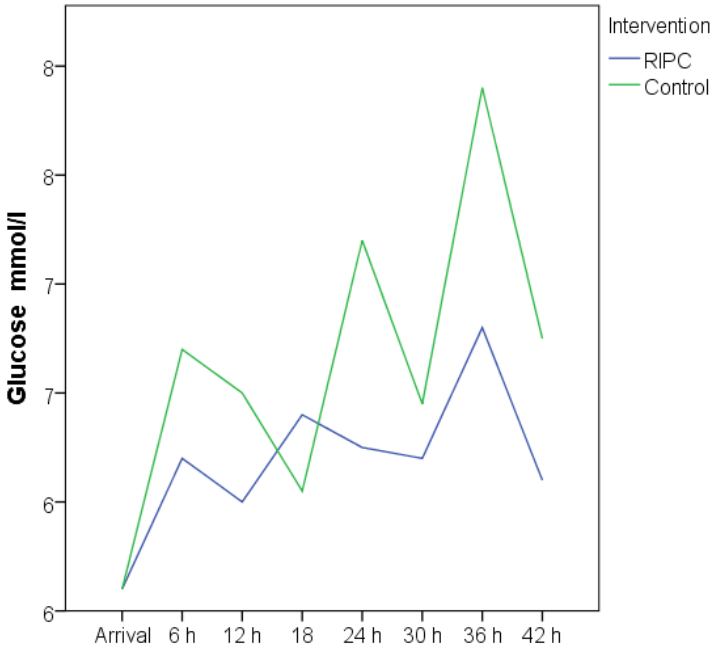

Figure 3.13. Glucose variation according to RIPC or control allocation across time points. Values expressed in medians. Abbreviations: h: hours; RIPC: remote ischaemic preconditioning.

	RIPC		Control		
Time point	**N**	**Median – IQ range**	**N**	**Median – IQ range**	**P value**
Arrival	25	1.2(0.9-1.9)	22	1.5(1.0-1.8)	0.488
6 h	20	1.3(1.1-1.7)	21	1.2(1.2-2.0)	0.969
12 h	23	1.2(0.8-1.8)	21	1.2(0.9-1.6)	0.841
18 h	16	1.03±0.28	20	1.16±0.36	0.252
24 h	10	1.05±0.35	15	1.15±0.29	0.430
30 h	9	1.2(1.0-1.3)	14	1.0(1.0-1.3)	0.643
36 h	12	1.1(0.8-1.3)	13	1.0(0.8-1.2)	0.650
42 h	8	1.2(0.9-1.4)	11	0.9(0.9-1.1)	0.206

Table 3.14. Descriptive data for lactate levels according to group allocation for each time point. Abbreviations: h: hours; RIPC: remote ischaemic preconditioning.

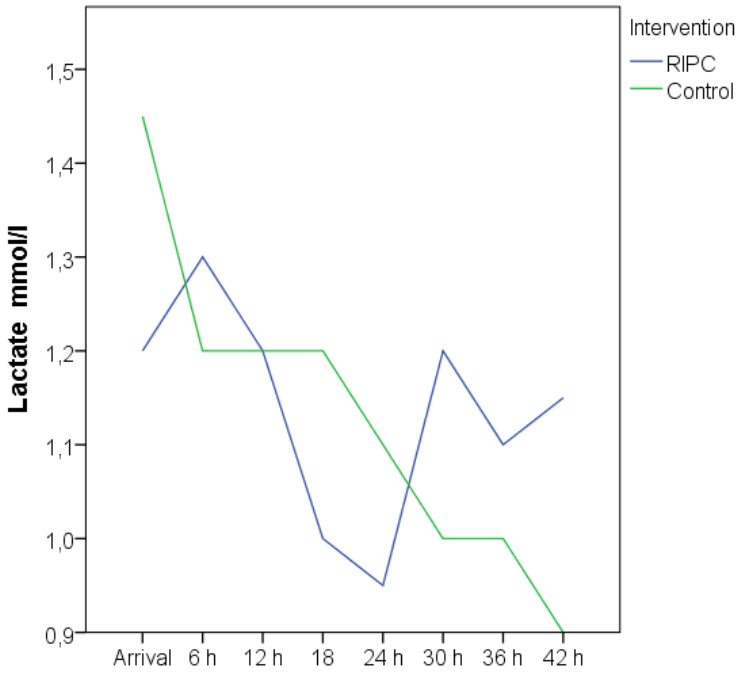

Figure 3.14. Lactate variation according to RIPC or control allocation across time points. Values expressed in medians. Abbreviations: h: hours; RIPC: remote ischaemic preconditioning.

3.2.4 Renal function – Urea and electrolytes

Urea (*Figure 3.15*) and creatinine (*Figure 3.16*) did not vary significantly according to group allocation. Electrolytes including Na, K, Cl, Phosphate and Mg did not present a significant difference either. All descriptive data can be seen on *Appendix 3*.

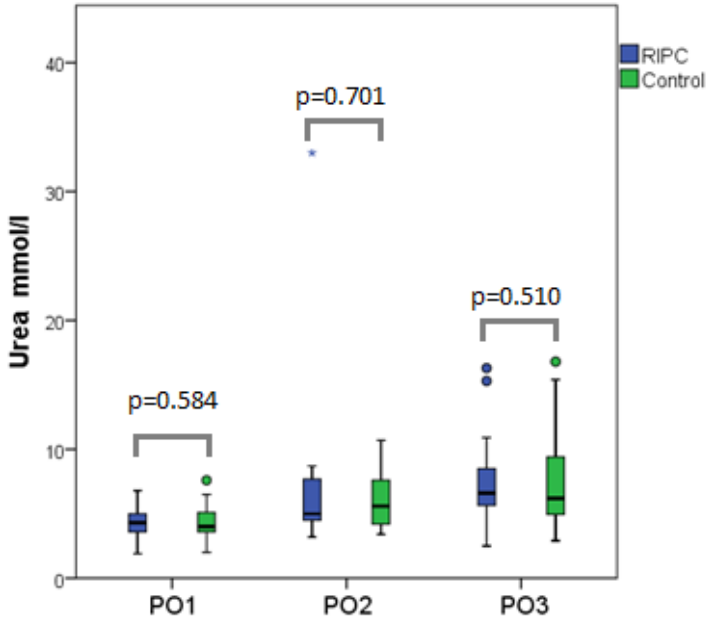

Figure 3.15. Urea variation according to RIPC or control allocation across time points. Abbreviations: h: hours; RIPC: remote ischaemic preconditioning.

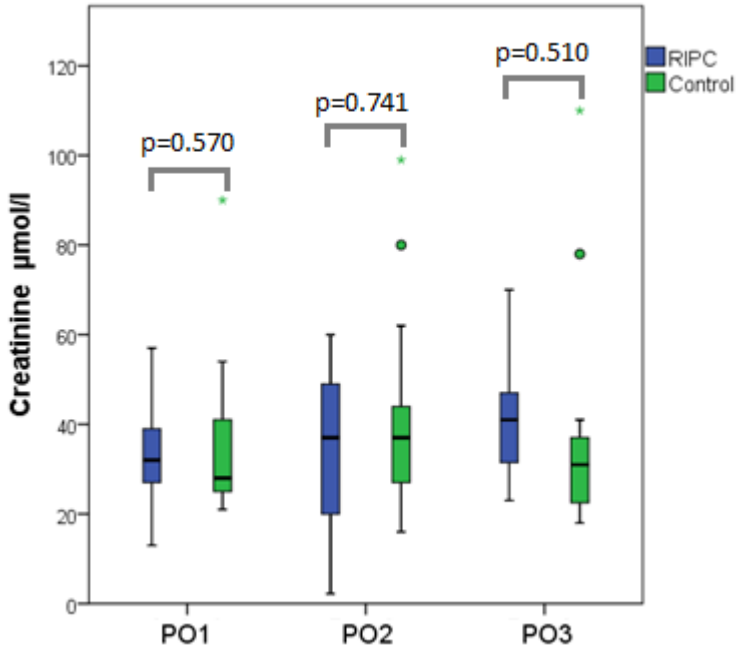

Figure 3.16. Creatinine variation according to RIPC or control allocation across time points. Abbreviations: h: hours; RIPC: remote ischaemic preconditioning.

3.2.5 Liver function tests

All parameters including alkaline phosphatase (*Figure 3.17*), aspartate transaminase (AST), alanine transaminase (ALT), bilirubin (*Figure 3.18*), albumin (*Figure 3.19*), and C reactive protein (CRP) (*Figure 3.20*) were not significantly different according to RIPC or control allocation. Descriptive data is summarised in *Appendix 3*.

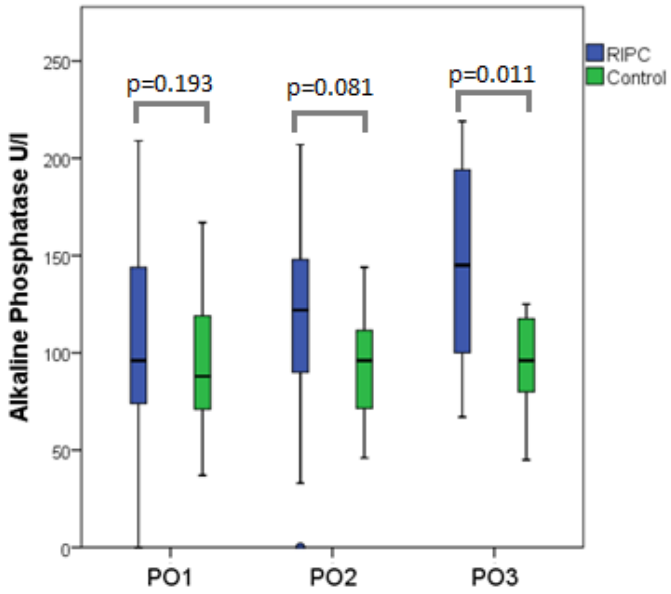

Figure 3.17. Alkaline phosphatase variation according to RIPC or control allocation across time points. Abbreviations: h: hours; PO: post-operative; RIPC: remote ischaemic preconditioning.

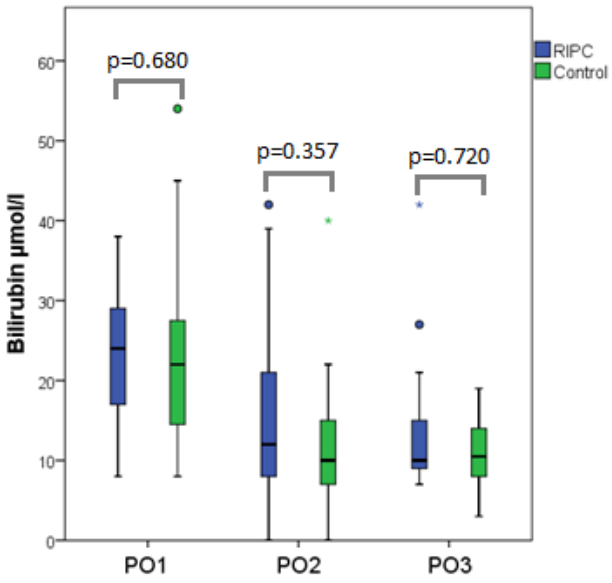

Figure 3.18. Bilirubin variation according to RIPC or control allocation across time points. Abbreviations: h: hours; PO: post-operative; RIPC: remote ischaemic preconditioning.

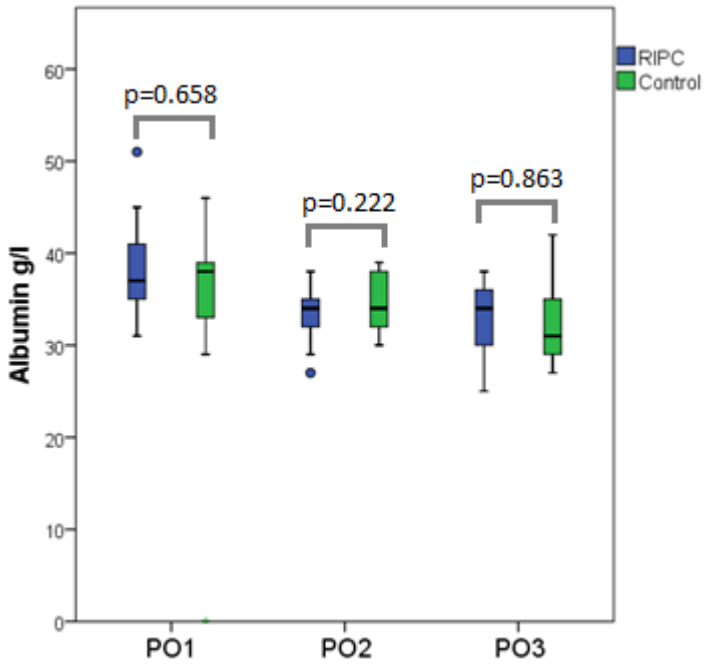

Figure 3.19. Albumin variation according to RIPC or control allocation across time points. Abbreviations: h: hours; PO: post-operative; RIPC: remote ischaemic preconditioning.

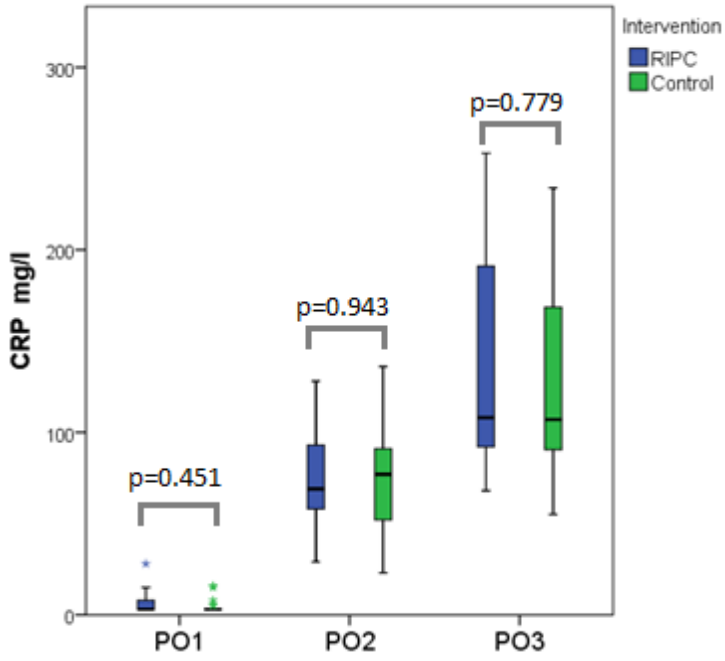

Figure 3.20. CRP variation according to RIPC or control allocation across time points. Abbreviations: CRP: C reactive protein; h: hours; PO: post-operative; RIPC: remote ischaemic preconditioning.

3.2.6 Whole blood count

Mean corpuscular haemoglobin (MCH) was significantly higher in the control group at PO2 (RIPC: 28.52±1.55 vs Control: 29.46±1.53, p=0.049) (*Figure 3.21*). All other parameters did not vary according to group allocation, all data is shown in *Appendix 3*.

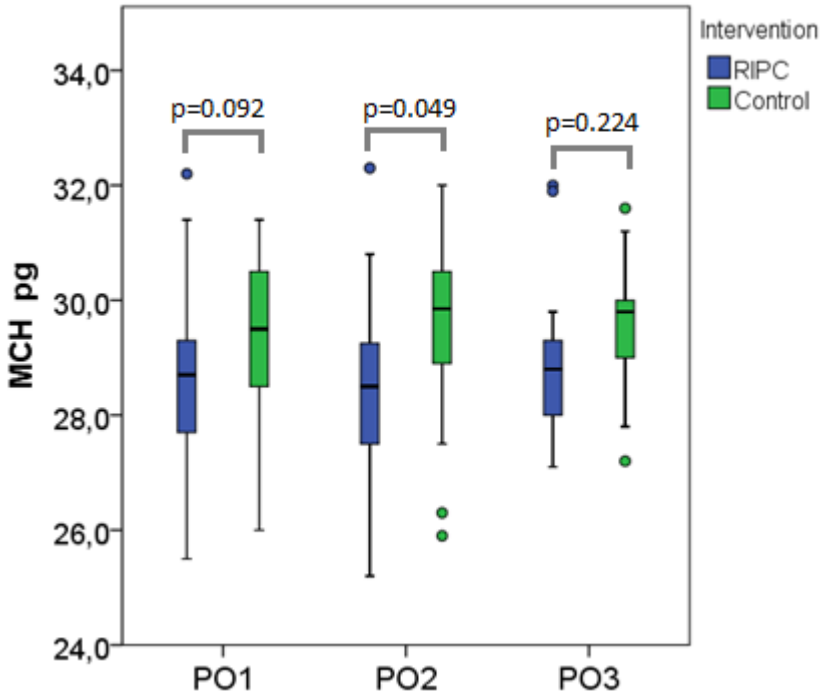

Figure 3.21. MCH variation according to RIPC or control allocation across time points. Abbreviations: h: hours; MCH: mean corpuscular haemoglobin; PO: post-operative; RIPC: remote ischaemic preconditioning.

3.2.7 Coagulation

Coagulation parameters including prothrombin time (PT) (*Figure 3.22*), partial thromboplastin time (APTT) (*Figure 3.23*) and fibrinogen (*Figure 3.24*) were not significantly different according to group allocation.

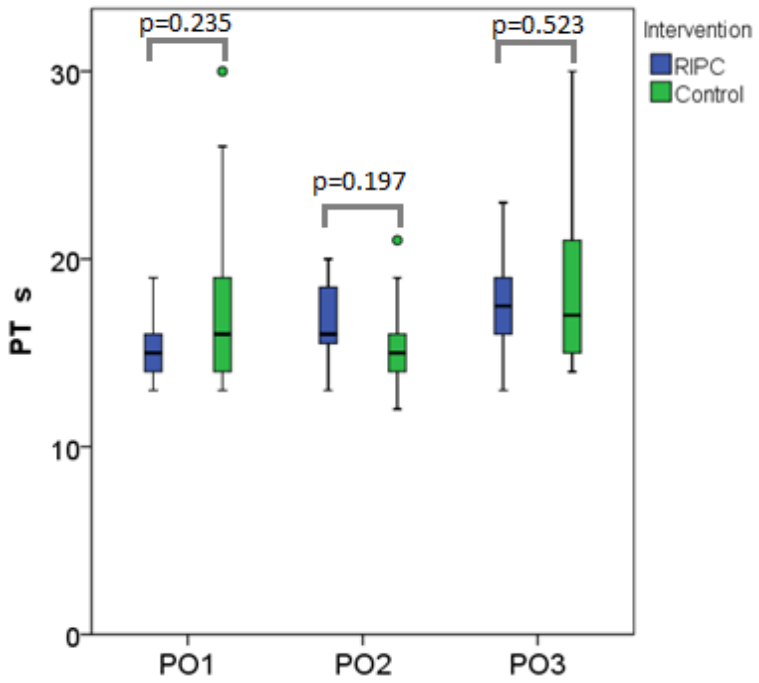

Figure 3.22. PT variation according to RIPC or control allocation across time points. Abbreviations: PO: post-operative; PT: prothrombine time; RIPC: remote ischaemic preconditioning; s: seconds.

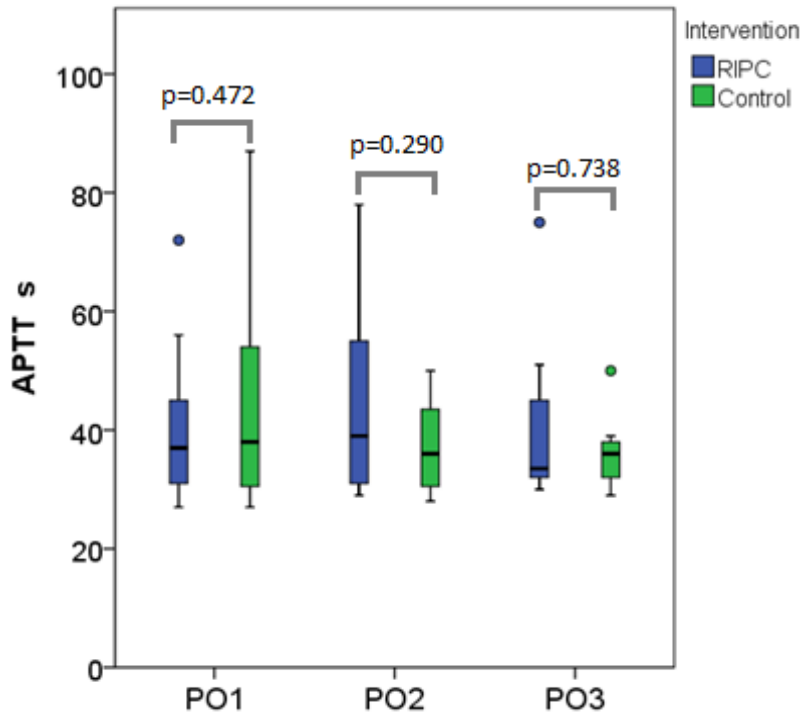

Figure 3.23. APPT variation according to RIPC or control allocation across time points. Abbreviations: APPT: activated partial thromboplastin time; PO: post-operative; RIPC: remote ischaemic preconditioning; s: seconds.

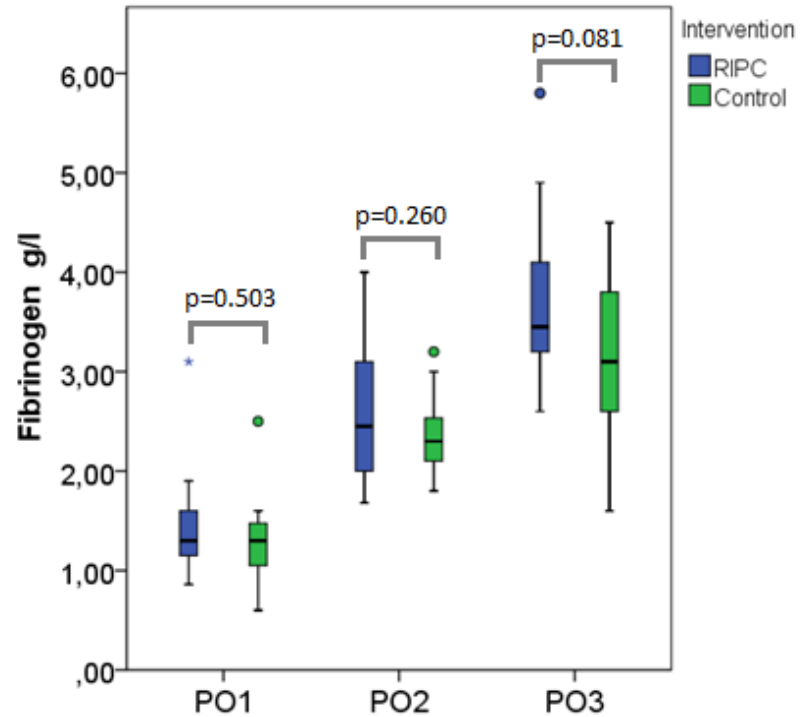

Figure 3.24. Fibrinogen variation according to RIPC or control allocation across time points. Abbreviations: PO: post-operative; RIPC: remote ischaemic preconditioning; s: seconds.

3.2.8 Limitations

Due to clinical circumstances all variables in this section do not have a pre-operative measurement which would have been useful as a baseline in order to follow post-operative changes.

3.2.9 Summary of key findings

- Length of PICU stay was shorter in the RIPC group.
- Arterial blood PCO_2 concentration was higher in the control group 42 hours after surgery.
- MCH was higher in the control group.

3.2.10 Discussion

Cardiac surgical procedures and all the components involved have an impact on cardiac and end organ functionality after surgery. During the post-operative period of CHD correction there is a very close surveillance of all systems within PICU. A record and analysis of these surveillance parameters allow an insight into the evolution of patients with any effect on them from a research intervention being the most relevant for patient outcome.

Patients in the RIPC group presented a shorter length of stay in PICU, no other clinical characteristics favoured the application of preconditioning. Most of the previously reported RIPC studies have not found differences in intensive care length of stay including all paediatric trials (*Table 1.8*), while only one study in adults reported a significantly shorter ICU stay after RIPC (Choi *et al*, 2011).

In spite of this, a meta-analysis that pooled together the data from 7 paediatric clinical trials concluded that RIPC significantly reduced ICU length of stay (Tie *et al*, 2014), this report was not confirmed in a later analysis by the same main authors which included 2 additional trials,

one of them focused on kidney injury (Tie *et al*, 2015). Both reports highlighted the heterogeneity of the analysed clinical trials.

ICU length of stay is an established predictor of outcome, with prolonged stay times being associated to immediate and long term effects after cardiac surgery (Mahesh *et al*, 2012). Although the present finding is in discordance to previous reports, this should be explored further.

Inotrope requirements tended to be higher in the control group, however differences did not reach statistical significance for any time points. RIPC clinical trials have mostly reported no differences in inotropic scores according to group allocation, however lower inotrope requirements have been reported after RIPC in adults (Wu *et al*, 2011). Likewise, several paediatric clinical trials found that RIPC resulted in a lower inotropic score after surgery (Cheung *et al*, 2006; Zhou *et al*, 2010; Luo *et al*, 2011).

Ventilation parameters did not vary between groups, previous paediatric studies reporting length of intubation did not find significant variations; six trials reported additional ventilatory parameters, with one including M-FiO_2 and peak inspiratory pressure (McCrindle *et al*, 2014), while the other reported indexes with no significant variation in any parameters after RIPC (Cheung *et al*, 2006; Zhou *et al*, 2010; Lee *et al*, 2012; Jones *et al*, 2013; Pepe *et al*, 2013).

Arterial blood gas reports showed higher PCO_2 in control patients at one time point only (42 hours after surgery); other parameters of the analysis were not significantly different, nevertheless increased PCO_2 could reflect respiratory issues. Although previous RIPC studies have recorded blood gas analysis results in order to calculate indexes, PCO_2 values have not been reported.

We found no variation in lactate or glucose levels; accordingly, one previous study reported no difference in glucose while several studies analysed lactate levels reporting there was no significant difference according to RIPC allocation (Lee *et al*, 2012; Pavione *et al*, 2012; Jones *et al*, 2013; Pepe *et al*, 2013; McCrindle *et al*, 2014).

Creatinine levels did not vary significantly between groups, this result supports previous findings in paediatric RIPC studies which found no differences in the marker at post-operative measurements (Lee *et al*, 2012; Pavione *et al*, 2012; Pepe *et al*, 2013; McCrindle *et al*, 2014).

Other renal function parameters including urea and electrolytes presented no significant variation; urea had been previously analysed in relation to RIPC finding no influence from the intervention (McCrindle *et al*, 2014).

MCH presented borderline significantly higher values in the control group; MCH expresses the average content of haemoglobin in red blood cells, it is used during the differential diagnosis of anaemia and is unlikely to be clinically relevant regarding RIPC. This variable has not been reported in this context before.

As previously described by Lee *et al*, 2012, there was no difference in Hb or haematocrit levels; all other post-operative blood count values were not significantly different, McCrindle *et al*, 2014 reported higher neutrophil and white blood cell count in the control group 48 hours after surgery.

There was no significant difference in liver function tests, CRP, or coagulation parameters in this study, similar results were obtained in other clinical trials (Lee *et al*, 2012; McCrindle *et al*, 2014).

Conclusion

Clinical parameter analysis revealed an important effect of RIPC with a significantly shorter PICU length of stay, all other parameters presented no significant difference. The implications and possible factors influencing this outcome should be explored.

Cardiac analysis

Chapter 4. Cardiac injury

4.1 Introduction

Children presenting with CHD who require surgical intervention are vulnerable to cardiac IRI as a consequence of the procedure itself and the use of CPB; advances in surgical techniques, perfusion, operative and perioperative care have improved survival and prognosis, however the unavoidable cardiac ischaemic lapse and exposure to systemic reactions continue to cause myocardial injury and to impact cardiac function after surgery and into adult life (Townsend *et al*, 2013).

Myocardial stress can lead to cardiomyocyte stretch, peptide and hormone synthesis and cellular death, with a release of its contents, triggering inflammatory mediators. Products of this process can then be detected in blood and used as indicators of injury (reviewed by Lorts *et al*, 2013). Cardiac troponin and B-type natriuretic peptide are habitually used in clinical practice as injury markers in adults and although their measurement is not routine in CHD paediatric patients they can offer an assessment of cardiac injury.

4.1.1 Cardiac Troponin

Troponin is part of the myocardium sarcomeric unit where it controls the interaction of myosin and actin filaments. Three subunits make up the troponin complex with troponin C acting as the calcium binding subunit, troponin T contributing to the thin filament attachment, and troponin I inhibiting the formation of the cross bridge between actin and myosin (reviewed by Lorts *et al*, 2013).

Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) are specific to the myocardium and are released after cardiomyocyte death, they are detectable for several days constituting one of the most sensitive and specific biomarkers for cardiac injury; the choice of cTnT or cTnI is mostly interchangeable and although most research involving them has been done regarding cardiac injury and myocardial infarction, the marker is useful in other circumstances including myocarditis and detecting transplant rejection (reviewed by Fathil *et al*, 2015).

In children troponin is not measured routinely, however it is a valuable marker in order to assess cardiac injury. A study found higher cTnT levels in newborn children with CHD which were linked to haemodynamic but not to ventricular function (Tarkowska and Furmaga-Jabłońska, 2012), similar results were found with higher cTnI in cyanotic and acyanotic CHD children when compared to healthy controls (Hafez *et al*, 2015).

Technology advances are leading to a recent shift to the use of high sensitivity cardiac troponin (hscTn) assays which are able to detect much lower concentrations of the protein and have been included in the most recent guidelines for fast detection by the European Society of Cardiology (Roffi *et al*, 2015).

4.1.2 B-type natriuretic peptide

Natriuretic peptides are hormones produced as a response to myocyte stretch by the heart and vascular endothelium, although the name B (brain)-type natriuretic peptide (BNP) was assigned, BNP is mainly synthesised in the ventricular myocardium (Hosoda *et al*, 1991), it is synthesised as an inert N-terminal fragment (N-terminal pro-BNP) and as the biologically active BNP (reviewed by Egom, 2015).

BNP has been established as a first line biomarker for the diagnosis of heart failure in adult patients with abundant evidence and is used in order to estimate disease severity and as an outcome predictor (Yancy *et al*, 2013).

In paediatric practice the use of BNP is much less common, interest has been demonstrated for the use of BNP in the management of CHD patients and small studies have suggested that BNP could be very suitable as a prognosis marker following paediatric cardiac surgery (Cantinotti *et al*, 2015); however no definite evidence, recommendation or reference values have been established for its clinical use (reviewed by Cantinotti *et al*, 2015).

It is to be noted that the inherent characteristics of CHDs could alter BNP values, it has been observed that BNP levels are related to the type of CHD, presenting higher values in complex conditions, in general involving left rather than right ventricle overload, with higher values in

conditions such as univentricular heart, TGA, VSD, AVSD, PDA and truncus arteriosus while values are lower in ASD, pulmonary stenosis and TOF, presenting a correlation with ventricular function (Holmgren *et al*, 2005; Koch *et al*, 2006).

In addition factors external to CHD may affect BNP levels including age, general well-being and comorbidities (reviewed by Cantinotti *et al*, 2015).

4.2 Aims

To analyse the pattern of cardiac markers cTnI and BNP on CHD patients before and after surgery using CPB and to analyse the effect or RIPC on the levels of these markers.

4.3 Materials and Methods

Blood Marker Analysis

Troponin-I and Brain Natriuretic Peptide (BNP)

Troponin-I and BNP levels were determined by Dr Peter Galloway´s team (Department of Clinical Biochemistry – Southern General Hospital and Glasgow Victoria Infirmary Hospital).

500 µl plasma samples were analysed using a chemiluminescent microparticle immunoassay (CMIA) for quantifying cardiac troponin-I in human plasma or serum and human BNP in ethylenediaminetetraacetic acid (EDTA) plasma respectively using the ARCHITECT *i*-System with STAT protocols.

CMIA assays work in a two-step approach, initially an antibody specific to the analyte (anti-Troponin-I or anti-BNP respectively) coated to paramagnetic microparticles binds to the antigen present in the sample. Magnets attract the microparticles and a wash takes place discarding unbound material.

During the second step, acridinium-labeled conjugate specific to the analyte (anti-BNP or anti-Troponin-I acridinium-labeled) is added. Further washing takes place in order to remove unbound substances. Pre-trigger solution is then added in order to release the conjugate into solution and to arrange the label for the chemiluminescent reaction. Magnets once again attract the microparticles, separating them from the label. Addition of a trigger solution follows, triggering the final phase of the chemiluminescent reaction which can be quantified as relative light units (RLUs), which present a direct link to the amount of the analyte present in the sample. RLUs are processed by the system´s software producing a concentration result. STAT, faster protocols refer to a shorter incubation time during the first step (reviewed by Quinn and Ambruster, 2013).

4.4 Statistical analysis

Troponin measurements did not present a normal distribution after analysis with histograms and the Shapiro Wilk test, therefore nonparametric analysis was undertaken.

Repeated samples Friedman´s analysis of variance was performed in order to test for differences between time points, post-hoc comparisons between time points were done using Wilcoxon signed rank tests with Bonferroni correction for multiple testing.

Correlations were tested using the nonparametric Spearman´s rank correlation test (r_s).

The association of cTnI PO1 measurements with independent variables were explored using a linear regression analysis for independent variables. Significant variables from univariate analyses were used in a multivariate model followed by backwards selection in order to establish predictor relationships.

Mann-Whitney tests were carried out in order to determine differences between groups (2 tailed). Statistical analysis was performed using SPSS Software version 23 at a significance level of 5%.

4.5 Results

Perioperative analysis

4.5.1 cTnI

Fifty children completed the study, demographic data and its analysis can be seen on *Table 3.1* and *3.2*. Thirty three patients presented complete cTnI measurements for all time points. Levels variated significantly between time points $X^2(2)=93.018$, p=<0.0001. Post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were performed resulting in a significance level at p<0.0083. *Table 4.1* shows median and interquartile range values. Differences were significant between all time points at p<0.0001. *Figure 4.1* presents the variation in cTnI levels according to time point.

Time point	Median – IQ range
Baseline	0.000 (0.000-0.003)
PO1	22.339 (7.411-43.283)
PO2	12.504 (1.775-19.980)
PO3	6.424 (0.946-12.555)

Table 4.1. Descriptive statistics for cTnI measurements according to time points. Abbreviations: PO1: post-operative 1; PO2: post-operative 2; PO3: post-operative 3.

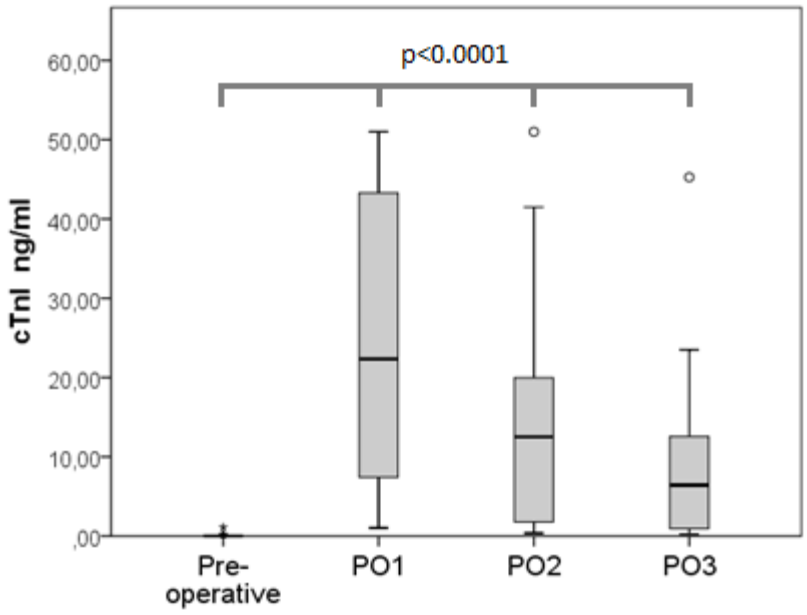

Figure 4.1. cTnI variation according to time point. Abbreviations: cTnI: cardiac troponin I; PO1: post-operative 1; PO2: post-operative 2; PO3: post-operative 3.

Higher cTnI levels for PO1 only were seen in female patients, however a non-significant tendency for longer cross clamp times in females was observed which could have influenced the result (*Figure 4.2*).

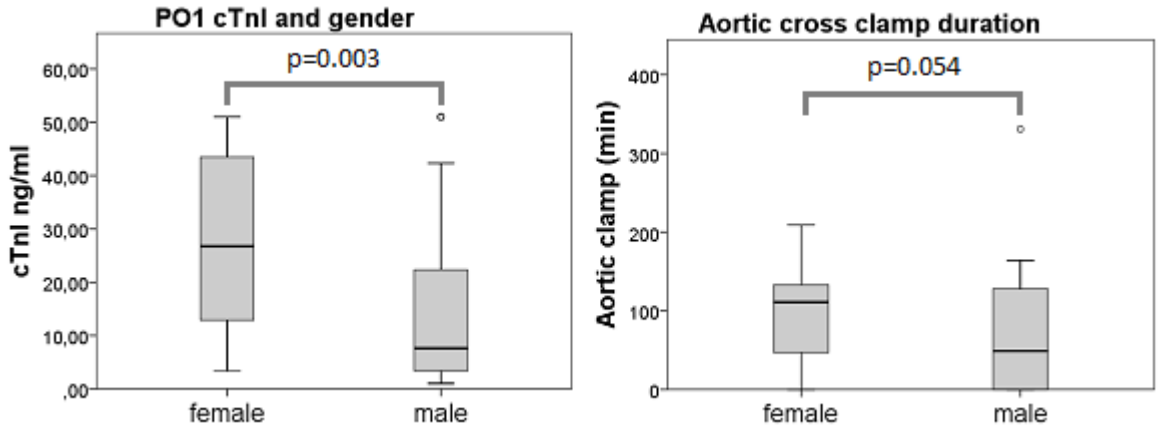

Figure 4.2. Variation of cTnI and aortic cross clamp duration according to gender. Abbreviations: cTnI: cardiac troponin I; min: minutes; PO1: post-operative 1.

Correlation of the time point with the highest cTnI values (PO1) was investigated with the independent variables age (r_s= -0.342, p=0.015), weight (r_s= -0.419, p=0.002), CPB (r_s= 0.499, p<0.0001), aortic clamp duration (r_s= 0.086, p<0.0001) and cyanosis status before surgery (r_s= -0.333, p=0.018); cTnI was significantly higher in cyanotic patients (p=0.020). *Figure 4.3* shows the correlation between CPB, aortic cross clamp duration and cTnI at PO1.

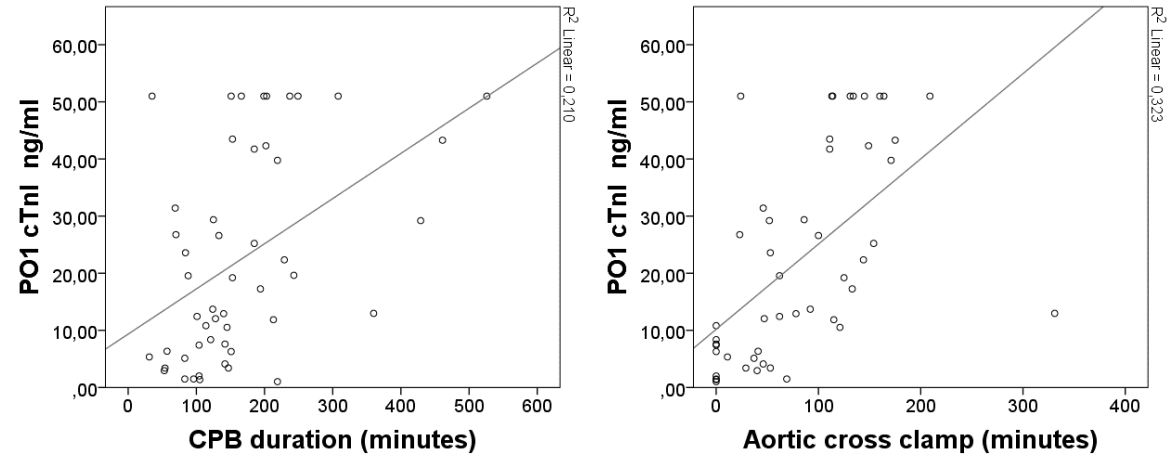

Figure 4.3. Plasma cTnI correlation to CPB and aortic cross clamp times. Abbreviations: cTnI: cardiac troponin I; PO: post-operative.

Multiple regression analysis resulted in a significant correlation of aortic cross clamp duration, cyanosis status and weight producing an adjusted R^2 value of 0.510 with $p<0.0001$, implying that 51% of the variation on PO1 cTnI was caused by cross clamp duration, cyanosis status and weight.

Post-operative cTnI levels correlated to ventilation time ($r_s=0.471$, $p=0.001$), length of PICU stay ($r_s=0.401$, $p=0.004$) and inotrope requirements during all time points; coefficients shown correspond to PO1 cTnI.

4.5.2 BNP

Thirty four patients presented complete BNP measurements for all time points; levels variated significantly between time points $X^2(2)=68.188$, $p<0.0001$. Post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were performed resulting in a significance level at $p<0.0083$. *Table 4.2* shows descriptive values. Differences were significant between all time points at $p<0.0001$ except between PO2 and PO3 values which were not statistically different ($p=0.896$). *Figure 4.4* presents the variation in BNP according to time points.

Time point	**Median – IQ range**
Baseline	41.0 (14.9-89.2)
PO1	81.5 (53.4-260.6)
PO2	527.2 (299.5-980.1)
PO3	547.2 (167.7-1154.2)

Table 4.2. BNP descriptive statistics according to time point variation. Abbreviations: PO1: post-operative 1; PO2: post-operative 2; PO3: post-operative 3.

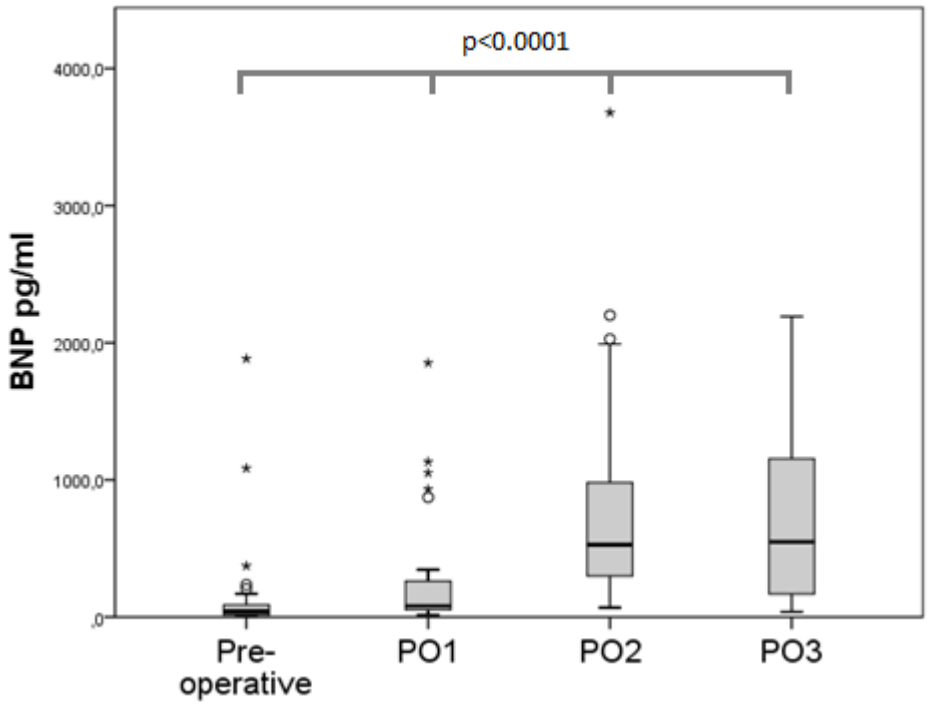

Figure 4.4. BNP variation according to time point. Abbreviations: PO1: post-operative 1; PO2: post-operative 2; PO3: post-operative 3.

BNP correlated negatively to gender at PO2 (r_s= -0.313, p=0.030) and PO3 (r_s= -0.382, p=0.021). Variation according to gender was significant for both time points with PO2: f: 603(383-896) vs m: 353(147-837), p=0.032; PO3: f: 820(390-1209) vs m: 273(93-865), p=0.023 (*Figure 4.5*).

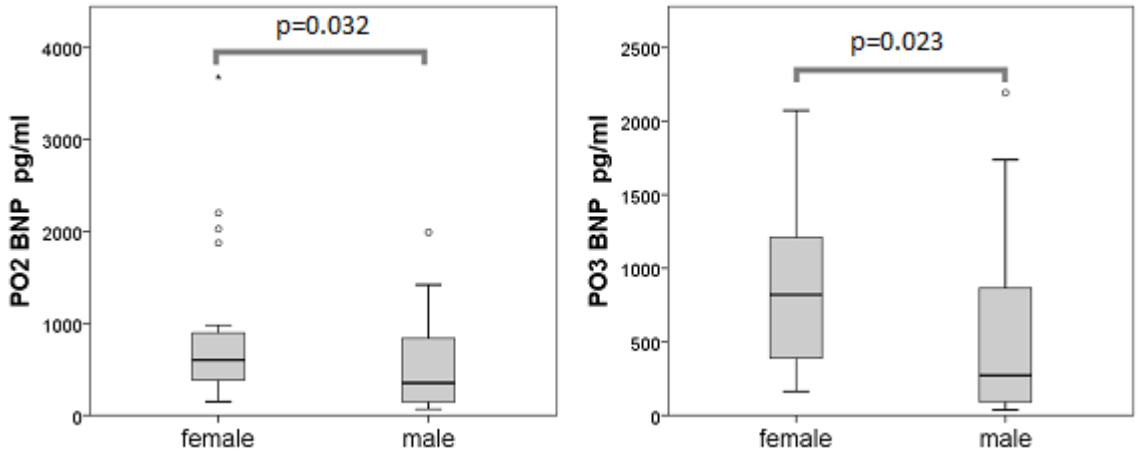

Figure 4.5. Plasma BNP levels according to gender. Abbreviations: BNP: B type natriuretic peptide; PO: post-operative.

A negative correlation was present with age at PO1 (r_s= -0.348, p=0.013), PO2 (r_s=-0.448, p=0.001), and PO3 (r_s= -0.435, p=0.008), and to weight at PO1 (r_s= -0.417, p=0.003), PO2 (r_s= -0.557, p p<0.0001) and PO3 (r_s= -0.541, p<0.0001) (*Figure 4.6*).

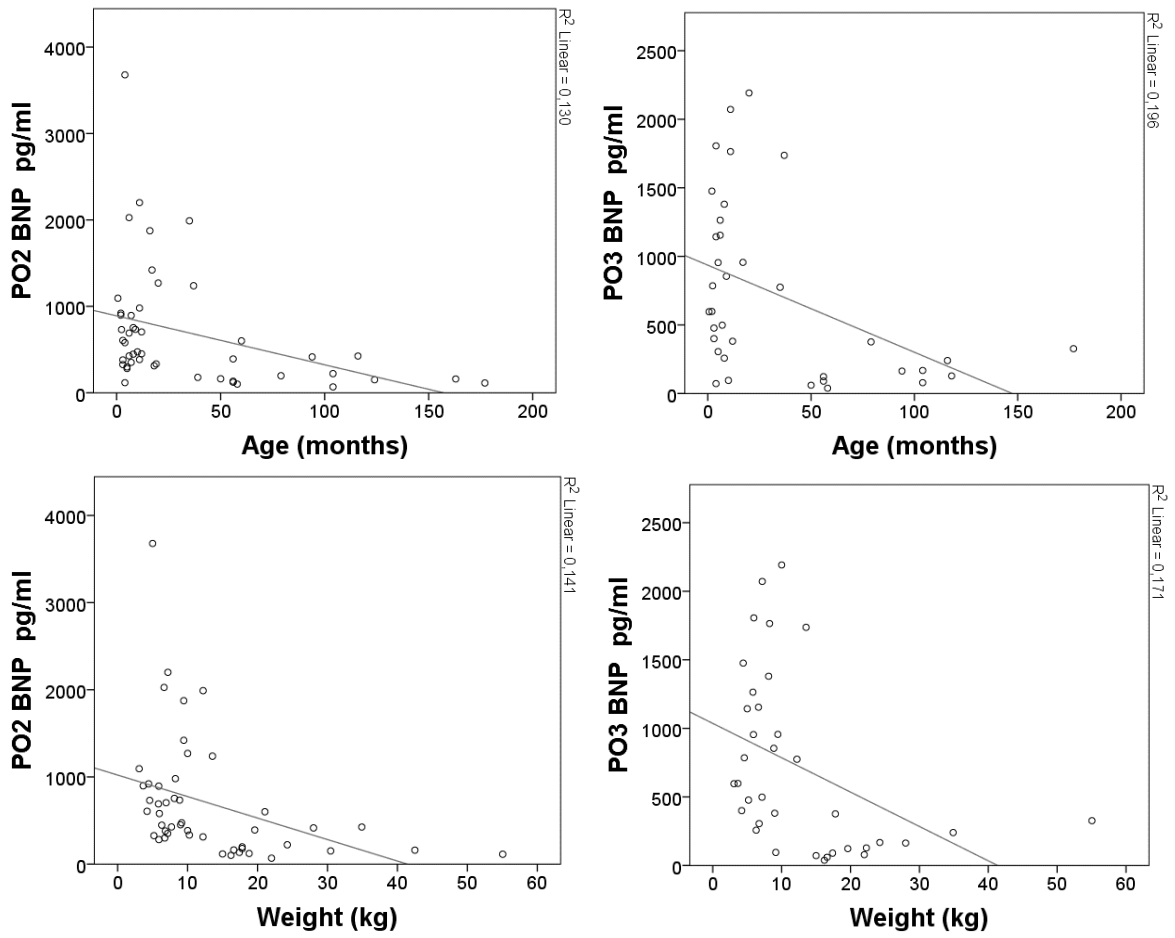

Figure 4.6. Plasma BNP correlation to age and weight. Significant positive correlations for PO2 and PO3 time points are shown. Abbreviations: BNP: B type natriuretic peptide; PO: post-operative.

BNP correlated significantly to CPB duration at PO1 (r_s =0.430, p=0.002), PO2 (r_s =0.393, p=0.006) and PO3 (r_s =0.480, p=0.003). Post-operative BNP also correlated to aortic cross clamp duration at time points PO1 (r_s=0.343, p=0.016), PO2 (r_s =0.507, p<0.0001) and PO3 (r_s =0.698, p<0.0001) (*Figure 4.7*).

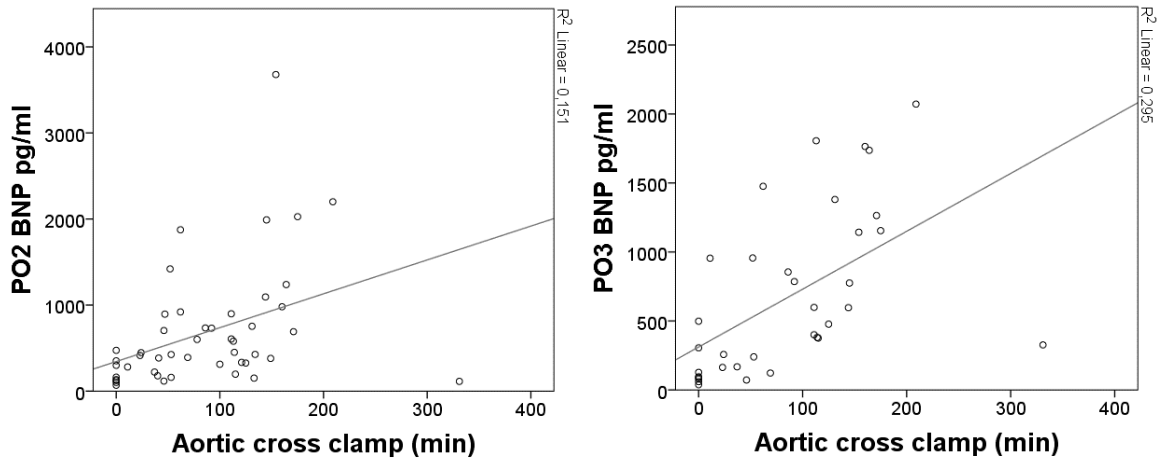

Figure 4.7. Plasma BNP correlation to aortic cross clamp duration. Significant positive correlations for PO2 and PO3 time points are shown. Abbreviations: PO: post-operative.

In addition BNP correlated to corresponding cTnI levels at time points PO1 (r_s=0.425, p=0.02), PO2 (r_s=00.596, p p<0.0001) and PO3 (r_s =0.740, p<0.0001) (*Figure 4.8*).

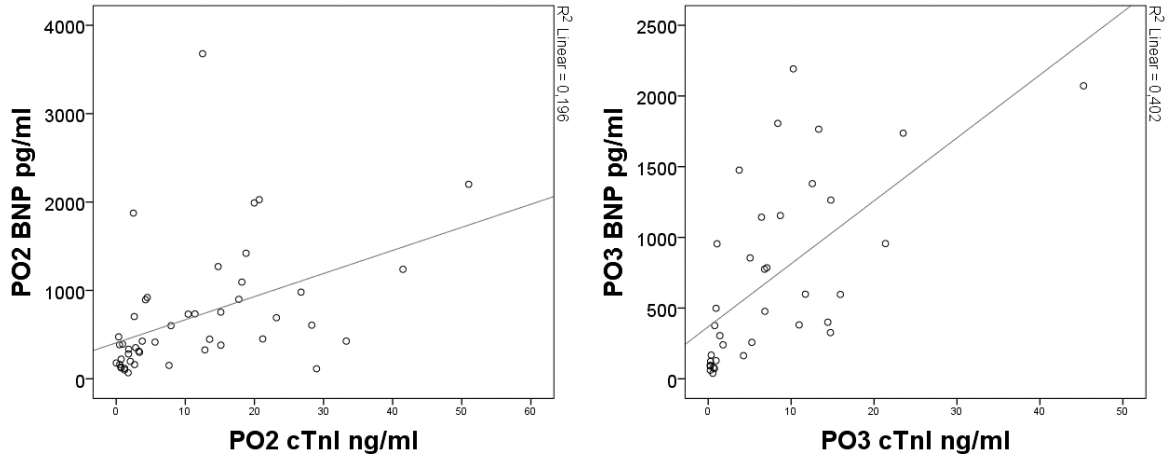

Figure 4.8. Plasma BNP correlation to cTnI levels. Significant positive correlations for PO2 and PO3 time points are shown. Abbreviations: PO: post-operative.

BNP correlated to ventilation times (r_s=0.557, p<0.0001), PICU stay (r_s=0.503, p<0.0001), and inotrope use during all time points; coefficients shown correspond to PO2 BNP.

RIPC analysis

In two cases assigned to RIPC parents asked for the intervention to be stopped before its completion due to discomfort, hence these two patients were excluded from analysis between Control and RIPC groups.

4.5.3 cTnI – RIPC

Baseline measurements of cTnI were similar in both groups, there was a tendency in the control group to present higher levels for PO1 and PO2 measurements, *Figure 4.9* shows the variation of cTnI according to intervention group.

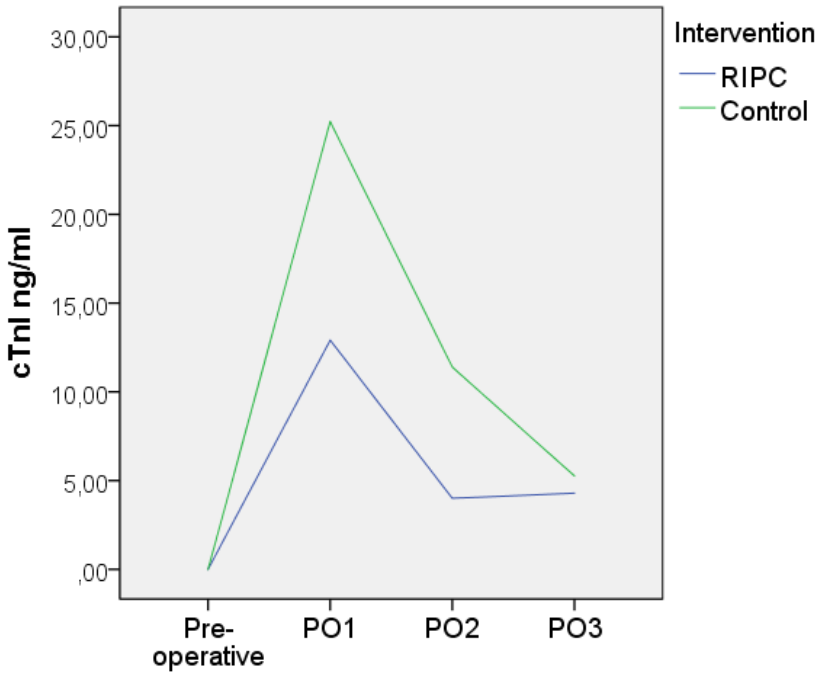

Figure 4.9. cTnI variation according to group allocation. Abbreviations: cTnI: cardiac troponin I; PO1: post-operative 1; PO2: post-operative 2; PO3: post-operative 3; RIPC: remote ischaemic preconditioning.

This difference however did not reach statistical significance. *Table 4.3* presents descriptive statistics for time points according to group allocation. *Figure 4.10* presents the comparison between intervention groups for each time point.

Variable	Intervention	N	Median – IQ range	P value
Baseline	RIPC	25	0.00(0.00-0.00)	0.978
	Control	22	0.00(0.00-0.00)	
PO1	RIPC	25	12.91(6.29-39.76)	0.450
	Control	23	25.22(8.37-43.48)	
PO2	RIPC	24	4.01(1.48-17.96)	0.439
	Control	21	11.39(2.81-18.79)	
PO3	RIPC	16	4.29(0.60-12.52)	0.659
	Control	19	5.26(0.95-12.56)	

Table 4.3. Descriptive statistics for cTnI levels according to group allocation for each time point. Abbreviations: cTnI: cardiac troponin I; PO1: post-operative 1; PO2: post-operative 2; PO3: post-operative 3.

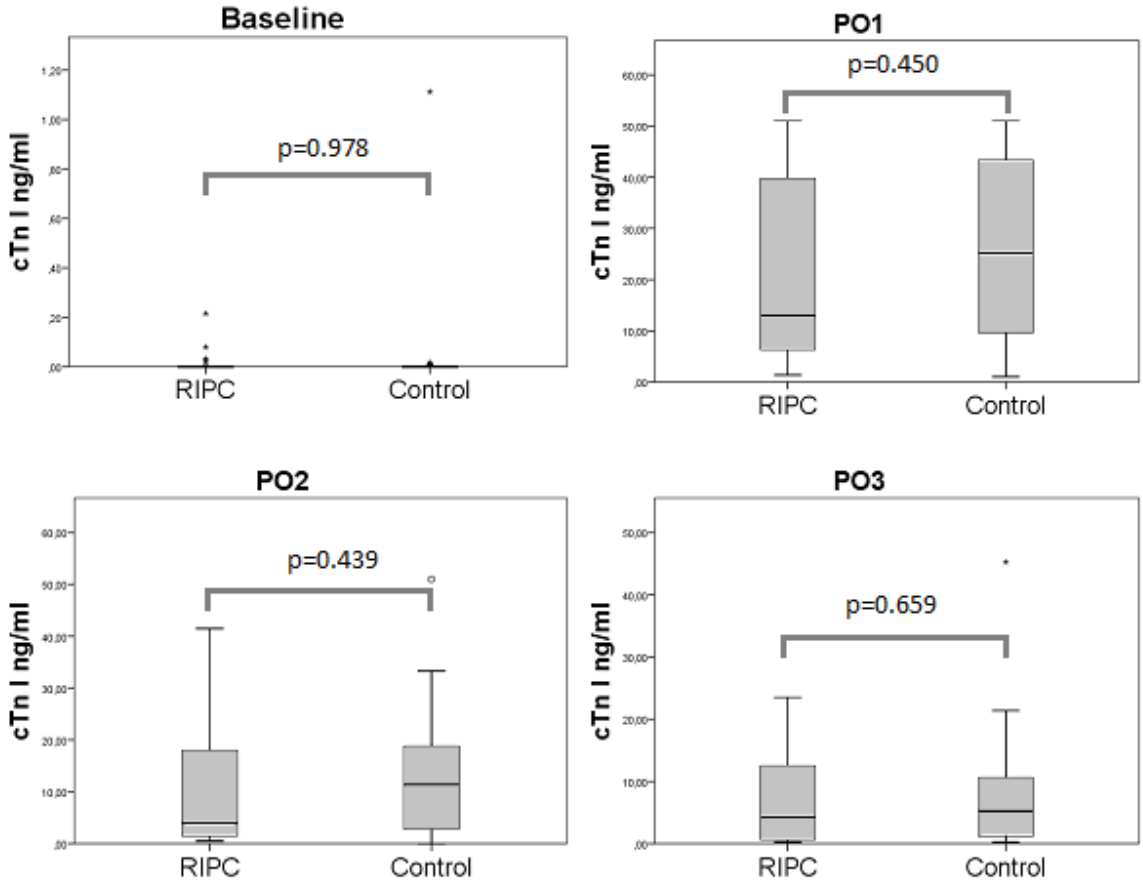

Figure 4.10. cTnI levels according to RIPC or Control allocation for each time point. Abbreviations: cTnI: cardiac troponin I; PO1: post-operative 1; PO2: post-operative 2; PO3: post-operative 3.

Analysis by categories of age and cyanotic status did not produce significant results for any time points.

The area under the curve (AUC) for cTnI release was calculated in order to compare total release between RIPC and control groups. AUC was not significantly different between RIPC and control groups (*Figure 4.11*) with RIPC: 393.05 (98.85-1038.73) vs Control: 596.10 (225.38-954.24) p=0.752.

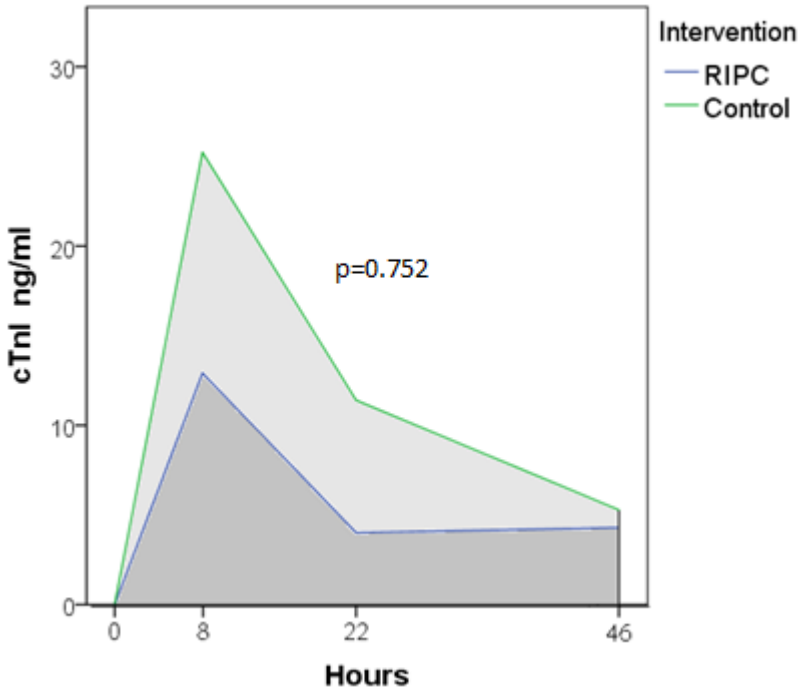

Figure 4.11. Area under the curve for cTnI values according to group allocation. cTnI levels are presented at baseline and at 8, 22 and 46 hours in average according to post-operative time points. Abbreviations: cTnI: cardiac troponin I. RIPC: remote ischaemic preconditioning.

4.5.4 BNP – RIPC

Baseline BNP measurements were similar in both groups and kept that tendency through post-operative measurements (*Figure 4.12*). *Table 4.4* presents descriptive data for BNP values according to group allocation.

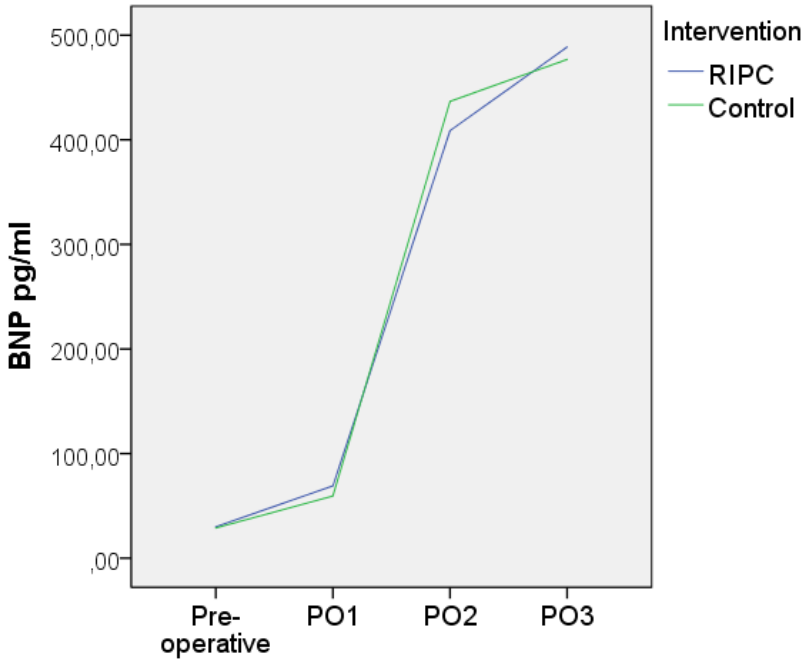

Figure 4.12. Variation of BNP values according to group allocation. Median values represented. Abbreviations: BNP: B type natriuretic peptide; PO: post-operative; RIPC: remote ischaemic preconditioning.

Variable	Intervention	N	Median – IQ range	P value
Baseline	RIPC	25	30.00 (16.40-85.40)	0.578
	Control	22	28.90 ()10.60-69.60	
PO1	RIPC	25	69.20 (27.30-88.30)	0.910
	Control	23	59.40(31.00-178.30)	
PO2	RIPC	24	408.70(160.90-812.75)	0.553
	Control	22	436.75(299.50-753.80)	
PO3	RIPC	16	488.65(84.45-869.95)	0.271
	Control	19	476.80(257.20-1154.20)	

Table 4.4. Descriptive statistics for BNP values according to group allocation. Data is expressed as mean – SD or median – IQ range as appropriate. Abbreviations: IQ: interquartile range; PO: post-operative; RIPC: remote ischaemic preconditioning; SD: standard deviation.

BNP measurements were not statistically different according to RIPC or control allocation for any time point (*Figure 4.13*).

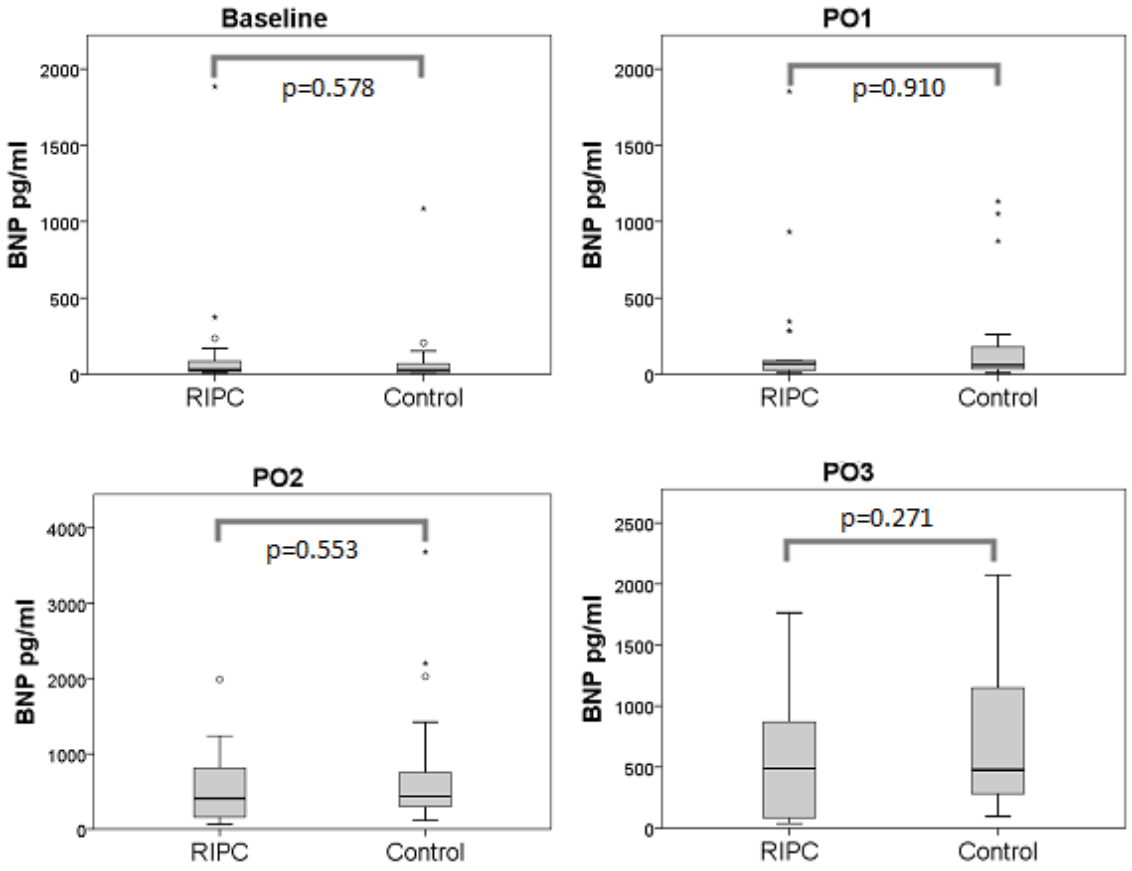

Figure 4.13. BNP levels according to group allocation for each time point. Abbreviations: BNP: B type natriuretic peptide; PO: post-operative; RIPC: remote ischaemic preconditioning.

4.6 Summary of key findings

- cTnI variated according to time points.
- Post-operative cTnI was higher in female patients.
- Post-operative cTnI correlated negatively to age and weight and positively to CPB and aortic clamp duration.
- Post-operative cTnI was higher in cyanotic patients.
- Cross clamp duration, cyanosis status and weight were predictors of post-operative cTnI levels.
- cTnI was not different according to RIPC or control allocation for any time point or in AUC.
- BNP varied across time points.
- Post-operative BNP was higher in females.
- Post-operative BNP correlated negatively to age and weight and positively to CPB and aortic clamp duration and to cTnI levels.
- BNP was not different according to intervention allocation.

4.7 Discussion

Troponin is an established marker used in the clinical environment as a diagnostic tool for cardiac ischaemia and injury. Most studies evaluating RIPC efficacy have measured an isoform of troponin.

This study analysed cTnI and BNP release in order to determine the effect of RIPC in cardiac injury after CHD corrective surgery using CPB.

cTnI measurements were higher during the early post-operative period, peaking at patient PICU arrival immediately after surgery (PO1) and started a lowering trend at the next morning measurement (PO2). Post-operative cTnI correlated to intubation time, PICU stay and inotrope requirements.

As expected the duration of the ischaemic insult and of the exposure to inflammatory triggers reflected by CPB and aortic cross clamp duration correlated to early post-operative levels of cTnI; in addition cTnI levels were higher in younger and lower weight patients, suggesting that

smaller patients are more susceptible to injury. Higher troponin levels have been reported in infants compared to older children after cardiac surgery using CPB (Modi *et al*, 2003; Gupta-Malhotra *et al*, 2013).

Cyanosis also increased the levels of cTnI after surgery, it should be noted that cyanotic patients did not present higher cTnI pre-operatively, suggesting an enhanced reaction to the IRI insult; this difference was no longer significant by the second day after surgery. A study looking at differences related to hypoxia found that cyanotic children presented worse IRI after surgery reflected on cTnI levels (Imura *et al*, 2001).

The relationship between these variables allowed for a regression model in which 51% of the variation on early post-operative PO1 was caused by the duration of cross clamp, cyanosis status and weight.

In the present study cTnI was surprisingly higher in female patients. An analysis of demographic characteristics revealed that female patients recruited presented an unplanned significantly lower weight; as cTnI levels also correlated to weight, the difference could be due to weight and not gender based. In addition to this, female patients tended to present higher cross clamp times during surgery, this difference did not reach statistical significance.

There was a tendency for higher cTnI levels in the control group at postoperative time points, however the difference was not significant. Age and cyanosis status have been identified as influencing factors on RIPC response (Konstantinov, 2013) hence, separate analysis according to age categories and cyanosis status was performed without reaching significant results for any of the groups. It should be noted that the majority of recruited patients were infants and subgroup analysis for other categories had reduced sample sizes.

In order to compare the change pattern according to group allocation cTnI AUC analysis was undertaken; the difference between groups was not significant.

During the analysed procedures an incision into the right atrium was required as part of the standard surgical protocol in order to access internal cardiac anatomy. In addition to this, certain procedures required ventricular tissue excision causing an insult that could result in a troponin rise independent of other injury factors and IRI, these procedures included correction of TOF,

sub-aortic or sub-pulmonary stenosis, pulmonary atresia, one case of truncus arteriosus and a case of right ventricular outlet tract obstruction. The distribution of patients who required such incision and those who did not was even between the two study arms.

Evidence of the effect of RIPC on the extent of cardiac injury represented by troponin release has produced conflicting results. Initial reports on adult and paediatric patients were promising and drew interest in an intervention without reports of adverse events and great potential.

Eight paediatric trials investigated the effect of RIPC in troponin release, three of these studies reported a significant reduction after the intervention (Cheung *et al*, 2006; Zhou *et al*, 2010; Luo *et al*, 2011); while five studies did not find a significant difference (see *Table 1.8*). The largest paediatric clinical trial so far, which recruited 299 patients reported no difference in troponin release after RIPC (McCrindle *et al*, 2014).

A similar pattern occurred with adult studies, with initial positive reports (see *Table 1.7*); nevertheless two recent multicentre studies recruiting 1612 and 1385 patients respectively found no significant difference in troponin release between RIPC and control groups (Hausenloy *et al*, 2015; Meybohm *et al*, 2015).

BNP is used as a marker of cardiac failure in the clinical setting, it is released after cardiomyocyte stretch and is an indicator of cardiac function; it has been identified as a predictor of patient outcome after surgery, correlating to inotropic requirements, ventilation times, hospital stay and mortality (Attaran *et al*, 2009). As BNP is not routinely measured in paediatric patients the descriptive data obtained from this study is of general interest.

The present study found a correlation of post-operative BNP levels to length of intubation, length of PICU stay and inotrope use. In contrast to cTnI patterns, BNP levels were above zero at baseline and kept increasing after surgery, with the last measured time point (PO3) being the highest. These higher BNP time points correlated to the duration of CPB and aortic cross clamp as well as to corresponding cTnI levels, implying that BNP increased according to the extent of injury in the myocardium.

BNP had a negative correlation to weight and age, with its values increasing in smaller and younger children at all post-operative time points. Analysis of BNP perioperative patterns in children presenting CHD compared to healthy controls showed an age dependent variation, with neonates presenting higher levels than older children even before surgery (Niedner *et al*, 2010; Cantinotti *et al*, 2013). In our study baseline BNP correlated negatively to weight but not age.

As BNP increased at PO2 and PO3, levels became significantly higher in female patients. Analysis of gender differences found that adult females presented higher BNP measures than males, however there were no gender differences in any age group under 19 years for healthy participants (Mir *et al*, 2006). Once again, the weight difference between male and female participants in our study could have influenced this gender bias.

RIPC had no significant effect in BNP levels at any time point, one previous paediatric clinical trial analysed the release of the inactive form of BNP, NTproBNP which is associated to BNP plasma levels; this study used a RIPC protocol on the day before surgery and reported lower levels of the marker in the RIPC group although it found no clinical benefit from the intervention (Pavione *et al*, 2012).

One adult RIPC clinical trial also looked at NTproBNP release (Lucchinetti *et al*, 2012), while a second study measured BNP levels (Karuppasami *et al*, 2011). Both adult studies reported no difference according to group allocation.

Conclusion

RIPC did not significantly reduce the markers of cardiac injury cTnI and BNP after cardiac surgery in CHD paediatric patients.

Chapter 5. Echocardiographic analysis

5.1 Introduction

Echocardiography is the most important tool in order to diagnose and assess the particular structures, functionality and haemodynamics in CHD, its findings allow therapeutic decisions and a definite follow-up of patient status.

Tissue Doppler imaging (TDI) detects frequency changes of ultrasound signals reflecting in moving objects; blood flow and myocardial contraction cause velocity variation in the heart, TDI analyses the longitudinal component of contraction through the cardiac cycle scanning fibres running from the atrio-ventricular annulus to the heart apex as they contract and relax providing velocities that reflect systolic and diastolic function (Henein and Gibson, 1999; reviewed by Kadappu and Thomas, 2015).

Of particular interest is the TDI analysis of right ventricular function, a previous study found a decrease in right ventricular velocity following surgical correction of CHD using CPB, reflecting a functional detrimental effect of IRI which in addition correlated with longer ventilation times required by patients presenting lower velocities (Vassalos *et al*, 2009).

5.2 Aims

To analyse cardiac function through echocardiographic parameters in CHD patients before and after surgery using CPB and to analyse the effect of RIPC on these parameters.

5.3 Materials and methods

Echocardiographic images, including TDI, were obtained and stored by experienced echocardiographers from patients before surgery while the patient was under anaesthesia (Pre-operative) and the morning after surgery (Post-operative). For clinical reasons it was only possible to obtain an immediate post-operative study in a few cases. *Table 5.1* presents the echocardiographic images obtained.

Image
Parasternal long axis 2D and M-mode
Parasternal long axis colour Doppler
Parasternal long axis anterior cut through pulmonary valve
Apical 4 chambers 2D
Apical 4 chambers 2D
Apical RV inflow-outflow 2D
Apical RV long axis
Apical 4 chambers Doppler
Apical 4 chambers colour TDI (3 cycles loop)
Apical 4 chambers pulse wave TDI (3 cycles)
LVOT PWD from the apex
Apical 2 chambers 2D
Apical 3 chambers 2D and colour
SAX at aortic valve level – 2D and colour
SAX at MV level 2D and colour
SAX at PV level 2D
SAX at the apex 2D
Subcostal RV inflow-outflow ("RAO") view 2D and colour
Subcostal RV SAX ("LAO") view 2D and colour

Table 5.1. Echocardiographic images obtained. Abbreviations: 2D: two dimensional; LAO: left anterior oblique; LVOT: left ventricular outflow tract; MV: mitral valve; PV: pulmonary valve; PWD: pulse wave Doppler; RAO: right anterior oblique; RV: right ventricle; SAX: short axis; TDI: tissue Doppler image.

Echocardiographic measurements were obtained offline by the principle investigator and Dr Ilina Maria, consultant paediatric cardiologist.

Echocardiographic parameters

Ejection fraction (EF) is the ratio of the stroke volume to ventricular end diastolic volume, it is a widely used measure of ventricular function (reviewed by Robotham *et al*, 1991), while fractional shortening (FS) measures the percentage change in the left ventricle minor axis diameter between end diastole and end systole (Bulwer *et al*, 2007). EF and FS are obtained in M-mode images.

Pulse wave Doppler in apical images showing a four chamber view are used to acquire mitral annular velocities, the early E wave represents the passive filling of the ventricle and the second A wave corresponds to the active filling of the ventricle during atrial systole (ventricular diastole); usually the E wave velocity is higher than the A wave (reviewed by Ashley and

Niebauer, 2004) (*Figure 5.1-I*). E and A waves are used to calculate the MV E/A ratio, a measure of diastolic function.

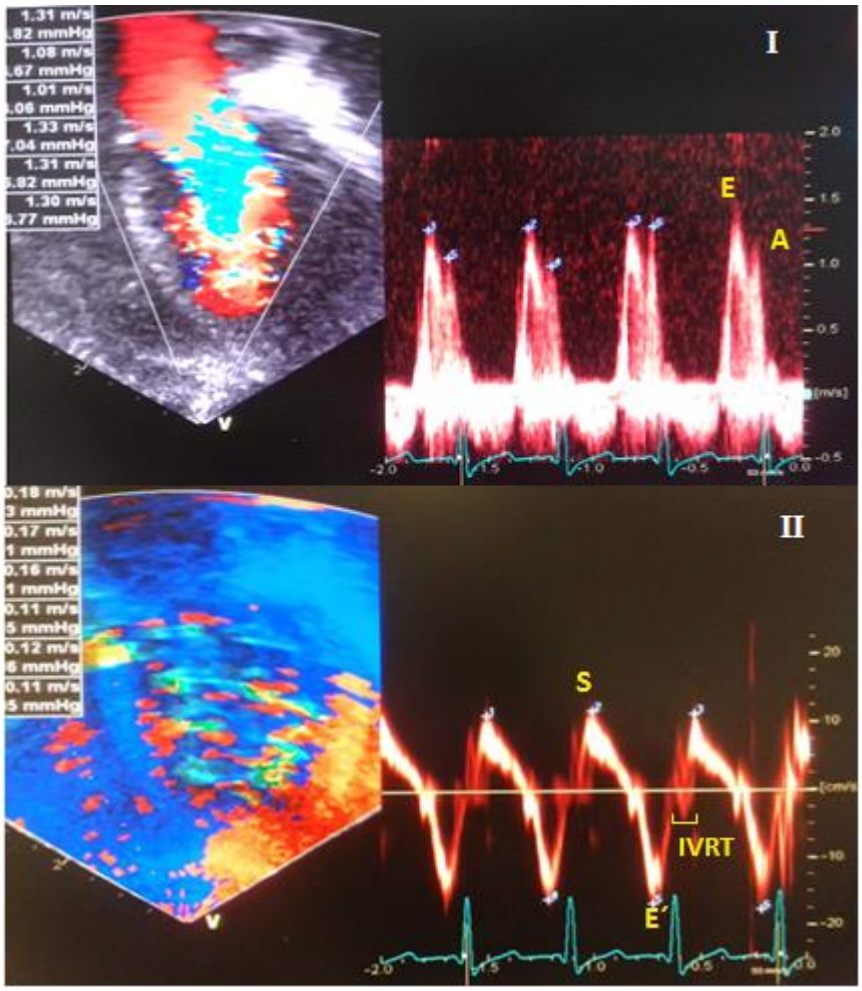

Figure 5.1. PWD and TDI echocardiographic images. I) PWD mitral image: waves representing the early (E) and active (A) phases of ventricle filling are shown. II) TDI tricuspid image: waves representing systolic (S) and early diastolic (E´) velocities, as well as IVRT measure are shown. Abbreviations: IVRT: isovolumetric relaxation time; PWD: pulse wave Doppler; TDI: tissue Doppler imaging. Images obtained from patients participating in the clinical trial.

TDI waveforms corresponding to the lateral part of the mitral valve, septal portion of the mitral valve, and from the tricuspid valve (lateral section) were used in order to measure the systolic (S) wave, early diastolic wave (E´) and IVRT (*Figure 5.1-II*) as reviewed by Nagueh *et al*, 2016. S measurements represent systolic function measures.

Mitral E´ waves from TDI wave images and E waves from PWD images were then used to calculate E/E´ ratios, additional diastolic measures. All variables measured in the study are summarised in *Table 5.2*.

Measurement	Abbreviation	Function
Fractional shortening	FS	Measure of LV systolic function
Ejection fraction	EF	Measure of LV systolic function
LV isovolumetric relaxation time	LV IVRT	Measure of LV diastolic function
Mitral inflow pulsed wave Doppler E to A velocities ratio	MV E/A ratio	Measure of LV diastolic function
Lateral mitral annulus systolic velocity	MV LAT S	Measure of LV systolic function
Lateral mitral annulus E to E' ratio	MV LAT E/E'	Measure of LV diastolic function
Septal mitral annulus systolic velocity	MV SEP S	Measure of LV systolic function
Septal mitral annulus E to E' ratio	MVSEP E/E'	Measure of LV diastolic function
Tricuspid annular systolic velocity	TV ANT S	Measure of RV systolic function

Table 5.2. Echocardiographic measurements obtained. Abbreviations: LV: left ventricle; RV: right ventricle; TV: tricuspid valve.

5.4 Statistical analysis

Echocardiographic variables were analysed for normality with histograms and the Shapiro Wilk test. Data is presented as mean and SD for normally distributed variables, or median and interquartile range for non-normal distribution.

Repeated measures ANOVA with Bonferroni correction for multiple testing, or the non-parametric repeated samples Friedman test with post-hoc comparisons using Wilcoxon signed rank test with Bonferroni correction for multiple testing were performed in order to test for differences between the three echocardiographic time points in a small number of patients.

Paired sample t-test or related samples Wilcoxon signed rank test were performed in order to analyse differences between preoperative and next morning time points for each variable.

Independent sample t-test or Mann-Whitney tests were carried out in order to determine differences between groups according to group allocation for each time point. Statistical analysis was performed using SPSS Software version 23 at a significance level of 5%.

5.5 Results

Fifty patients completed the study, however it was not possible to obtain all echocardiographic measurements in all cases due to individual anatomical variation and clinical situation.

A small number of patients had an immediate post-operative analysis performed, however the sample size was not deemed sufficient to include in the analysis, results for patients presenting three complete measures at pre, immediate post-operative and next morning post-operative time points can be found in *Appendix 4*.

5.5.1 Perioperative analysis

All recruited patients were analysed for pre-operative and next morning (post-operative) echocardiographic imaging, however for anatomical and clinical reasons it was not always possible to obtain all measures, *Table 5.3* shows descriptive information for each variable.

Variable	Time point	N	Mean - SD	Median – IQ range	P
FS	Pre-operative	27		36(28-40)	0.011
	Post-operative			31(17-36)	
EF	Pre-operative	16		66(56-74)	0.036
	Post-operative			61(37-68)	
MV LAT S	Pre-operative	26		0.05(0.04-0.07)	0.041
	Post-operative			0.06(0.04-0.07)	
MV SEP S	Pre-operative	18	0.0456±0.120		0.919
	Post-operative		0.0452±0.160		
TV ANT S	Pre-operative	20		0.073(0.056-0.090)	0.003
	Post-operative			0.040(0.040-0.053)	
IVRT	Pre-operative	27	48.37±15.717		0.154
	Post-operative		43.74±9.184		
MV E/A	Pre-operative	13		1.21(1.08-1.53)	0.075
	Post-operative			1.76(1.27-1.89)	
MV LAT E/E´	Pre-operative	17		11.883(9.229-16.00)	0.356
	Post-operative			11.409(6.981-15.892)	
MV SEP E/E´	Pre-operative	15	11.995±3.837		0.055
	Post-operative		15.141±5.264		

Table 5.3. Descriptive statistics for echocardiographic variables according to time points. Data is expressed as mean – SD or median – IQ range as appropriate. Abbreviations: EF: ejection fraction; FS: fractional shortening; IVRT: left ventricle isovolumetric relaxation time; MV E/A: mitral inflow E to A velocities ratio; MV LAT E/E´: lateral mitral annulus E to E' ratio; MV LAT S: lateral mitral annulus systolic velocity; MV SEP E/E´: septal mitral annulus E to E' ratio; MV SEP S: septal mitral annulus systolic velocity; PO: post-operative; TV ANT S: tricuspid annular systolic velocity. MV SEP S: septal mitral annulus systolic velocity

The measures of LV systolic function EF, FS and MV LAT S presented significant variation after surgery, while MV SEP S did not differ significantly (*Figure 5.2*).

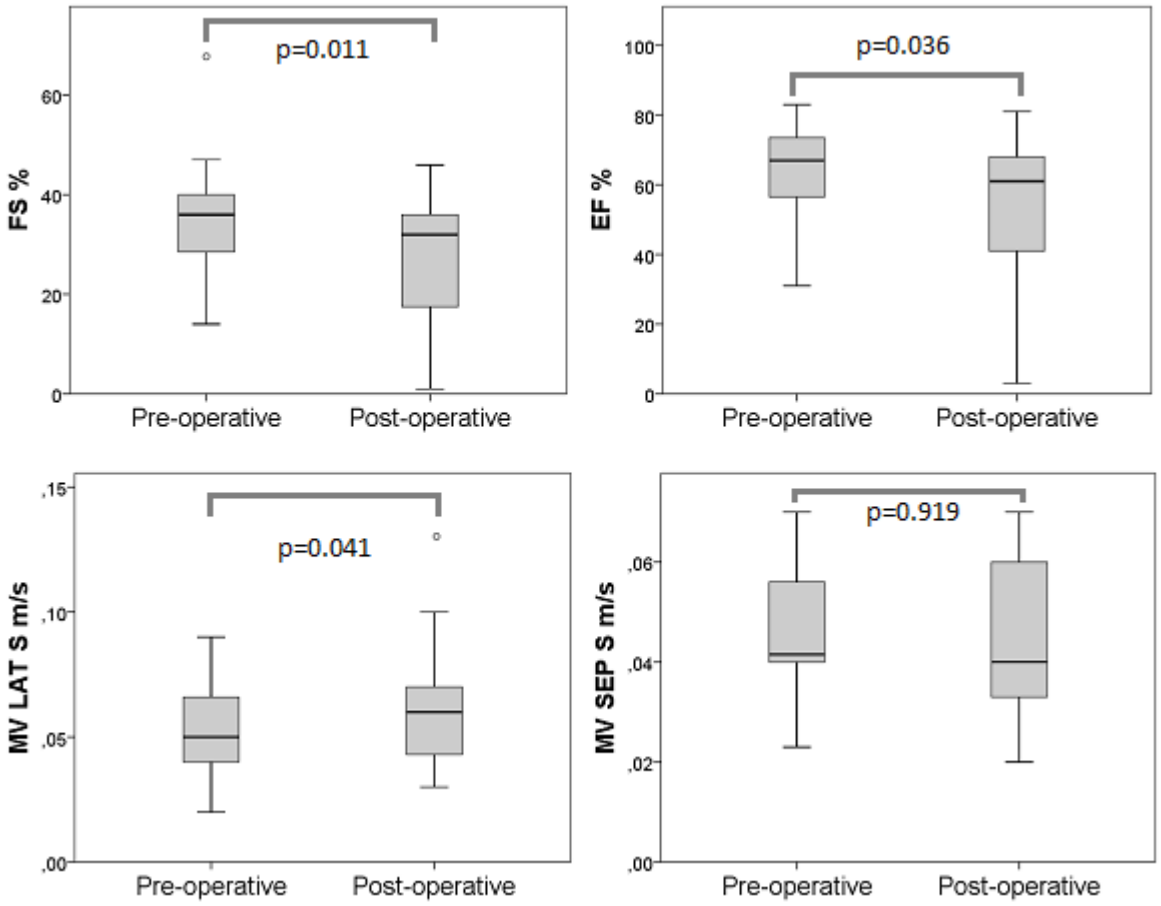

Figure 5.2. Echocardiographic measures of LV systolic function before and after surgery. Abbreviations: EF: ejection fraction; FS: fractional shortening; MV LAT S: lateral mitral annulus systolic velocity; MV SEP S: septal mitral annulus systolic velocity; PO: post-operative.

Diastolic function measures were not significantly changed after surgery (*Figure 5.3*).

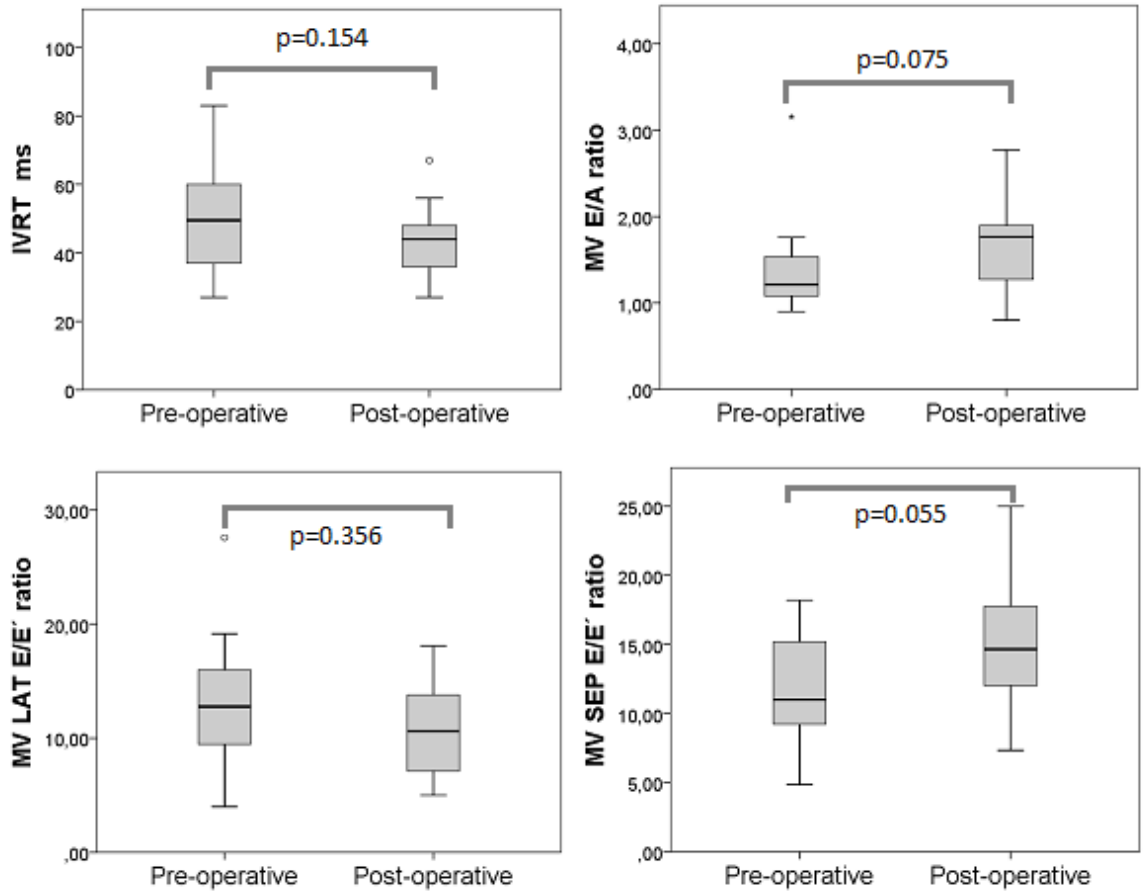

Figure 5.3. Echocardiographic measures of LV diastolic function before and after surgery. Abbreviations: IVRT: isovolumetric relaxation time; MV E/A: mitral inflow pulsed wave Doppler E to A velocities ratio; MV LAT E/E´: lateral mitral annulus E to E' ratio; MV SEP E/E´: septal mitral annulus E to E' ratio; PO: post-operative.

The RV systolic function measure TV ANT S was significantly reduced after surgical procedures (*Figure 5.4*).

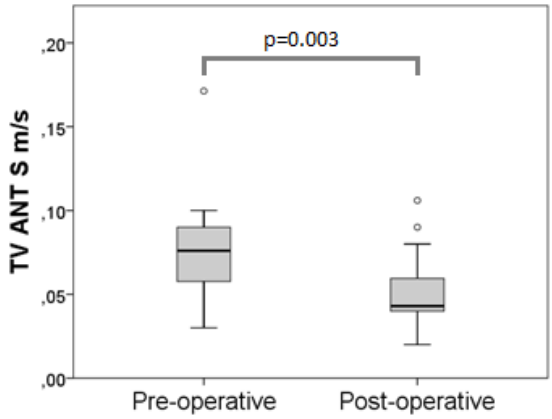

Figure 5.4. Echocardiographic measure of RV systolic function before and after surgery. Abbreviations: PO: post-operative; TV ANT S: tricuspid annular systolic velocity.

Correlations of preoperative measurements were explored with gender, age and weight without finding any associations. Cyanosis correlated to post-operative MV LAT E/E´ (r=0.521, p=0.009), MV SEP S (r=0.644, p=0.001) and TV ANTS (rs=0.567, p=0.004). Differences in these three variables according to cyanotic status were significant with MV LAT E/E´: cyanotic: 9.34±3.80 vs non-cyanotic 13.98±4.16, p=0.009; MV SEP S: cyanotic: 0.034±0.011 vs non-cyanotic: 0.055±0.015, p=0.001, and TV ANTS: cyanotic: 0.040(0.030-0.040) vs non-cyanotic 0.050(0.040-0.070), p=0.006 (*Figure 5.5*).

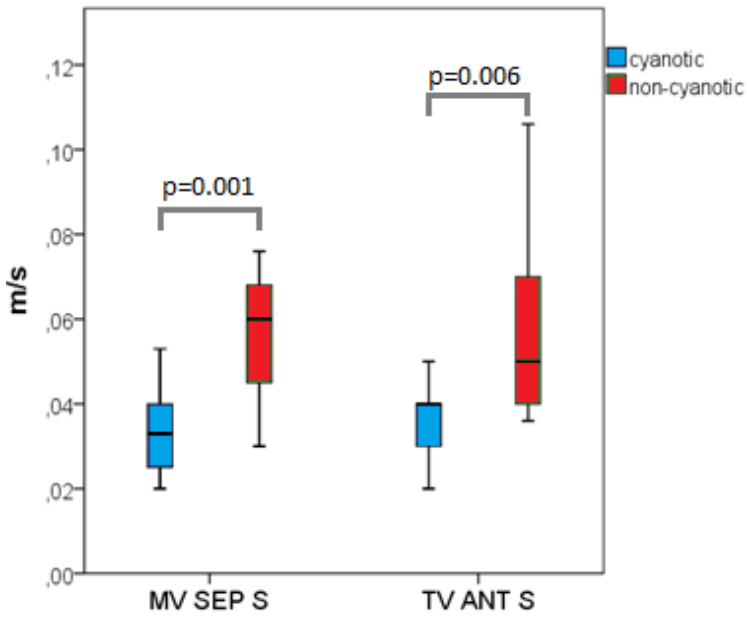

Figure 5.5. MV SEP S and TV ANT S variation according to cyanosis status. Abbreviations: MV SEP S: septal mitral annulus systolic velocity; TV ANT S: tricuspid annular systolic velocity.

MV LAT S presented a negative correlation to aortic cross clamp duration (r_s= -0.390, p=0.044); CPB duration correlated negatively to MV E/A (r_s= -0.506, p=0.027), and MV LAT S (r_s= -0.413, p=0.029).

A significant negative correlation was present between PO1 cTnI levels and MV SEP S (r_s= -0.513, p=0.015), and MV LAT S velocities after surgery (r_s= -0.443, p=0.018) (*Figure 5.6*).

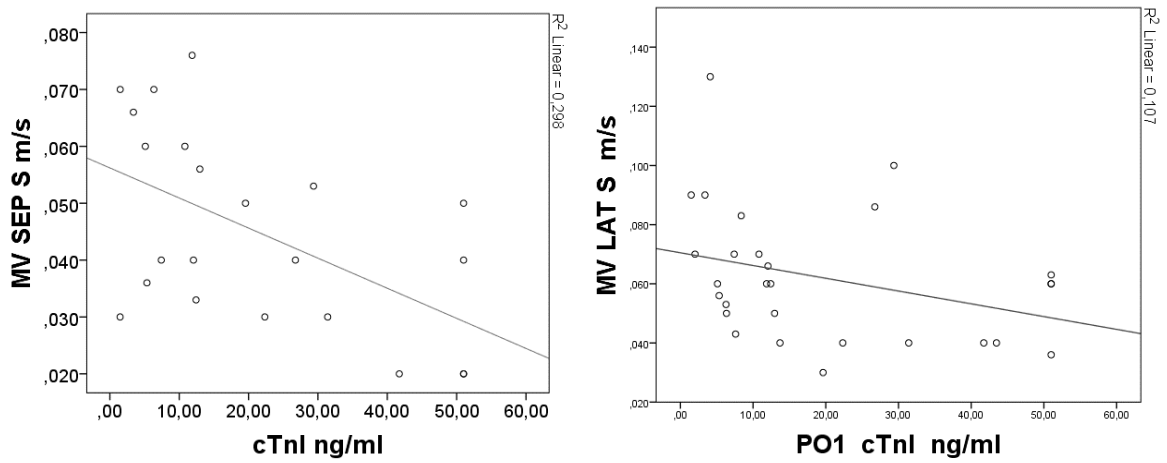

Figure 5.6. Correlation of post-operative cTnI with MV SEP S and MV LAT S velocities after surgery. Abbreviations: MV SEP S: septal mitral annulus systolic velocity; TV ANT S: tricuspid annular systolic velocity.

In addition PO1 BNP correlated negatively to MV LAT S (r_s= -0.442, p=0.019), MV SEP S (r_s= -0.615, p=0.002), and TV ANTS (r_s= -0.456, p=0.025) (*Figure 5.7)*. Correlation of PO2 BNP to these variables remained significant, however it was weaker.

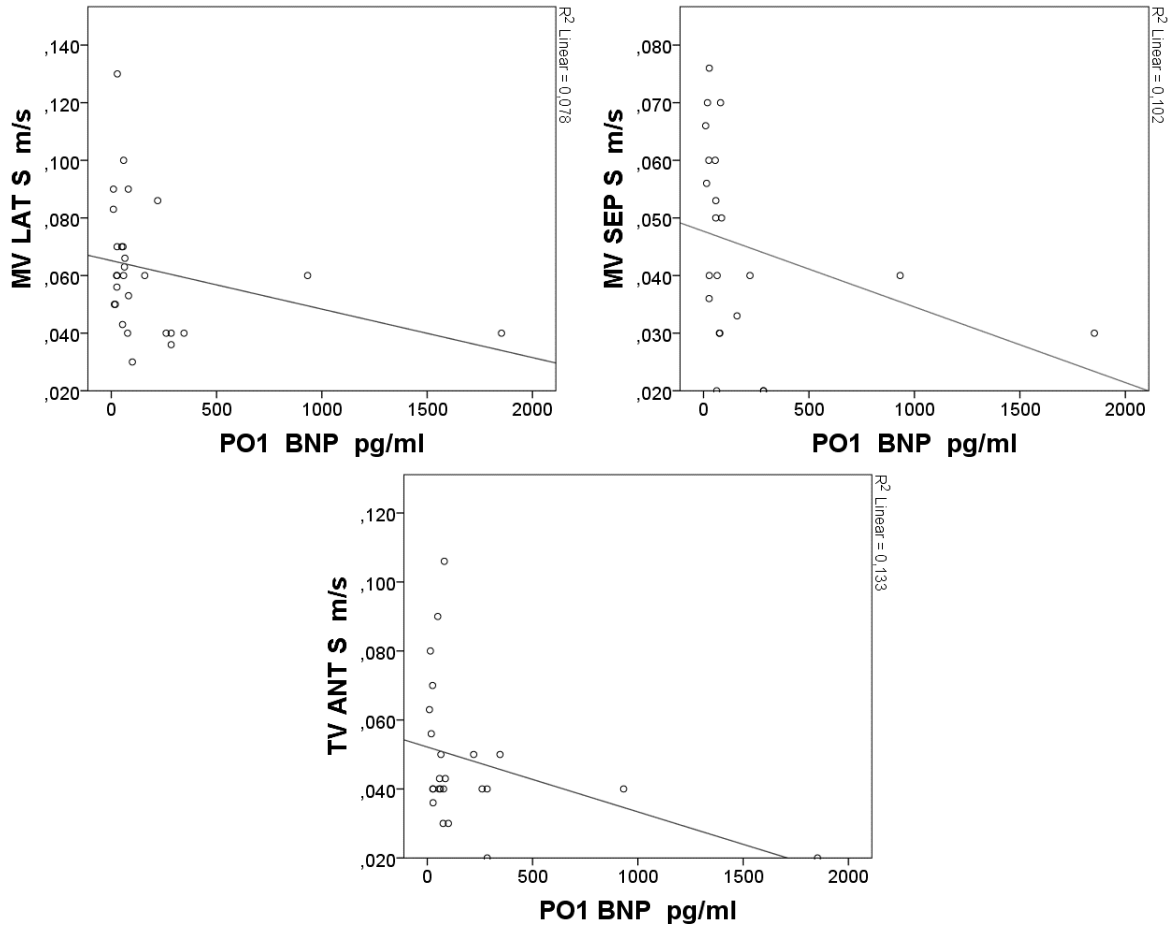

Figure 5.7. Correlation of post-operative 1 BNP with MV SEP E/E´ ratio after surgery. Abbreviations: BNP: B type natriuretric peptide; MV SEP S: septal mitral annulus E to E' ratio; PO2: post-operative 2.

5.5.2 RIPC analysis

Echocardiographic measurements were compared according to RIPC or control allocation; there was no significant difference at the baseline analysis for any of the variables according to group allocation. *Table 5.4* shows descriptive statistics for post-operative echocardiographic measurements.

Variable	Intervention	N	Mean - Std deviation	Median – IQ range	P value
FS	RIPC	19		32 (26-37)	0.529
	Control	14		29 (17-37)	
EF	RIPC	15		65 (54-68)	0.815
	Control	9		62 (57-69)	
MV LAT S	RIPC	16		0.055(0.040-0.068)	0.162
	Control	11		0.063(0.060-0.083)	
MV SEP S	RIPC	12	0.042±0.017		0.542
	Control	9	0.047±0.017		
TV ANT S	RIPC	13		0.040(0.040-0.050)	0.794
	Control	9		0.040(0.040-0.070)	
IVRT	RIPC	16	42.75±11.463		0.759
	Control	12	43.83±6.926		
MV E/A	RIPC	12	1.767±0.451		0.188
	Control	7	1.417±0.663		
MV LAT E/E´	RIPC	14	11.375±4.155		0.660
	Control	9	12.244±5.155		
MV SEP E/E´	RIPC	11	16.035±5.054		0.304
	Control	8	13.582±4.868		

Table 5.4. Descriptive statistics of post-operative echocardiographic variables according to intervention. Data is expressed as mean – SD or median – IQ range as appropriate. Abbreviations: EF: ejection fraction; FS: fractional shortening; LV IVRT: left ventricle isovolumetric relaxation time; MV E/A: mitral inflow pulsed wave Doppler E to A velocities ratio; MV LAT E/E´: lateral mitral annulus E to E' ratio; MV LAT S: lateral mitral annulus systolic velocity; MV SEP E/E´: septal mitral annulus E to E' ratio; MV SEP S: septal mitral annulus systolic velocity; PO: post-operative; TV ANT S: tricuspid annular systolic velocity. MV SEP S: septal mitral annulus systolic velocity

All measured post-operative echocardiographic variables did not present significant differences according to intervention allocation for RV systolic function measures (*Figure 5.8*), RV diastolic function measures (*Figure 5.9*), and LV systolic function measures (*Figure 5.10*).

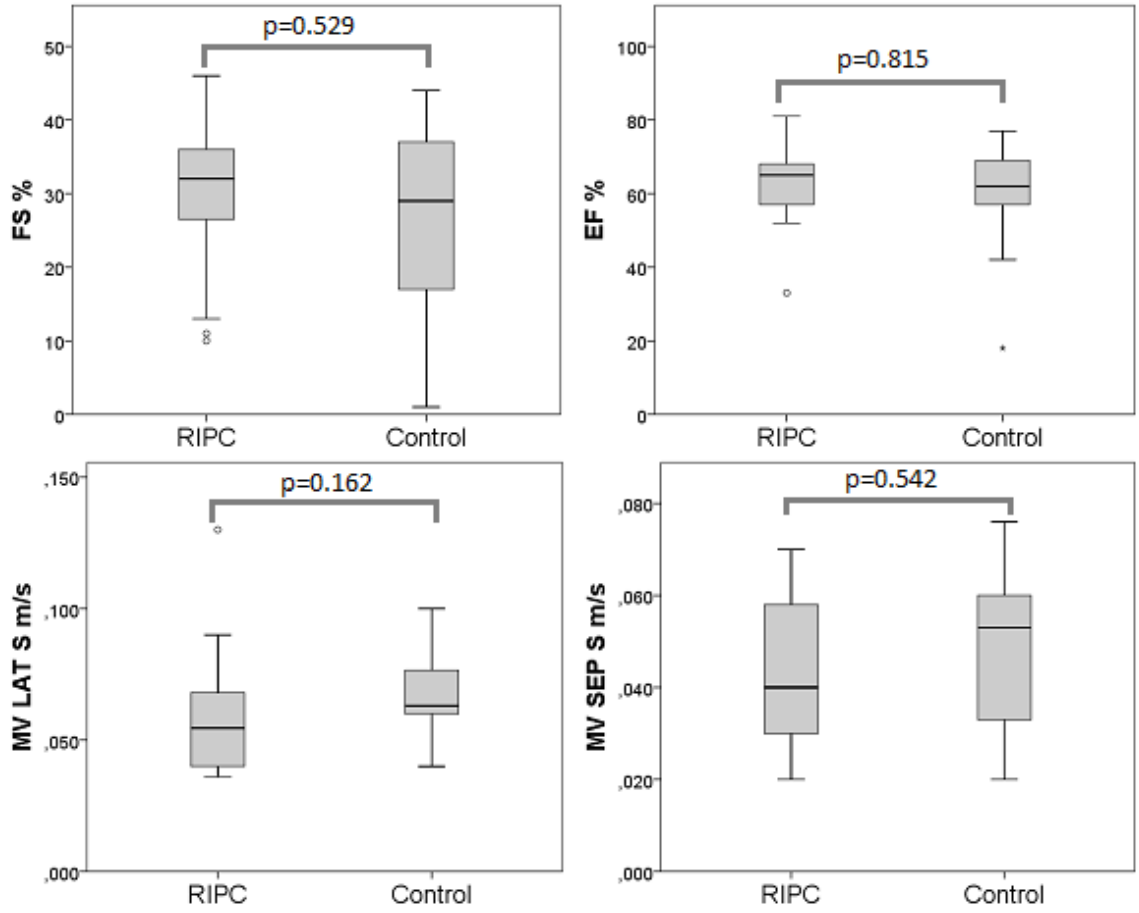

Figure 5.8. Post-operative echocardiographic measures of LV systolic function according to group allocation. Abbreviations: Abbreviations: EF: ejection fraction; FS: fractional shortening; MV LAT S: lateral mitral annulus systolic velocity; MV SEP S: septal mitral annulus systolic velocity; PO: post-operative; RIPC: remote ischaemic preconditioning.

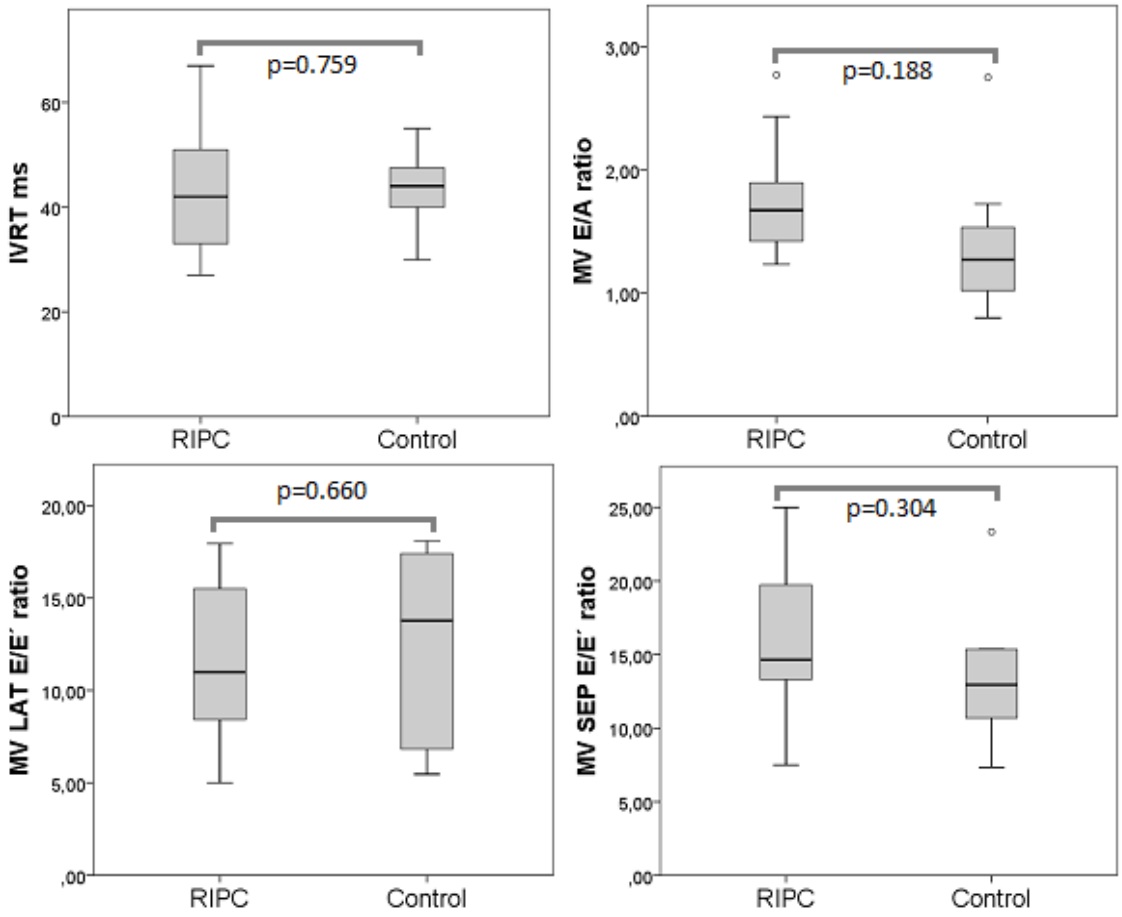

Figure 5.9. Post-operative echocardiographic measures of LV diastolic function according to group allocation. Abbreviations: IVRT: isovolumetric relaxation time; MV E/A: mitral inflow pulsed wave Doppler E to A velocities ratio; MV LAT E/E´: lateral mitral annulus E to E' ratio; MV SEP E/E´: septal mitral annulus E to E' ratio; PO: post-operative; RIPC: remote ischaemic preconditioning.

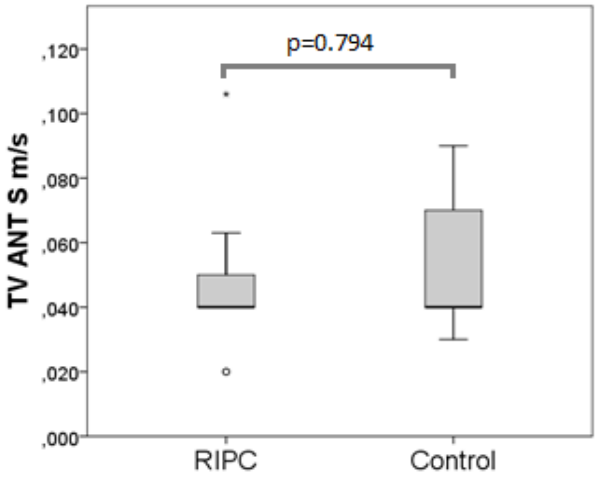

Figure 5.10. Post-operative echocardiographic measure of RV systolic function according to group allocation. Abbreviations: PO: post-operative; TV ANT S: tricuspid annular systolic velocity; RIPC: remote ischaemic preconditioning.

5.6 Summary of key findings

- MV LAT E/E´ ratio, MV SEP S and TV ANT S were lower in cyanotic patients after surgery.
- Post-operative MV LAT S was reduced in correlation to aortic cross clamp duration.
- Post-operative MV E/A ratio and MV LAT S were reduced according to CPB duration.
- Post-operative MV SEP S and MV LAT S correlated negatively to post-operative cTnI.
- Post-operative MV LAT S, MV SEP S and TV ANTS correlated negatively to post-operative BNP.
- EF and FS were reduced, while MV LAT S increased after surgery.
- TV ANT S was significantly reduced after surgery.
- Echocardiographic parameters did not vary according to RIPC or control allocation.

5.7 Limitations

It was not always possible to obtain all necessary images for all patients; hence there is variation in the amount of measures available for analysis of each variable and the target sample number set for this study was not achieved.

5.8 Discussion

Echocardiographic imaging is used routinely during the clinical diagnosis and follow up of CHD paediatric patients. TDI imaging has increased its applications and has proven particularly useful in order to evaluate RV function in CHD patients (Lytrivi *et al*, 2005). TDI measures have even been proposed as a predictor of post-operative outcome including inotropic requirement (Groban *et al*, 2010; Imai *et al*, 2011).

This study looked at echocardiographic measurements in order to determine the effects of RIPC in cardiac function.

Echocardiographic parameters vary depending on the type of CHD that patients present. MV SEP S, MV LAT E/E´ and TV ANTS were lower in cyanotic compared to acyanotic patients after surgery, as these differences were not present at baseline measures this decline in cardiac

function in cyanotic patients could be due to a different response to surgical procedures; as described before cyanotic patients also presented higher cTnI levels, reflecting a higher vulnerability to injury.

It was to be expected that all measures of functionality would be affected by the surgical procedure. Aortic clamp duration correlated negatively to lateral mitral systolic velocity while CPB affected MV E/A ratio as well. In addition both lateral and septal mitral systolic velocities correlated negatively to cTnI release post-operatively while BNP correlated to mitral and tricuspid systolic velocities. These links suggest that echocardiographic parameters are affected by the extent of myocardial injury.

In this study the measures of LV function EF and FS where reduced post-operatively while there was no decline in LV TDI velocities, with MV LAT S presenting even higher measures than before surgery, there was no significant difference in any of the ratios evaluating LV diastolic function, while the RV velocity TV ANT S showed a marked decline in the post-operative period.

A previous small study including 18 adult patients (9 CPB and 9 off-pump procedures) determined that E/A ratio was not affected by surgery in CPB or off-pump cases while EF increased in both groups (Malouf *et al*, 2006).

A larger study including 32 patients presenting preserved preoperative LV function going through CABG procedures reported no deterioration of LV systolic function after surgery, with an improvement in left diastolic function; on the other hand RV function was affected after surgery (Diller *et al*, 2008). This decline caused by cardiac surgery in tricuspid function has a long term duration, with velocities still being decreased after a year of CABG procedures (Alam *et al*, 2003).

Previous analysis in paediatric patients requiring surgical correction of septal defects determined that LV systolic variables dropped during the immediate post-operative period but recovered by the next day after surgery, while a significantly reduced TV ANT S was sustained by the first post-operative day (Vassalos *et al*, 2009). Our post-operative analysis corresponds to the day after surgery, a timing by which LV parameters could have recovered, while TV ANT S showed decrease, corroborating previous findings.

The sample size of the present study was initially calculated based on the significant decrease of TV ANT S in the data by Vassalos *et al* within our research group, in order to evaluate if RIPC could reduce this difference significantly. Although we recruited the required number of patients, due to clinical circumstances it was not possible to obtain the image necessary for TV ANT S measurement in all patients. For this reason this analysis would not have enough power to detect the specified difference in our calculation and the results must be considered exploratory.

RIPC had no effect in the echocardiographic parameters analysed. RIPC clinical trials have not usually explored echocardiographic parameters. As EF is a well-established measure of ventricular function, it was used in most adult RIPC clinical trials as an indicator of pre-operative LV cardiac function in order to compare baseline characteristics between groups and as means of exclusion criteria when function was low, however in most cases differences after surgery were not reported.

Karuppasami *et al*, 2011, reported no difference in EF according to RIPC before or after bypass in adult patients making no reference to pre and post-operative changes. One study followed up patients three months after surgery, finding improvement in EF for all patients compared to pre-operative values, with RIPC patients presenting a significantly higher long term improvement (Xie *et al*, 2012).

A large study including 72 patients in an echocardiographic analysis subgroup found no difference in pre vs post-operative EF or significant variation between RIPC or control groups (Rahman *et al*, 2010).

Conclusion

Echocardiographic changes after surgical interventions in CHD paediatric patients have not been extensively explored, characteristics of specific CHD pathologies and post-operative circumstances made it difficult to repeat measures during this study. RIPC did not result in a significant functional improvement according to echocardiographic parameters.

Blood marker analysis

Chapter 6. Inflammatory mediators

6.1 Introduction

Major surgeries and prolonged anaesthesia trigger inflammatory responses which are intensified by CPB due to non-physiological exposure to foreign surfaces, hypothermia, unusual shear stress, variation of intravascular pressures, blood gas composition and ischaemia, these insults are able to trigger detrimental reactions rendering the patient vulnerable to cardiac injury and systemic inflammatory response syndrome (SIRS). SIRS presents itself within the first 24 hours after surgery and involves oedema, thromboembolism, coagulation disorders and organ dysfunction that could affect the heart, brain, lungs and kidneys. SIRS is usually self-limited and temporary, however it can result in severe complications (reviewed by Warren *et al*, 2009; Kharbanda, 2010; Lawson *et al*, 2013).

The inflammatory reaction triggered by CPB starts as soon as the blood has been exposed to the CPB circuit and decays with time; cellular components including endothelial cells, neutrophils, monocytes, lymphocytes and platelets are activated while humoral mediators such as inflammatory cytokines including TNF-α and interleukins (IL-1, IL-2, IL-4, IL-6, IL-8 and IL-10) act. In addition the contact system, intrinsic and extrinsic coagulation cascades, classic and alternative complement pathways and fibrinolytic systems act leading to coagulopathies, tissue oedema and transient organ dysfunction (reviewed by Warren *et al*, 2009; Pągowska-Klimek *et al*, 2015).

In a second stage after CPB, IRI is established in the heart and endotoxins are believed to be released either after circuit contamination or from the portal circulation due to hypoperfusion of the intestine leading to augmented permeability. Endotoxins cause complement activation as well as NO and cytokine release (reviewed by Warren *et al*, 2009; Kats *et al*, 2011).

Neutrophil activation, haemolysis, ischaemia and reperfusion boost oxidative stress, a main factor contributing to inflammation and starting apoptotic cascades which can have an impact in organ function and recovery (reviewed by Zakkar *et al*, 2015).

Paediatric procedures for CHD represent an even greater challenge for CPB control as the system which cannot be completely adapted for patient size has to deal with small blood volumes resulting in higher haemodilution, higher pump flow rates and often longer times; in addition anatomical variations such as atresia, duplication of vessels and aortopulmonary collaterals complicate the establishment and control of the process (reviewed by Whiting *et al*, 2015).

SIRS in paediatric patients involves an increase in capillary leakage and fluid build-up in tissues, increased pulmonary vascular resistance after surgery has been identified as a complication affecting cardiac function and leading to mortality (reviewed by McGuinness *et al*, 2008).

6.1.1 Cytokines

Numerous cytokines are involved in the process of IRI, among the main molecules TNF-α is considered as one of the most potent pro-inflammatory cytokines; produced by macrophages and monocytes, it mediates several oxidative stress responses during ischaemia and reperfusion and has been shown to exacerbate IRI augmenting infarct size (reviewed by Zhou *et al*, 2015). Conservative strategies aimed to reduce injury have targeted TNF-α obtaining reduced oxidative stress and inflammatory signalling resulting in an attenuation of IRI after inhibition of TNF-α (Gu *et al*, 2006; Pei *et al*, 2015). TNF- α was found to induce gene expression leading to inhibition of the RISK pathway contributing to injury, inhibition of TNF- α reduced IRI injury *in vitro* and *in vivo* (Xing *et al*, 2016).

IL-6 presents dual activity mediating immune reactions while supressing other inflammatory cytokines including TNF-α, contributing to the resolution of acute and chronic inflammatory processes (Tilg *et al*, 1997). IL-6 together with IL-8 are upregulated during cardiac surgery and CPB with increased levels observed in relation to IRI (Kawamura *et al*, 1993); on the other hand it has been found to mediate cardiac protection after exercise preconditioning (McGinnis *et al*, 2015).

IL-8 is released by monocytes, T cells and the endothelium, it recruits leukocytes and has been linked to respiratory distress syndrome in hypoxic circumstances (Hirani *et al*, 2001). IL-8 levels correlated witch cTnI values after CABG procedures, implying that IL-8 could contribute to the

occurrence of cardiac injury (Wan *et al*, 1999), in addition blocking IL-8 resulted in an infarct size reduction in an IRI model (Boyle *et al*, 1998).

IL-10 has been described as one of the most powerful anti-inflammatory cytokines, it is produced in a wide range of immune cells including B and T cells, neutrophils, eosinophils, dendritic, mast and natural killer cells. It acts directly and indirectly on the innate and adaptive immune systems inhibiting the release of cytokines, as well as intervening in antigen presentation and cellular proliferation (reviewed by Saxena *et al*, 2015). The main mechanism for its anti-inflammatory activity is believed to be the inhibition of NF-κB resulting in downregulation of all the factors downstream (Wang *et al*, 1995).

Animal studies have demonstrated the role of IL-10 in cardiac protection as it inhibited TNF-α production, NO and I-CAM activity during ischaemia and reperfusion of the heart (Yang *et al*, 2000). Furthermore, a study in a mouse model found that IL-10 activity resulted in infarct size reduction after RIPC stimulation (Cai *et al*, 2013).

The anti-inflammatory properties of IL-10 are thought to be one of the mechanisms of steroid inhibition of CPB induced inflammation, which are commonly used in the clinical setting; a study revealed that steroids upregulated IL-10 and abolished inflammatory cytokines after CPB resulting in heart and lung protection (Giomarelli *et al*, 2003).

Experience in CPB induced inflammation has established a correlation between the duration of cardiac ischaemia and the levels of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8, establishing the myocardium as their main source of production, while IL-10 levels were correlated to bypass duration, mainly originated from the liver and presented definite anti-inflammatory properties (Wan *et al*, 1997).

6.1.2 Cellular adhesion molecules

Leukocyte recruitment takes place during inflammation through three steps: leukocyte rolling in the endothelium, leukocyte arrest and transmigration. Rolling is mediated by low affinity receptors selectins or low affinity integrins interacting with cell adhesion molecules such as ICAM-1 or vascular cell adhesion molecule 1 (VCAM-1) which bind to the leukocyte slowing it down, this and chemokine activity cause integrins to be activated, presenting a high affinity which allows a strong bond to VCAM-1 or ICAM-1 causing arrest of the leukocyte within the endothelium, followed by migration into the tissue (reviewed by Cook-Mills *et al*, 2011).

ICAM-1

ICAM-1 or CD54 is a transmembrane glycoprotein within the immunoglobulin superfamily, it has five immunoglobulin domains and can be spliced into several isoforms, it is expressed in low levels in the surface of endothelial cells, platelets, fibroblasts, epithelial cells, neutrophils and other types of leukocytes and rises after stimulation of toll like receptors (TLRs) and cytokine receptors, in order to contribute to the extravasation of neutrophils from circulating blood into tissues by interacting with β2-integrins (reviewed by Lyck and Enzmann, 2015).

VCAM-1

VCAM-1 or CD106, another member of the immunoglobulin superfamily presents several immunoglobulin like domains with two possible splice variations. VCAM-1 is expressed in endothelial cells, macrophages, dendritic cells, spleen, thymus and Kupffer cells as well as in bone marrow stromal cells, playing an important role in leukocyte recruitment, in homing early haematological progenitor cells to the stroma in the bone marrow and due to its wide distribution it is linked to several pathologic processes. VCAM-1 can be upregulated by cytokines, oxidative stress, TLR activity and shear stress, very late activation antigen-4 (VLA-4) is the best known integrin working with VCAM-1 during leukocyte recruitment (reviewed by Schlesinger and Bendas, 2015).

Cardiac involvement

VCAM-1 and ICAM-1 have been identified as possible markers of endothelial activation, this application has however not been established as measurements of their soluble forms have not always correlated to cellular concentrations, in addition expression of the molecules is different according to the origin of the endothelium in culture experiments (Videm and Albrigtsen, 2008).

Research on the participation of ICAM-1 and VCAM-1 on cardiac IRI has not been extensive, however studies have demonstrated their influence in animal models. A study with knockout mice showed that myocardial injury and neutrophil accumulation were markedly reduced by ICAM-1 or neutrophil CD18 deficiency after ischaemia and reperfusion (Palazzo *et al*, 1998). Furthermore ICAM-1 regulation influenced the occurrence of IRI in a study analysing the activity of micro RNA 144 which resulted in decreased ICAM-1 and consequently reduced leukocyte adhesion to the endothelium *in vitro*. On the other hand, *in vivo* reduced expression of ICAM-1 in the myocardium persisted and related to smaller infarct size and troponin concentration (Liu *et al*, 2015).

VCAM- 1 involvement was shown in a rat model of IRI, in which a higher expression within the affected and healthy myocardium was observed through the use of a molecular imaging technique employing high field magnetic resonance (Grieve *et al*, 2013).

Human studies looking at the expression pattern of soluble adhesion molecules during cardiac surgery and CPB have been performed, finding a significant increase after bypass in all the molecules including ICAM-1 and VCAM-1, with ICAM-1 regressing to baseline levels by the first postoperative day while VCAM-1 remained raised (Boldt *et al*, 1998).

Further research showed ICAM-1 and VCAM-1 were increased in patients after CPB in association to augmented endothelial activation, with both adhesion molecules presenting different kinetics; VCAM-1 showed the highest values which were correlated to aortic clamp duration as well as heparin and protamine doses while ICAM-1 variated according to more variables including age and administration of additional medication (Eikemo *et al*, 2004).

VCAM-1 levels have been found to increase in the culture media of endothelial cells rather than within the cells as other adhesion molecules, which could make it an adequate circulating

marker; correspondingly soluble VCAM-1 levels were raised significantly during the first 3 postoperative hours in the serum of patients going through cardiac surgery with CPB suggesting it could be a suitable early marker for endothelial activation (Andresen *et al*, 2002).

Analysis in paediatric patients has demonstrated higher preoperative values of ICAM-1 and VCAM-1 compared to adult patients, surprisingly these values were reduced during and after bypass and remained below the baseline preoperative values in children (Boldt *et al*, 1995).

One *in vitro* study in a hypoxia preconditioning model using aortic rat endothelial cells found that preconditioning prevented free radical induction of ICAM-1, resulting in a reduction in neutrophil adhesion to the endothelium which could contribute to cardiac protection during RIPC (Beauchamp *et al*, 1999).

6.2 Aims

To assess he influence of RIPC on the levels of inflammatory mediators in blood and to describe their pattern before and after CHD surgery using CPB.

6.3 Materials and Methods

Levels of inflammatory markers were measured in plasma samples at baseline and three post-operative time points at PICU arrival (PO1), morning after surgery (PO2) and second morning after surgery (PO3). ELISA DuoSet® assays from R&D Systems were used to measure blood sample markers according to the manufacturer´s protocol. For details on materials and methods see *Section 2.8*.

6.4 Statistical analysis

Repeated samples Friedman analysis of variance was performed in order to test for differences between time points, post-hoc comparisons between time points were done using Wilcoxon signed rank test with Bonferroni correction for multiple testing.

Correlation analysis of post-operative measurements to demographic characteristics including weight, age, gender and cyanosis status, as well as to post-operative cTnI, CPB and aortic clamp duration were tested using the nonparametric Spearman´s rank correlation test.
Independent samples t-test or Mann-Whitney tests were carried out in order to determine differences between groups according to data distribution.
Statistical analysis was performed using SPSS Software version 23 at a significance level of 5% (2 tailed).

6.5 Results

Fifty children completed the study, demographic data and its analysis can be seen on *Table 3.1* and *3.2*.

Perioperative analysis

6.5.1 IL-6

Descriptive statistics for IL-6 plasma levels according to time point are shown in *Table 6.1*.

Time point	N	Median – IQ range
Baseline	12	5.47 (4.68-33.43)
PO1	40	343.54(170.68-900.00)
PO2	38	98.52(32.41-221.99)
PO3	27	53.27(27.87-82.64)

Table 6.1. Descriptive statistics for plasma IL-6 levels according to time point. Data expressed in median and IQ range. Abbreviations: IQ: interquartile; PO: post-operative.

Three patients presented complete measures for all time points, this sample was not deemed big enough to perform any analysis. Exclusion of the most incomplete time point Baseline allowed the analysis of 18 patients with complete measures between three time points (PO1, PO2 and PO3) which resulted in a significant variation ($X^2(2)=24.704$, $p<0.0001$). Post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were performed resulting in a significance level at $p<0.017$. The difference was significant between all time points at $p<0.0001$. *Figure 6.1* presents the variation in IL-6.

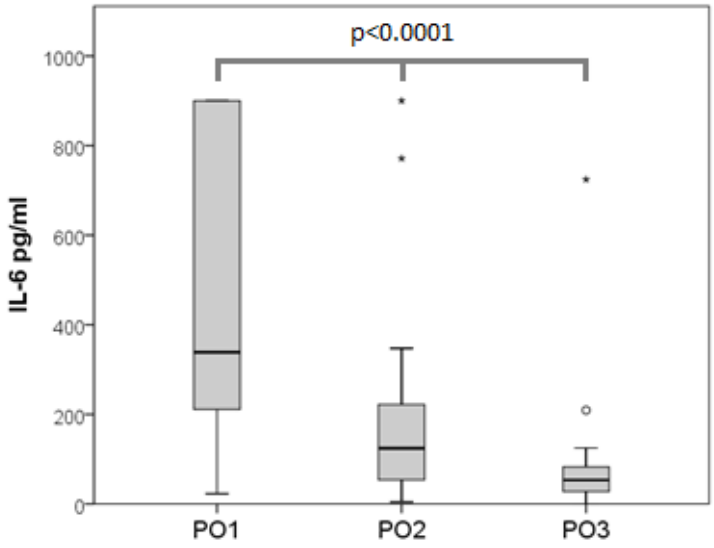

Figure 6.1. Plasma IL-6 levels according to time point. Abbreviations: IL-6: interleukin 6; PO: post-operative.

There was a significant negative correlation of PO-1 IL-6 levels to age (r_s= -0.377, p=0.017), and a positive correlation of CPB to PO1 (r_s=0.369, p=0.019) (*Figure 6.2*).

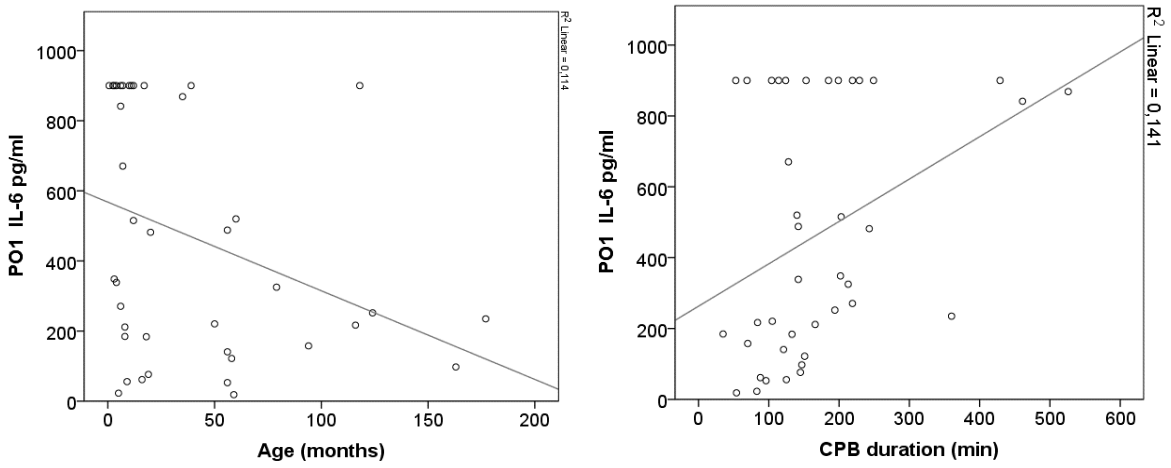

Figure 6.2. Correlation of PO1 IL-6 to age and CPB duration. Abbreviations: IL-6: interleukin 6; PO1: post-operative 1.

6.5.2 IL-8

Descriptive statistics for IL-8 plasma levels according to time point are shown in *Table 6.2*.

Time point	N	Median – IQ range
Baseline	48	5.47 (4.68-33.43)
PO1	50	343.54(170.68-900.00)
PO2	49	98.52(32.41-221.99)
PO3	35	53.27(27.87-82.64)

Table 6.2. Descriptive statistics for plasma IL-8 levels according to time point. Data expressed in median and IQ range. Abbreviations: IL-8: interleukin 8; IQ: interquartile; PO: post-operative.

Thirty three patients presented complete measures for all time points, variation was significant between time points ($X^2(2)=72.377$, $p<0.0001$). Post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were performed resulting in a significance level at $p<0.0083$. The difference was significant between all time points. *Figure 6.3* presents the variation in IL-6.

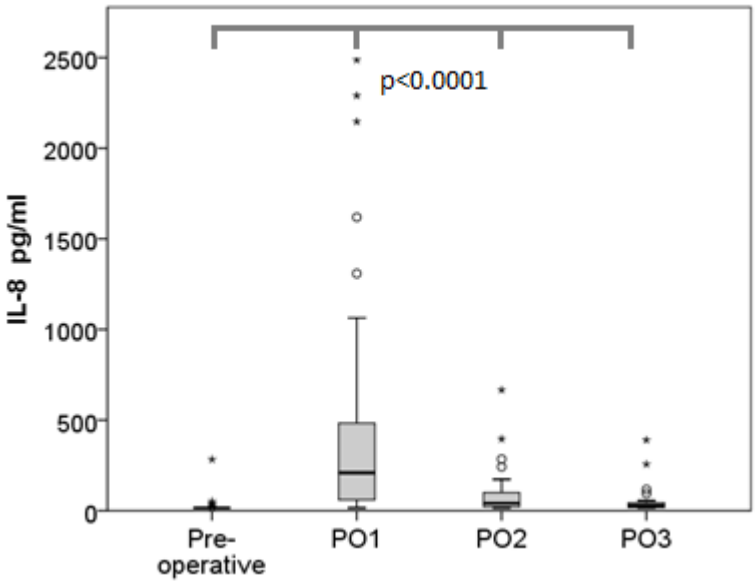

Figure 6.3. Plasma IL-8 levels according to time point. Abbreviations: IL-8: interleukin 8; PO: post-operative.

There was a significant negative correlation of weight to PO1 (r_s= -0.515, p<0.0001) and PO2 (r_s= -0.329, p=0.021); age correlated to IL-8 PO1 (r_s= -0.485, p<0.0001) and PO2 levels (r_s= -0.333, p=0.019) (*Figure 6.4*).

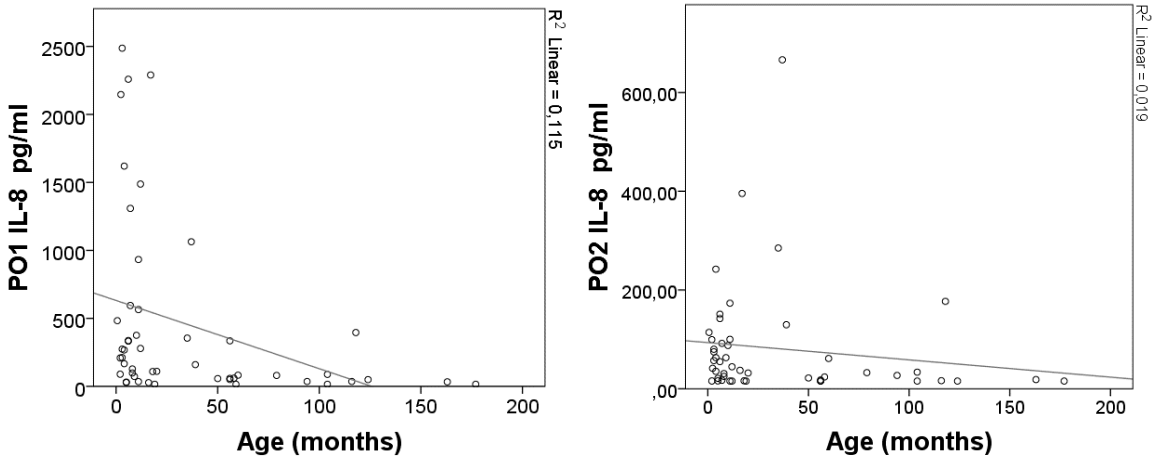

Figure 6.4. Correlation of PO1 and PO2 IL-8 to age. Abbreviations: IL-8: interleukin 8; PO1: post-operative 1.

A positive correlation of CPB to PO1 (r_s=0.457, p<0.0001), PO2 (r_s=0.500, p= p<0.0001) and PO3 IL-8 (r_s=0.475, p=0.004) was present (*Figure 6.5*).

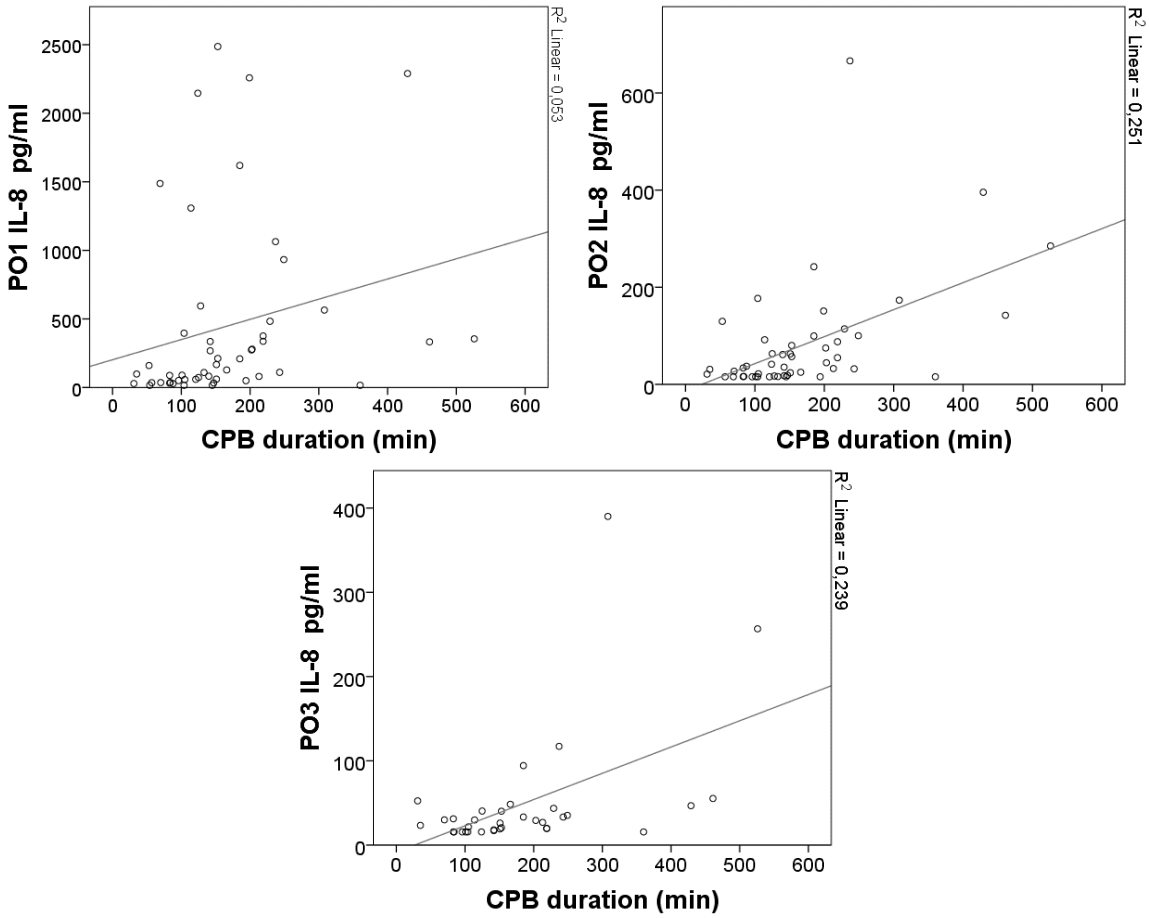

Figure 6.5. Correlation of CPB duration to IL-8 at PO1, PO2 and PO3 time points. Abbreviations: IL-8: interleukin 8; PO: post-operative.

In addition there was a positive correlation of aortic cross clamp duration to PO1 (r_s=0.306, p=0.033), PO2 (r_s=0.360, p=0.012) and PO3 (r_s=0.432, p=0.011) (*Figure 6.6*).

IL-8 levels correlated to cTnI measurements at corresponding timepoints PO1 (r_s=0.437, p=0.002), PO2 (r_s=0.528, p<0.0001) and PO3 (r_s=0.445, p=0.007) (*Figure 6.7*).

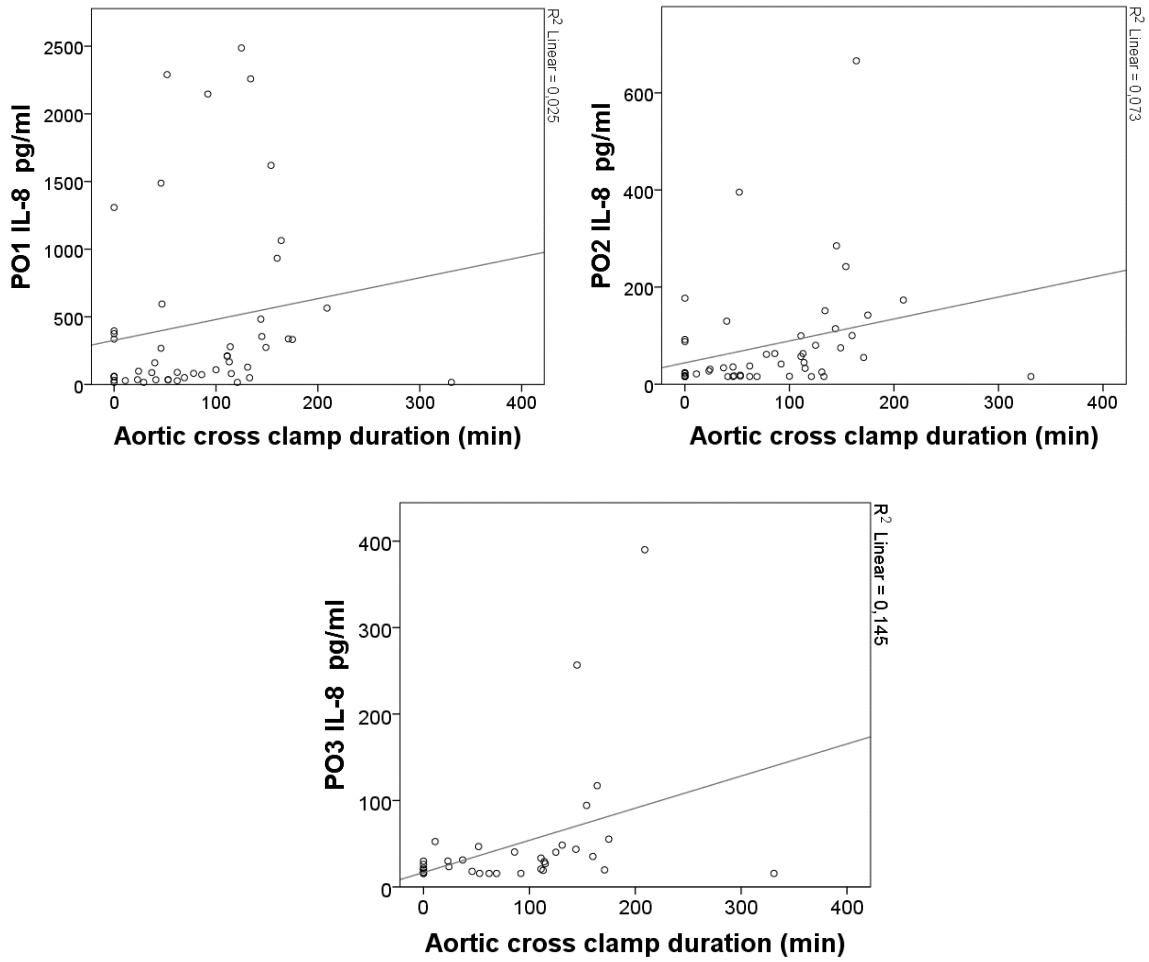

Figure 6.6. Correlation of aortic cross clamp duration to IL-8 at PO1, PO2 and PO3 time points. Abbreviations: IL-8: interleukin 8; PO: post-operative.

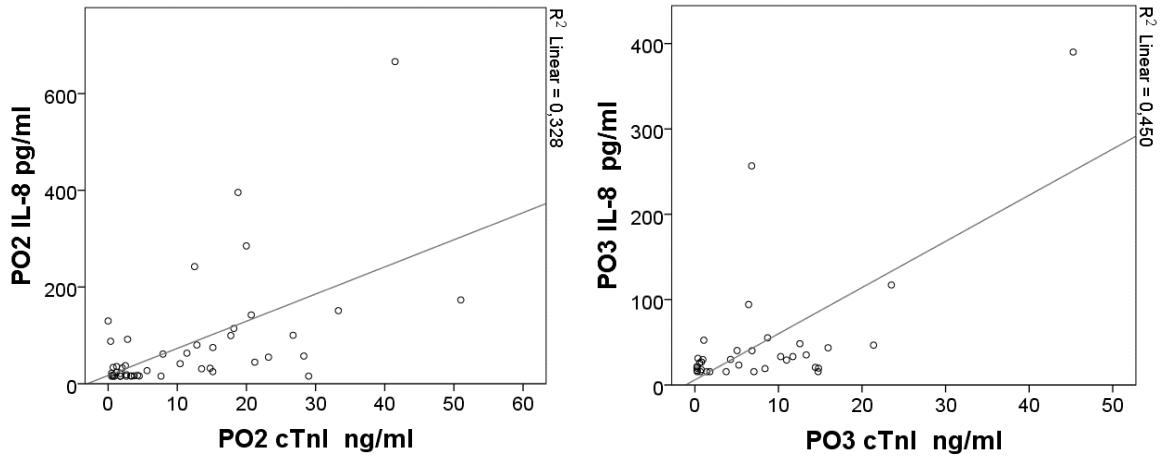

Figure 6.7. Correlation of IL-8 to cTnI levels at PO2 and PO3 time points. Abbreviations: cTnI: cardiac troponin I; IL-8: interleukin 8; PO: post-operative.

6.5.3 IL-10

Descriptive statistics for IL-10 plasma levels according to time point are shown in *Table 6.3*.

Time point	N	Median – IQ range
Baseline	47	18.64 (15.63-94.14)
PO1	50	224.49 (92.31-462.0.5)
PO2	48	36.06 (15.63-141.95)
PO3	35	27.63 (15.63-64.98)

Table 6.3. Descriptive statistics for plasma IL-10 levels according to time point. Data expressed in median and IQ range. Abbreviations: IL-10: interleukin 10; IQ: interquartile; PO: post-operative.

Thirty three patients presented complete measures for all time points, variation was significant between time points ($X^2(2)=48.105$, $p<0.0001$) (*Figure 6.8*). Post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were performed resulting in a significance level at $p<0.0083$. The difference was significant only between Baseline- PO1, PO1-PO2, and PO1-PO3 at $p<0.0001$.

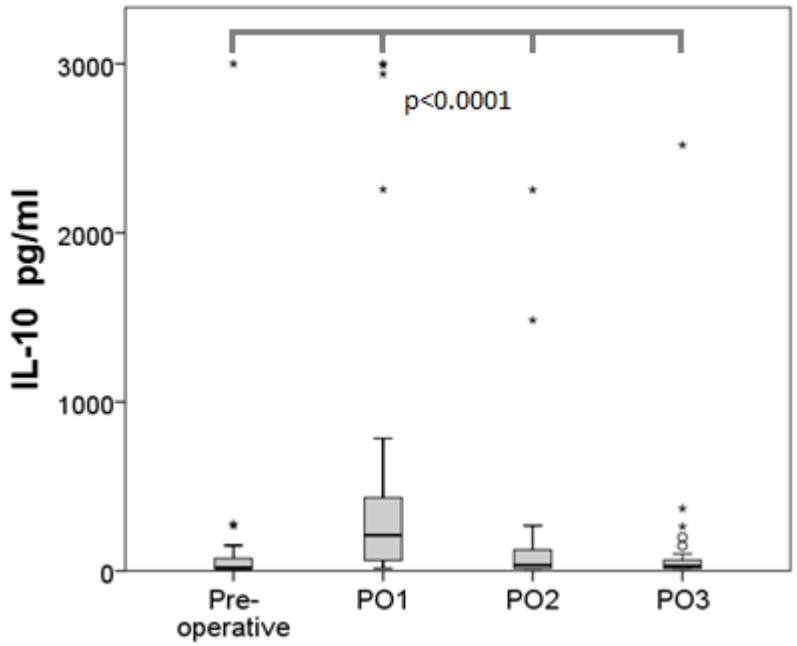

Figure 6.8. Plasma IL-10 levels according to time point. Abbreviations: IL-10: interleukin 10; PO: post-operative.

There was no significant correlation to any of the analysed variables.

6.5.4 TNF-α

Descriptive statistics for TNF-α plasma levels according to time point are shown in *Table 6.4*.

Time point	N	Median – IQ range
Baseline	31	28.99 (7.81-560.82)
PO1	47	140.69 (28.26-473.42)
PO2	43	62.18 (7.81-296.25)
PO3	31	34.64 (7.81-101.96)

Table 6.4. Descriptive statistics for plasma TNF-α levels according to time point. Data expressed in median and IQ range. Abbreviations: IQ: interquartile; PO: post-operative.

Fourteen patients presented complete TNF-α measurements for all time points; levels did not variate significantly between time points ($X^2(2)=4.240$, $p=0.237$).

Exclusion of the most incomplete time point PO3 allowed the analysis of 26 patients with complete measures for three time points which resulted in a significant variation ($X^2(2)=10.298$, $p=0.006$) (*Figure 6.9*). Post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were performed resulting in a significance level at $p<0.017$. The difference was significant between PO1 and PO2 time points at $p<0.0001$.

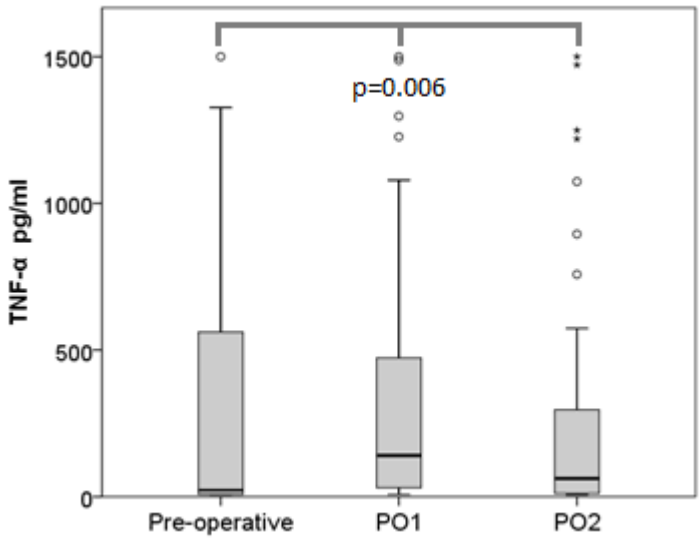

Figure 6.9. Plasma TNF-α levels according to time point. Abbreviations: PO: post-operative; TNF-α: tumor necrosis factor α.

There was no significant correlation of TNF-α levels to any of the explored variables.

6.5.5 ICAM-1

Descriptive statistics for ICAM-1 plasma levels according to time point are shown in *Table 6.5*.

Time point	N	Median – IQ range
Baseline	43	55.19 (18.73-141.78)
PO1	50	39.72 (15.60-96.06)
PO2	47	61.74 (20.42-127.94)
PO3	33	42.85 (17.60-124.61)

Table 6.5. Descriptive statistics for plasma ICAM-1 levels according to time point. Data expressed in median and IQ range. Abbreviations: ICAM-1: intercellular adhesion molecule 1; IQ: interquartile; PO: post-operative.

Twenty seven patients presented complete measures for all time points, variation was not significant between time points ($X^2(2)=5.178$, p=0.159) (*Figure 6.10*).

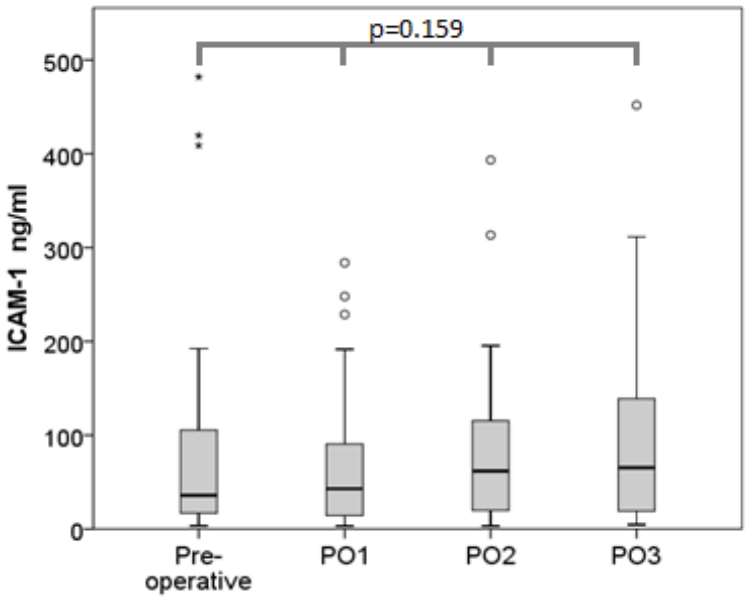

Figure 6.10. Plasma ICAM-1 levels according to time point. Abbreviations: ICAM-1: cellular adhesion molecule 1; PO: post-operative.

There was a weak positive significant correlation between aortic clamp duration and ICAM-1 levels at PO1 ($r_s=0.308$, p=0.031), as well as to cTnI values at the corresponding time point ($r_s=0.282$, p=0.048) (*Figure 6.11*).

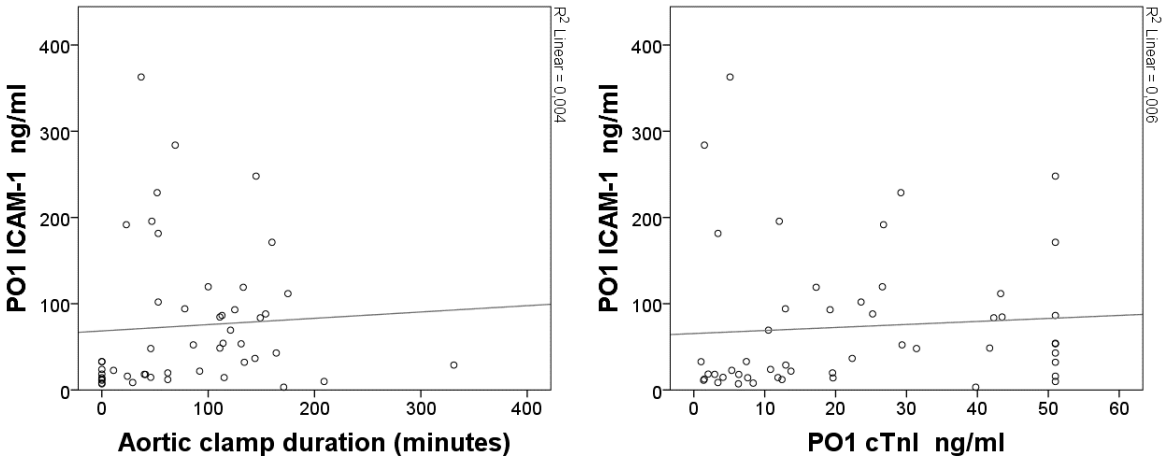

Figure 6.11. Correlation of PO1 ICAM-1 to aortic clamp duration and PO1 cTnI levels. Abbreviations: CPB: cardiopulmonary bypass; ICAM-1: intercellular adhesion molecule 1; PO: post-operative.

6.5.6 VCAM-1

Descriptive statistics for VCAM-1 plasma levels according to time point are shown in *Table 6.6*.

Time point	N	Median – IQ range
Baseline	43	337.74 (235.90-430.46)
PO1	50	282.47 (188.29-360.75)
PO2	47	353.64 (228.73-480.43)
PO3	33	268.07 (197.04-424.60)

Table 6.6. Descriptive statistics for plasma VCAM-1 levels according to time point. Data expressed in median and IQ range. Abbreviations: VCAM-1: vascular cell adhesion molecule 1; IQ: interquartile; PO: post-operative.

Twenty seven patients presented complete measures for all time points, variation was significant between time points ($X^2(2)=19.044$, $p<0.0001$) (*Figure 6.12*). Post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were performed resulting in a significance level at $p<0.0083$. The difference was significant between Baseline – PO1 and PO1 – PO2 at $p<0.0001$.

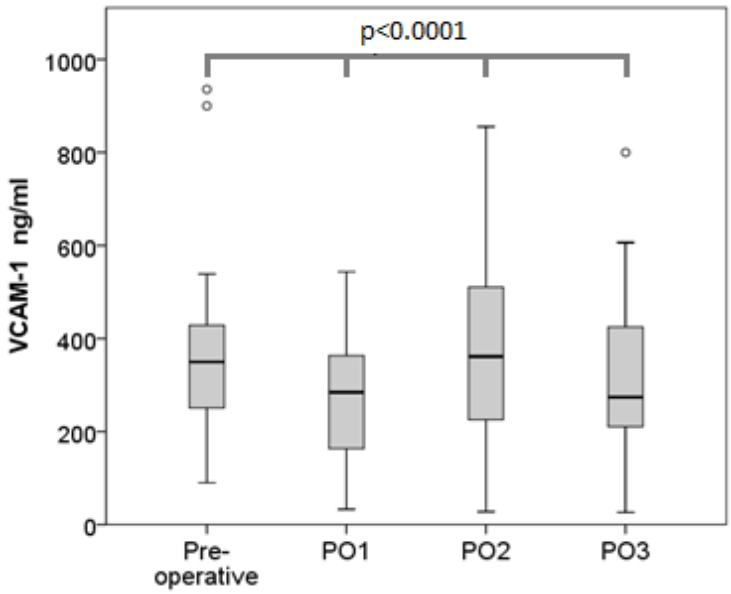

Figure 6.12. Plasma VCAM-1 levels according to time point. Abbreviations: VCAM-1: vascular cell adhesion molecule 1; PO: post-operative.

Weight presented a significant positive correlation to VCAM-1 levels at PO1 (r_s=0.534, p<0.0001) and PO3 (r_s=0.464, p=0.006). Age also correlated to PO1 (rs=0.473, p=0.001) and PO3 (r_s=0.378, p=0.030) (*Figure 6.13*).

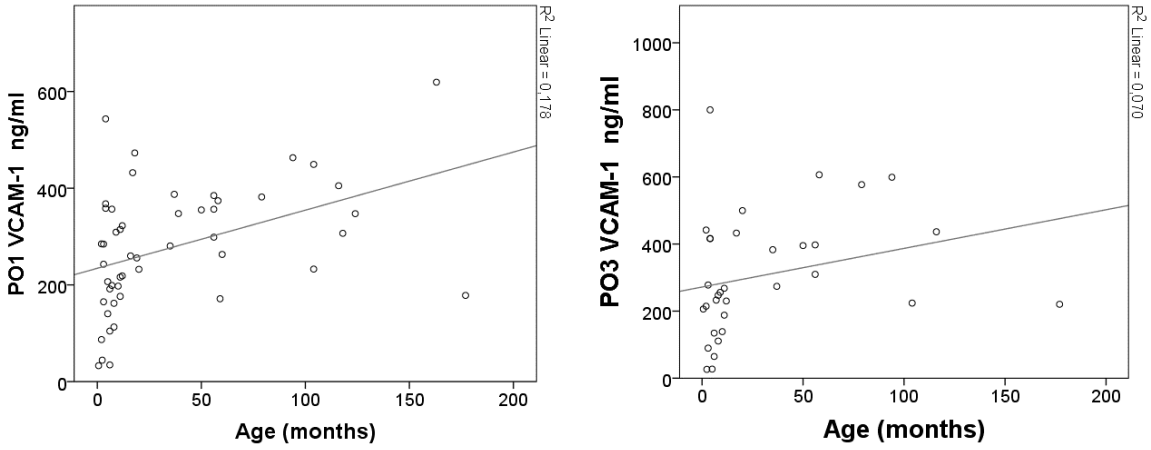

Figure 6.13. Correlation of CPB duration to VCAM-1 at PO1 and PO3 time points. Abbreviations: CPB: cardiopulmonary bypass; VCAM-1: vascular cell adhesion molecule 1; PO: post-operative.

RIPC analysis

Two cases were excluded from analysis for RIPC as parents asked for the intervention to be stopped before its completion due to discomfort.

6.5.7 IL-6 – RIPC

IL-6 measurements were similar in RIPC and control groups for all time points. *Figure 6.14* shows the variation of IL-6 according to intervention group.

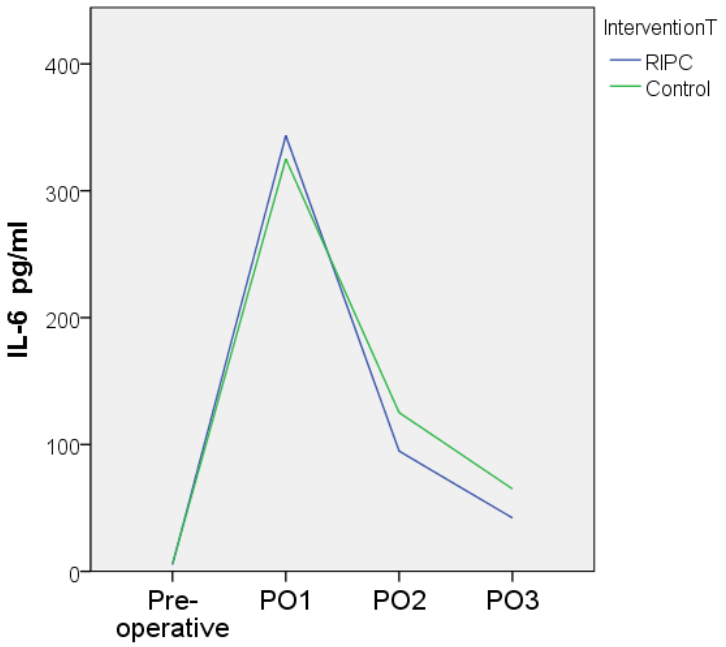

Figure 6.14. Plasma IL-6 levels variation for time points according to group allocation. Median values are represented. Abbreviations: IL-6: interleukin 6; PO: post-operative; RIPC: remote ischaemic preconditioning.

Table 6.7 presents descriptive statistics for each time point according to group allocation; there was no significant difference between control and RIPC groups at any time point (*Figure 6.15*).

Variable	Intervention	N	Median – IQ range	P value
Baseline	RIPC	4	5.26 (4.69-402.40))	0.730
	Control	5	5.96 (4.69-52.99)	
PO1	RIPC	20	343.54 (169.30-769.52)	0.728
	Control	19	325.09 (157.61-900.00)	
PO2	RIPC	18	94.78 (32.41-222.00)	0.443
	Control	16	125.14 (66.77-321.61)	
PO3	RIPC	12	42.10 (20.21-144.05)	0.611
	Control	13	65.01 (51.50-76.97)	

Table 6.7. Descriptive statistics for plasma IL-6 values according to group allocation for each time point. Data is expressed in median and IQ range. Abbreviations: IL-6: interleukin 6; IQ: interquartile; PO: post-operative; RIPC: remote ischaemic preconditioning.

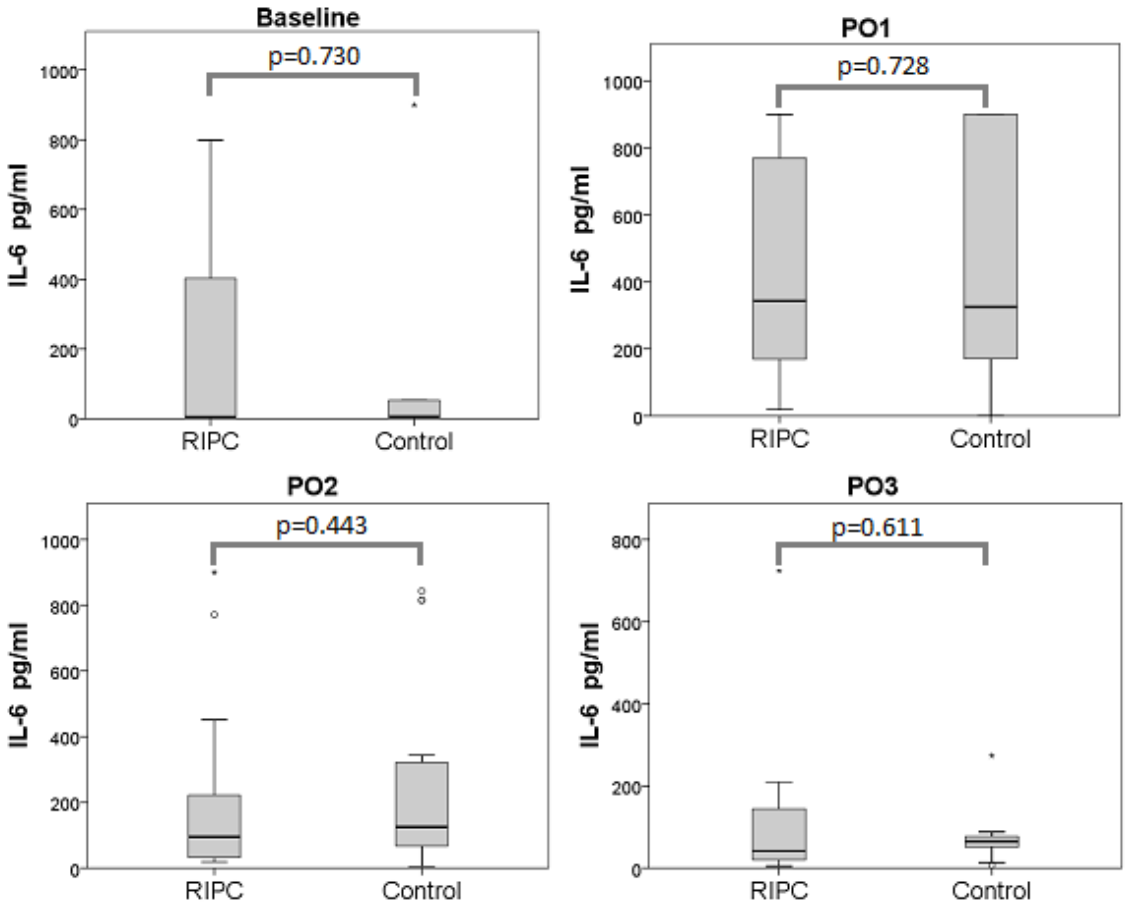

Figure 6.15. Plasma IL-6 levels according to group allocation for each time point. Abbreviations: IL-6: interleukin 6; PO: post-operative; RIPC: remote ischaemic preconditioning.

6.5.8. IL-8 – RIPC

IL-8 measurements were similar in RIPC and control groups for all time points. *Figure 6.16* shows the variation of IL-8 according to intervention group.

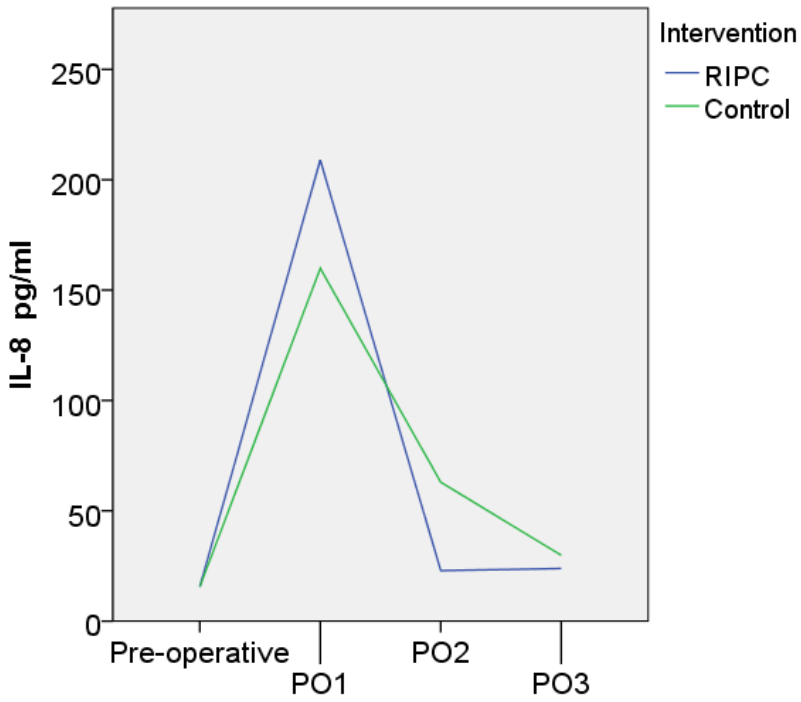

Figure 6.16. Plasma IL-8 levels variation for time points according to group allocation. Median values are represented. Abbreviations: IL-8: interleukin 6; PO: post-operative; RIPC: remote ischaemic preconditioning.

Descriptive information is summarised in *Table 6.8;* there was no significant difference according to group allocation for any time point (*Figure 6.17*).

Variable	Intervention	N	Median – IQ range	P value
Baseline	RIPC	25	15.63 (15.63-15.90)	0.331
	Control	21	15.623 (15.63-16.97)	
PO1	RIPC	25	208.92 (36.37-354.68)	0.312
	Control	13	159.90 (80.62-564.54)	
PO2	RIPC	24	22.88 (15.98-68.08)	0.096
	Control	23	62.99 (25.01- 142.23)	
PO3	RIPC	16	23.86 (16.40-39.46)	0.443
	Control	18	29.86 (19.72-46.72)	

Table 6.8. Descriptive statistics for plasma IL-8 values according to group allocation for each time point. Data is expressed in median and IQ range. Abbreviations: IL-8: interleukin 8; IQ: interquartile; PO: post-operative; RIPC: remote ischaemic preconditioning.

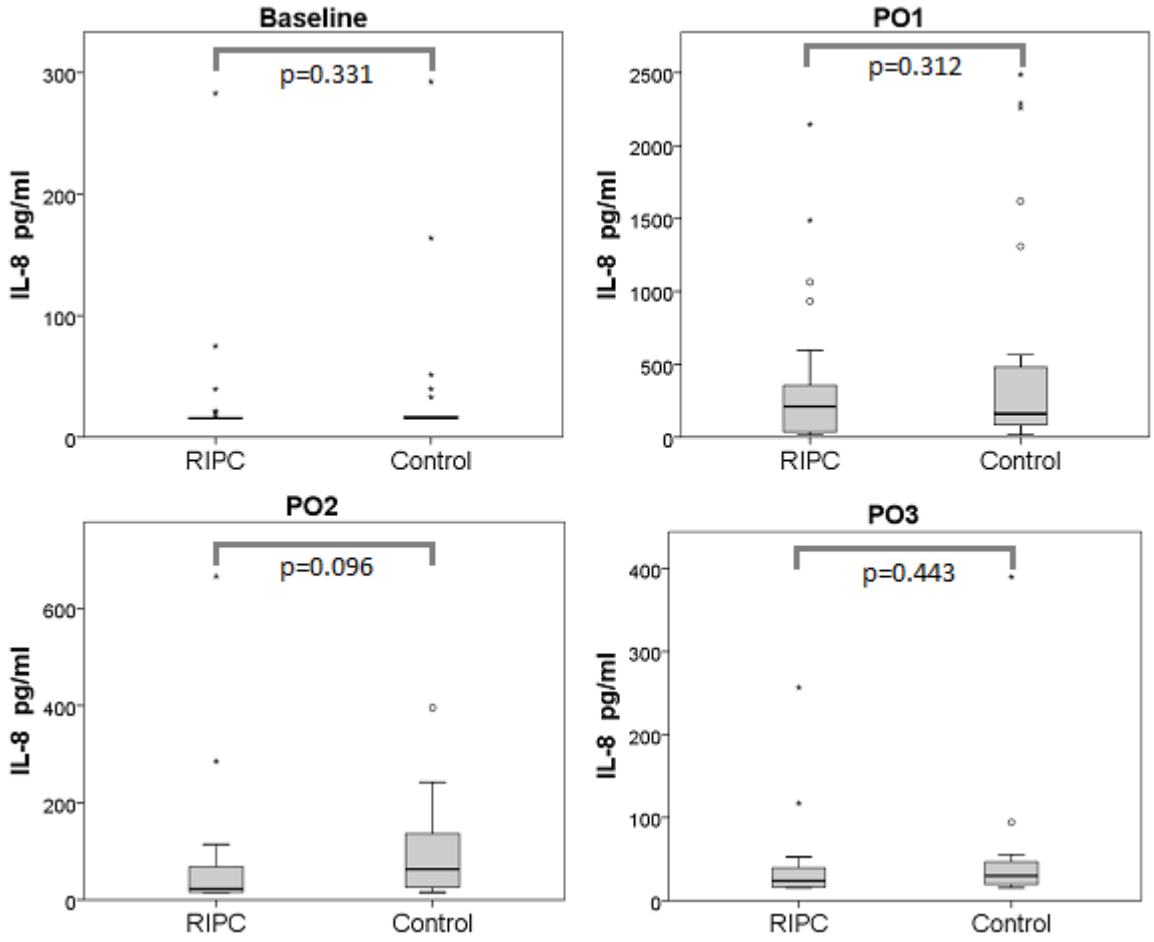

Figure 6.17. Plasma IL-8 levels according to group allocation for each time point. Abbreviations: IL-8: interleukin 8; PO: post-operative; RIPC: remote ischaemic preconditioning.

6.5.9 IL-10 – RIPC

IL-10 levels presented a similar pattern in RIPC and control groups. *Figure 6.18* shows IL-10 variation according to intervention group.

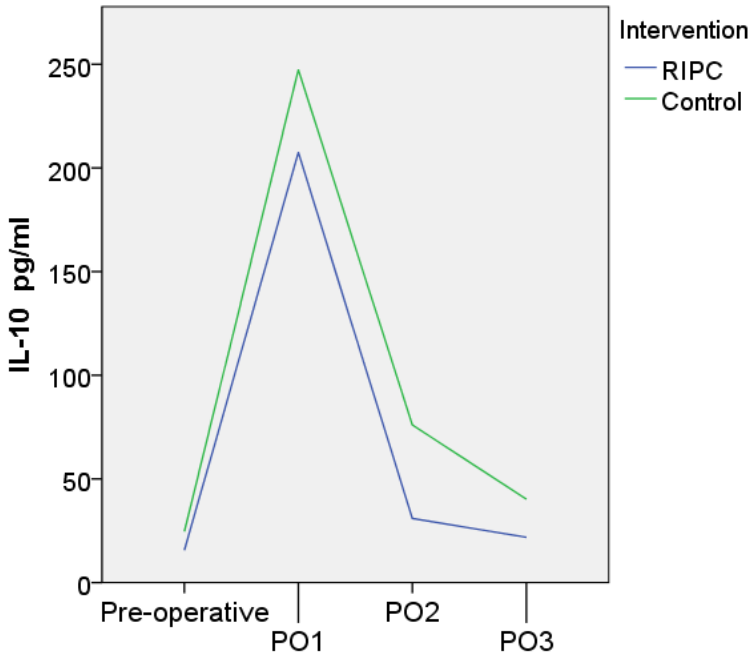

Figure 6.18. Plasma IL-10 levels variation for time points according to group allocation. Median values are represented. Abbreviations: IL-10: interleukin 6; PO: post-operative; RIPC: remote ischaemic preconditioning.

Table 6.9 presents descriptive statistics for each time point according to group allocation; there was no significant difference between control and RIPC groups at any time point (*Figure 6.19*).

Variable	Intervention	N	Median – IQ range	P value
Baseline	RIPC	24	15.63 (15.63-45.67)	0.245
	Control	21	24.64 (15.63-129.99)	
PO1	RIPC	25	207.50 (64.13-396.37)	0.570
	Control	23	247.31 (92.31-779.29)	
PO2	RIPC	24	30.92 (15.63-124.59)	0.167
	Control	22	76.01 (28.20-243.28)	
PO3	RIPC	16	21.86 (15.63-54.66)	0.621
	Control	18	40.22 (15.63-71.24)	

Table 6.9. Descriptive statistics for plasma IL-10 values according to group allocation for each time point. Data is expressed in median and IQ range. Abbreviations: IL-10: interleukin 10; IQ: interquartile; PO: post-operative; RIPC: remote ischaemic preconditioning.

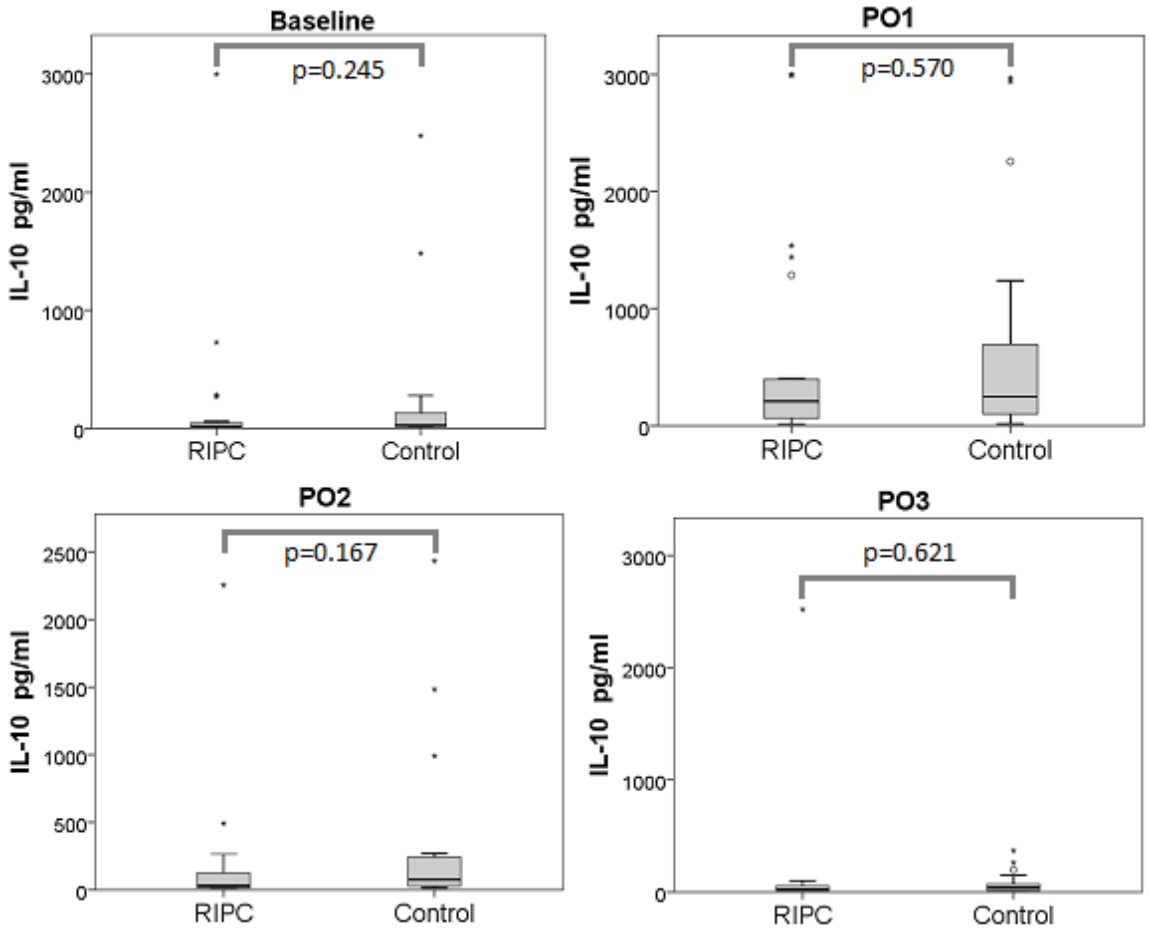

Figure 6.19. Plasma IL-10 levels according to group allocation for each time point. Abbreviations: IL-10: interleukin 8; PO: post-operative; RIPC: remote ischaemic preconditioning.

6.5.10 TNF-α – RIPC

Levels of TNF-α tended to be higher in the control group compared to RIPC at post-operative time points. *Figure 6.20* shows TNF-α levels according to intervention group.

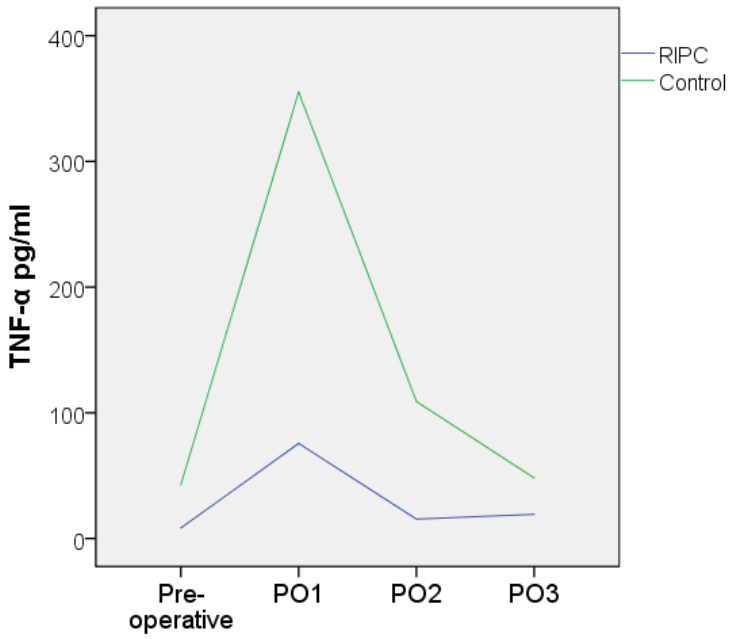

Figure 6.20. Plasma TNF-α levels variation according to group allocation. Median values are represented. Abbreviations: PO: post-operative; RIPC: remote ischaemic preconditioning; TNF-α: tumour necrosis factor α.

Table 6.10 presents descriptive information according to group allocation for each time point.

Variable	Intervention	N	Median – IQ range	P value
Baseline	RIPC	16	8.39 (7.81-106.03)	0.110
	Control	14	42.83 (10.00-1022.62)	
PO1	RIPC	24	75.64 (18.17-331.96)	0.084
	Control	21	355.22 (58.77-600.04)	
PO2	RIPC	20	15.42 (7.81-114.86)	0.020
	Control	21	108.98 (42.28-301.19)	
PO3	RIPC	15	19.23 (7.81-56.11)	0.148
	Control	15	48.18 (20.24-195.32)	

Table 6.10. Descriptive statistics for plasma TNF-α values according to group allocation for each time point. Data is expressed in median and IQ range. Abbreviations: IQ: interquartile; PO: post-operative; RIPC: remote ischaemic preconditioning; TNF-α: tumour necrosis factor α.

TNF-α levels tended to be higher in the control group at all time points, reaching statistical significance at PO2 (*Figure 6.21*).

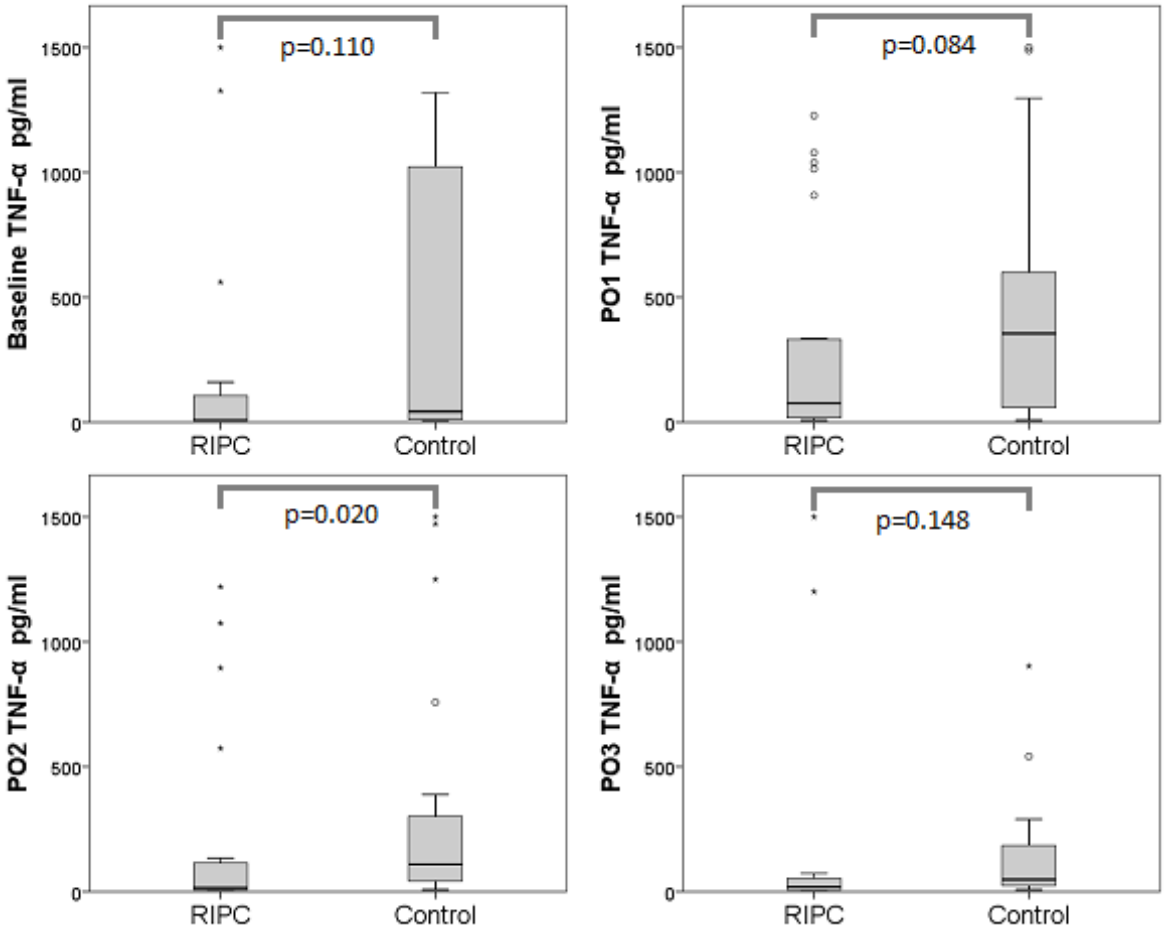

Figure 6.21. Plasma TNF-α levels according to intervention group allocation for each time point. Abbreviations: PO: post-operative; RIPC: remote ischaemic preconditioning; TNF-α: tumour necrosis factor α.

6.5.11 ICAM-1 – RIPC

ICAM-1 levels according to group allocation are shown in *Figure 6.22*.

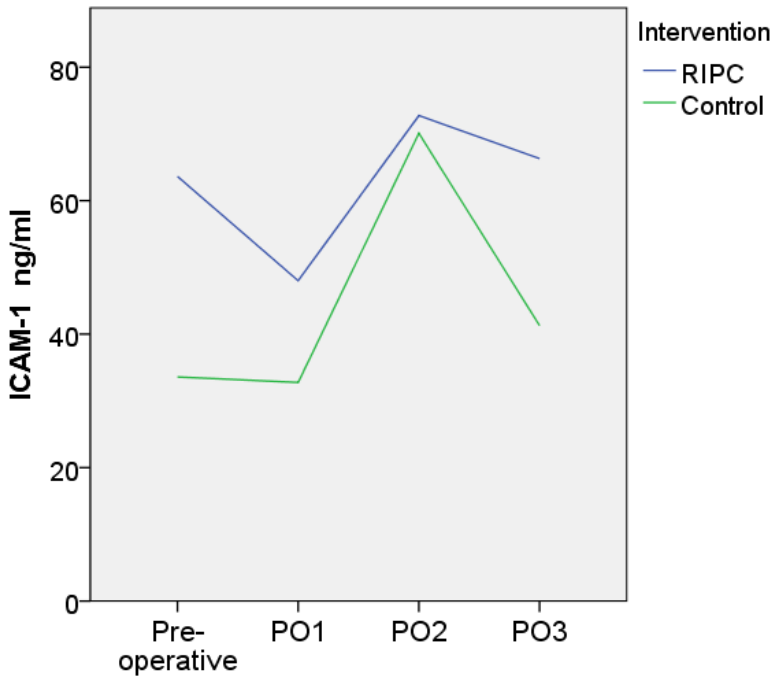

Figure 6.22. Plasma ICAM-1 levels variation for time points according to group allocation. Median values are represented. Abbreviations: ICAM-1: intercellular adhesion molecule 1; PO: post-operative; RIPC: remote ischaemic preconditioning.

Table 6.11 shows descriptive statistics for ICAM-1 levels according to group allocation for each time point.

Variable	Intervention	N	Median – IQ range	P value
Baseline	RIPC	22	63.63 (22.48-141.78)	0.433
	Control	19	33.58 (16.40-149.20)	
PO1	RIPC	25	48.01 (18.00-101.95)	0.861
	Control	23	32.75 (15.93-93.01)	
PO2	RIPC	24	72.75 (21.14-130.32)	0.820
	Control	21	70.10 (20.56-112.17)	
PO3	RIPC	15	66.31 (11.93-171.74)	0.766
	Control	17	41.28 (18.96-120.66)	

Table 6.11. Descriptive statistics for plasma ICAM-1 values according to group allocation for each time point. Data is expressed in median and IQ range. Abbreviations: ICAM-1: intercellular adhesion molecule 1; IQ: interquartile; PO: post-operative; RIPC: remote ischaemic preconditioning.

Variations in ICAM-1 measurements were not significant for any time points (*Figure 6.23*).

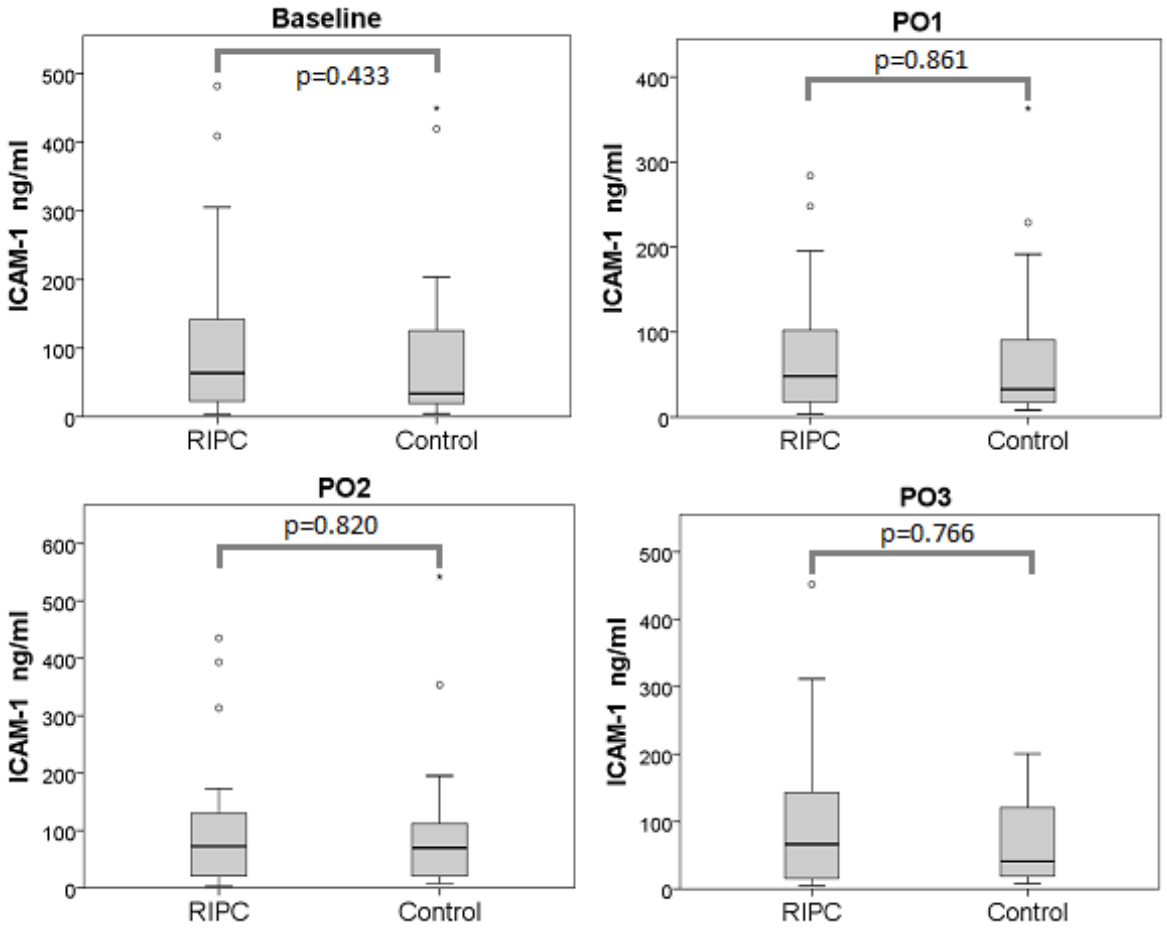

Figure 6.23. Plasma ICAM-1 levels according to group allocation for each time point. Abbreviations: ICAM-1: intercellular adhesion molecule 1; PO: post-operative; RIPC: remote ischaemic preconditioning.

6.5.12 VCAM-1 – RIPC

VCAM-I levels followed a similar pattern in RIPC and control groups. *Figure 6.24* presents the variation of VCAM-I according to time point and group allocation.

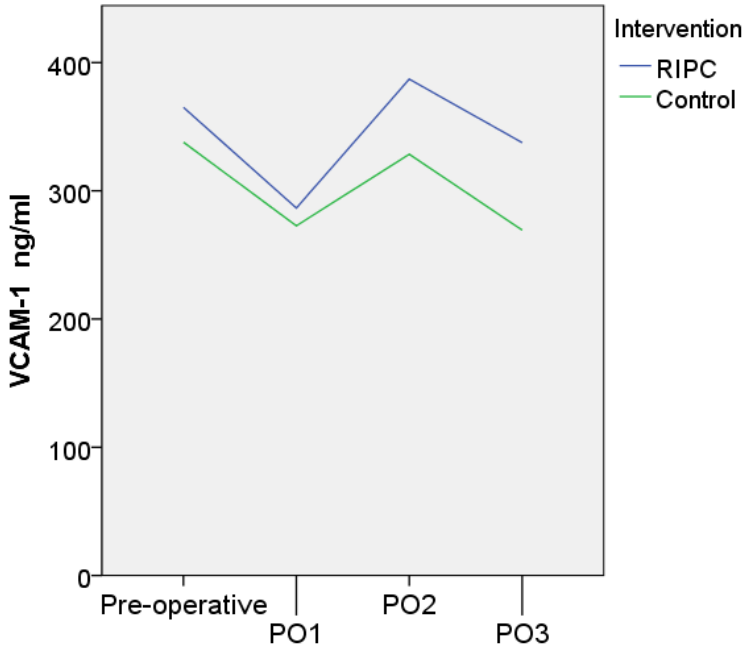

Figure 6.24. Plasma VCAM-1 levels variation for time points according to group allocation. Mean values are represented. Abbreviations: VCAM-1: vascular cell adhesion molecule 1; PO: post-operative; RIPC: remote ischaemic preconditioning.

Table 6.12 presents descriptive information on VCAM-1 values according to group allocation for each time point.

Variable	Intervention	N	Mean – SD	Median – IQ range	P value
Baseline	RIPC	22		344.53 (235.08-473.12)	0.794
	Control	19		337.74 (247.74-430.46)	
PO1	RIPC	25	286.58 ± 137.09		0.717
	Control	23	272.69 ± 125.70		
PO2	RIPC	24	387.11 ± 195.19		0.259
	Control	21	328.55 ± 139.37		
PO3	RIPC	15	337.54 ± 196.15		0.294
	Control	17	269.31 ± 165.61		

Table 6.12. Descriptive statistics for plasma VCAM-1 values according to group allocation for each time point. Data is expressed in median and IQ range. Abbreviations: VCAM-1: vascular cell adhesion molecule 1; IQ: interquartile; PO: post-operative; RIPC: remote ischaemic preconditioning.

There was no significant variation of VCAM-1 according to RIPC of control allocation for any time point (*Figure 6.25*).

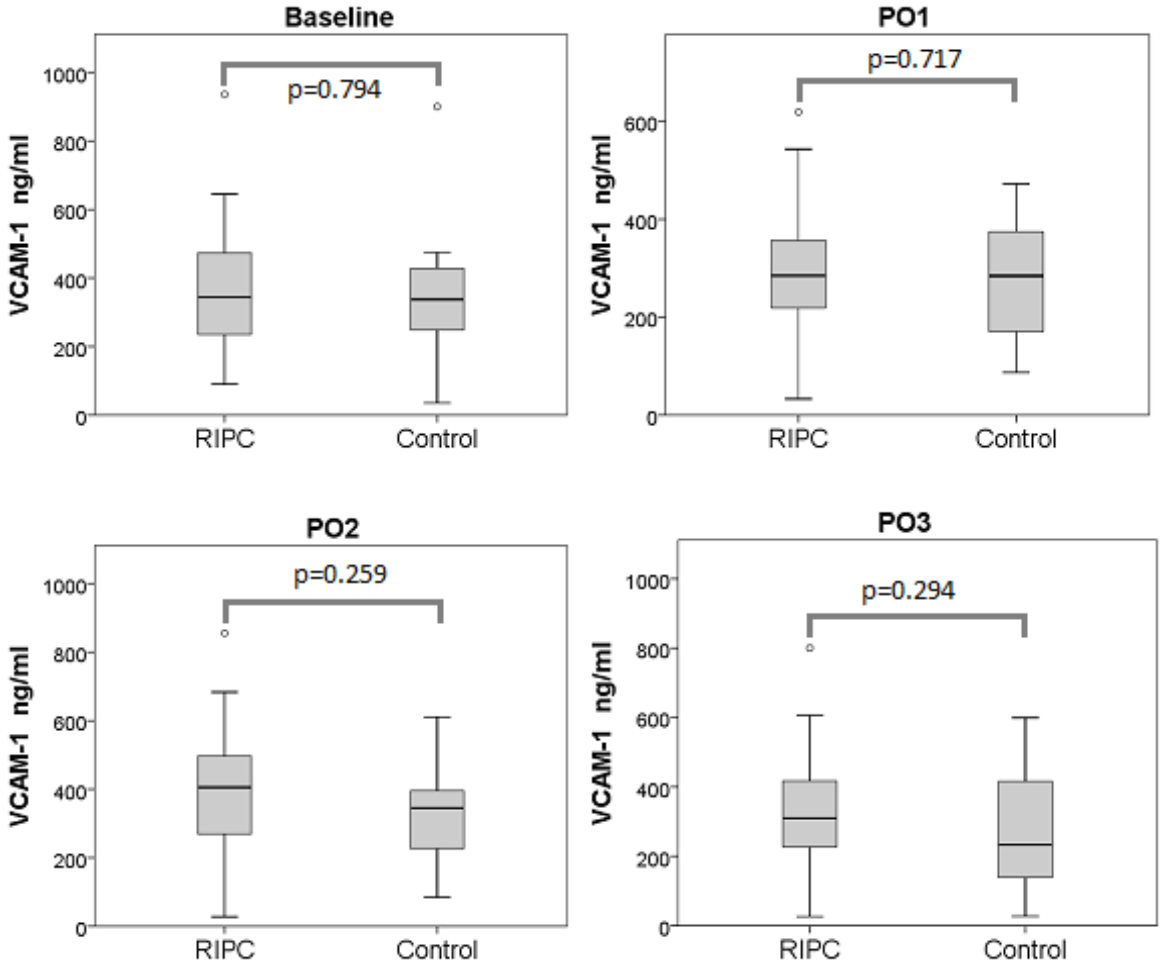

Figure 6.25. Plasma VCAM-1 levels according to group allocation for each time point. Abbreviations: VCAM-1: vascular cell adhesion molecule 1; PO: post-operative; RIPC: remote ischaemic preconditioning.

6.6 Summary of key findings

- Variation between time points was significant for all markers except for TNF-α and ICAM-1.
- Age had a negative correlation with IL-6 and IL-8 while there was a positive correlation to VCAM-1.
- Weight correlated negatively to IL-8 and positively to VCAM-1.
- CPB duration presented a positive correlation to IL-6, IL-8.
- Aortic clamp duration presented a positive correlation to IL-8 and ICAM-1.
- cTnI levels correlated to IL-8 and ICAM-1.
- TNF-α levels were significantly lower in the RIPC group at PO2, values for all other markers were not significantly different between RIPC and controls at any time point.

6.7 Limitations

Plasma sample volumes were limited and there was not enough to complete analysis of all markers at every time point for all patients causing a reduced sample size in some cases.

6.8 Discussion

Inflammatory responses are activated during cardiac surgery by several triggers including the trauma inflicted, with the main source of inflammation being the CPB circuit.

Cytokines such as IL-6, IL-8, IL-10 and TNF-α peak during the early post-operative period while they mediate further responses (reviewed by Warren *et al*, 2009). The contribution of IL-6, IL-8 and TNF-α to IRI has been established with increased activity linked to injury, with protective effects achieved through their inhibition (Boyle *et al*, 1998; Kawamura *et al*, 1993; Xing *et al*, 2016).

In this study the anti-inflammatory cytokine IL-10 showed a similar activity to its pro-inflammatory counterparts. Levels of these cytokines peaked during the immediate post-operative period, dropped considerably by the second post-operative measure on the morning after surgery, and were close to baseline levels by the second post-operative day.

ICAM-1 and VCAM-1 presented a different pattern, there were measurable values before surgery which dropped during the immediate post-operative period to rise again at the first post-operative day and drop below baseline values by the second post-operative day.

Activity of the adhesion molecules ICAM-1 and VCAM-1 after adult cardiac procedures using CPB was increased after surgery, with ICAM-1 regressing to baseline levels one day after surgery while VCAM-1 remained raised (Boldt *et al*, 1998). Paediatric experience however reported a different pattern with both molecules presenting higher pre-operative values than adult patients, which dropped after CPB, remaining below pre-operative levels (Boldt *et al*, 1995).

ICAM-1 and VCAM-1 are released after endothelial activation and higher pre-operative levels could be due to the underlying cardiac pathology, left to right shunts with pulmonary hypertension and reduced pulmonary circulation in cyanotic patients have been linked to higher levels of ICAM-1 and VCAM-1 before surgery (Yildirim *et al*, 2013).

IL-6 and IL-8 were increased in younger patients, while VCAM-1 values were higher in older patients, this supports an age based difference. A weight component was also present with a negative correlation to IL-8 and a positive correlation to VCAM-1.

IL-8 presented a correlation to aortic cross clamp duration and to cTnI levels, IL-8 had previously been found to correlate to troponin values after CABG surgery (Wan *et al*, 1999), while infarct size reduction was achieved after blocking its activity in an IRI model (Boyle *et al*, 1998), suggesting a link of IL-8 activity to the extent of injury in the myocardium.

Although the immediate post-operative measure of ICAM-1 was its lowest, it correlated to aortic clamp duration and cTnI as well, these correlations were very weak.

Evidence on inflammatory mediators in relation to RIPC has produced mixed results. A RIPC paediatric trial found no difference for IL-6 and IL-8 concentration, while TNF-α was reduced and IL-10 presented higher levels in RIPC patients (Cheung *et al*, 2006).

A second study found higher levels of IL-6, IL-8, IL-10 and TNF-α in RIPC patients at baseline, values became lower in the RIPC group compared to controls after surgery for IL-6, IL-8 and TNF- α , while IL-10 levels where higher in the RIPC group (Zhou *et al*, 2010).

Finally a study analysing IL-8 and IL-10 concentrations, found both cytokines peaked 4 hours after CPB to then decrease levels, there was no difference between RIPC and control groups (Pavione *et al*, 2012).

An adult study found no difference in cytokine levels according to group allocation for IL-6, IL-8, IL-10 and TNF-α, finding below detection levels for IL-10 and TNF-α (Karuppasamy *et al*, 2011). Higher concentration of IL-8 and TNF- α were reported in a second study for the RIPC group before CPB, there was an increase in the cytokines after bypass, however no difference was detected between groups (Albretch *et al*, 2013).

Cellular adhesion molecules have not been investigated in link to RIPC human studies before, evidence of ICAM-1 reduction was found in a rat model of renal IRI after the application of RIPC in relation to renal function improvement (Hussein *et al*, 2016).

Our analysis resulted in higher TNF-α values in the control group for one time point only (PO2). Measurements for all other mediators resulted in non-significant variations. There was however a tendency for notably higher postoperative measurements in the control group for IL-6, IL-8, IL-10 and TNF- α, suggesting a possible effect from the intervention that did not reach significance.

Conclusion

Although mediators such as cytokines and cell adhesion molecules explored in this study are involved in the pathophysiology of IRI and previous evidence suggests their participation during RIPC, their involvement was not evident according to the results of the present investigation as only TNF- α presented higher significant levels at one time point on the control group while every other marker analysed did not vary significantly.

Chapter 7. Cystatin C

7.1 Introduction

The inflammatory systemic effects and oxidative stress occurring as a consequence of cardiac surgery and CPB result in acute kidney injury (AKI) in up to 40% of patients going through cardiac surgical procedures (reviewed by Mao *et al*, 2013). During the CPB process the kidneys are not rendered ischaemic, however there is reduced perfusion which makes them vulnerable specially in case of high metabolic rate or poor oxygen delivery; kidney function can be particularly affected in complex procedures requiring long CPB times (Mehta *et al*, 2006; Lassnigg *et al*, 2008).

It has been established that stroke, early and long term mortality are associated to AKI occurrence after CPB which highlights the importance of attenuating the side effects of the procedure (Pickering *et al*, 2015). Paediatric patients exposed to CPB present a vulnerability that increases at very young ages and is correlated with bypass duration, the rate of kidney injury has lately been found to be higher than previously reported in this group, increasing mechanical ventilation time and hospital length of stay (Li *et al*, 2011; Aydin *et al*, 2012).

Early detection of AKI is crucial in order for management to be effective while the injury is reversible. Creatinine is used in the clinical setting as a marker of renal function constituting the main indicator for the identification of AKI with even small changes in creatinine levels having an impact in the outcome of cardiac surgery patients. In the downside, creatinine sensitivity is low, its response to renal injury is not prompt enough and can be affected by non-renal factors. In spite of this it continues to be the main marker for renal function due to its availability (reviewed by Najafi 2014). CPB implies further challenges as haemodilution makes creatinine values harder to detect, making the calculation of a reduction ratio a valid option (Takaki *et al*, 2015).

These circumstances have made it necessary to identify more precise, early stage markers and cystatin C has been identified as a candidate. Cystatin C is a cysteine protease inhibitor produced in all nucleated cells which goes through glomerular filtration and not tubular filtration as creatinine, it does not participate in protein binding and is not affected by factors such as gender,

age or muscle mass (reviewed by Laterza *et al*, 2002; Kiessling *et al*, 2014). Nevertheless discerning reports on the influence of gender, age and race have been published (Köttgen *et al*, 2008).

Cystatin C has been found to be an accurate predictor of renal function and glomerular filtration rate during the perioperative period of patients going through cardiac surgery (Kiessling *et al*, 2014). In children an early prediction of AKI after cardiac surgery was possible through cystatin C measurement, providing a better estimate of glomerular filtration rate than creatinine with a link to bypass parameters, cardiac injury and oxygen delivery, presenting a better association to other biomarkers (Vassalos *et al*, 2011; Hassinger *et al*, 2012; Zappitelli *et al*, 2015).

RIPC in renal function

In addition to cardiac protection it has been hypothesised that RIPC could reduce the detrimental renal effects of CPB. Conflicting reports have been produced with evidence of a definite reduction of AKI occurrence linked to RIPC in adult patients going through elective cardiac surgery (Zimmerman *et al*, 2011) and no benefit in patients going through complex valvular surgery (Choi *et al*, 2011).

A meta-analysis of 10 clinical trials found a lower incidence of AKI after RIPC by a fixed effect model and no difference by the random effects model concluding there was no benefit from RIPC on renal markers, AKI, ICU and hospital stay or mortality after cardiovascular procedures; similar results were seen when only cardiac surgeries were considered, however the analysis highlighted inconsistencies between studies and technical limitations (Li *et al*, 2013).

A later meta-analysis including 13 trials similarly failed to demonstrate a clear benefit from RIPC, further emphasising the need to develop more research in order to produce clear recommendations (Yang *et al*, 2014). Since then a large multicentre trial found that patients with a high risk of renal damage who received RIPC presented a lower rate of AKI and need for renal replacement therapy compared to patients without the intervention (Zarbock *et al*, 2015).

Paediatric trials have found no benefit from RIPC in renal function with one large study including 113 patients going through correction of complex CHD reporting no difference in AKI occurrence, cystatin C or neutrophil gelatinase-associated lipocalin (NGAL) levels

(Pedersen *et al*, 2012). A second study analysing cardiac, renal and neurological effects in cyanotic neonates presented similar results with no difference in NGAL levels after preconditioning (Jones *et al*, 2013).

7.2 Aims

To describe the levels of Cystatin C in paediatric CHD patients going through cardiac surgery using CPB and to assess the effect of RIPC in these levels.

7.3 Materials and Methods

Levels of Cystatin C were measured in plasma samples before surgery (baseline) and three post-operative time points at PICU arrival (PO1), morning after surgery (PO2) and second morning after surgery (PO3). The ELISA DuoSet® assay (DY1196) from R&D Systems was used according to the manufacturer´s instructions in order to quantify Cystatin C in plasma specimens. For details on materials and methods see *Section 2.8*.

7.4 Statistical analysis

Repeated samples Friedman analysis of variance was performed in order to test for differences between time points, post-hoc comparisons between time points were done using Wilcoxon signed rank tests with Bonferroni correction for multiple testing.

Correlation analysis of post-operative measurements to demographic characteristics including weight, age, gender and cyanosis status, as well as to post-operative cTnI and CPB and aortic clamp duration were tested using the Pearson test (r) for normally distributed data or Spearman´s rank correlation test (r_s) for non-parametric data.

Independent samples t-test or Mann-Whitney tests were carried out in order to determine differences between RIPC or control groups for each time point according to data distribution.

Statistical analysis was performed using SPSS Software version 23 at a significance level of 5% (2 tailed).

7.5 Results

Fifty children completed the study, demographic data and its analysis can be seen on *Table 3.1* and *3.2*.

7.5.1 Perioperative analysis

Descriptive statistics for Cystatin C plasma levels according to time point are shown in *Table 7.1*.

Time point	N	Median – IQ range
Baseline	43	1.14 (0.85-1.33)
PO1	50	0.99 (0.66-1.26)
PO2	47	0.95 (0.76-1.36)
PO3	33	1.10 (0.87-1.52)

Table 7.1. Descriptive statistics for plasma Cystatin C levels according to time point. Data expressed in median and IQ range. Abbreviations: PO: post-operative.

Twenty six patients presented complete measures for all time points, variation was significant between time points ($X^2(2)=9.831$, $p=0.020$). Post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were performed resulting in a significance level at $p<0.0083$. The differences were not significant between any of the time points (*Figure 7.1*).

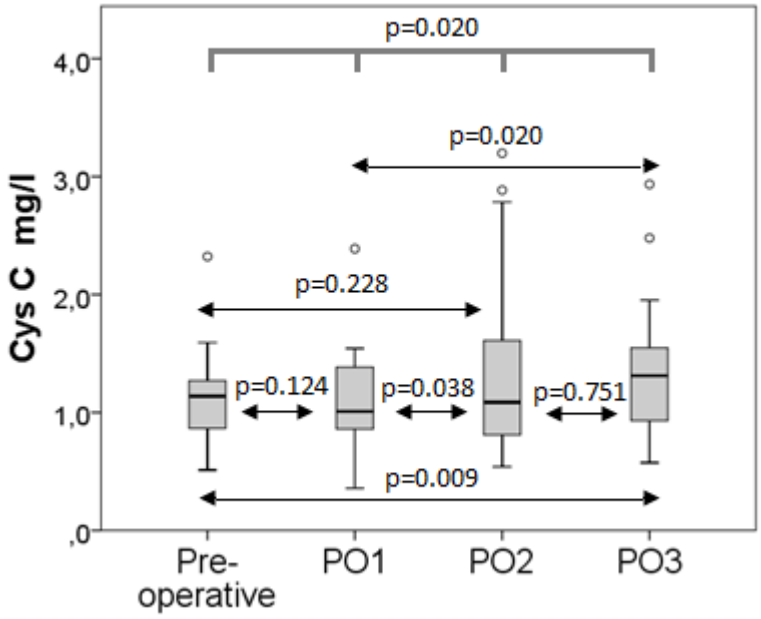

Figure 7.1. Plasma Cystatin C levels according to time point. Repeated measures Friedman test was significant, after post hoc analysis with Bonferroni corrections at a significance level of p<0.0083 differences between pairs were not significant. Abbreviations: Cys C: Cystatin C; PO: post-operative.

Correlations were explored in relation to demographic variables including weight, age, gender and cyanosis status as well as CPB and aortic clamp duration, cTnI and creatinine levels. Weight presented a significant negative correlation to PO2 (r_s= -0.412, p=0.004) and PO3 (r_s= -0.579, p<0.0001); age also correlated to PO2 (r_s= -0.350, p=0.016), and PO3 (r_s= -0.481, p=0.005) (*Figure 7.2*).

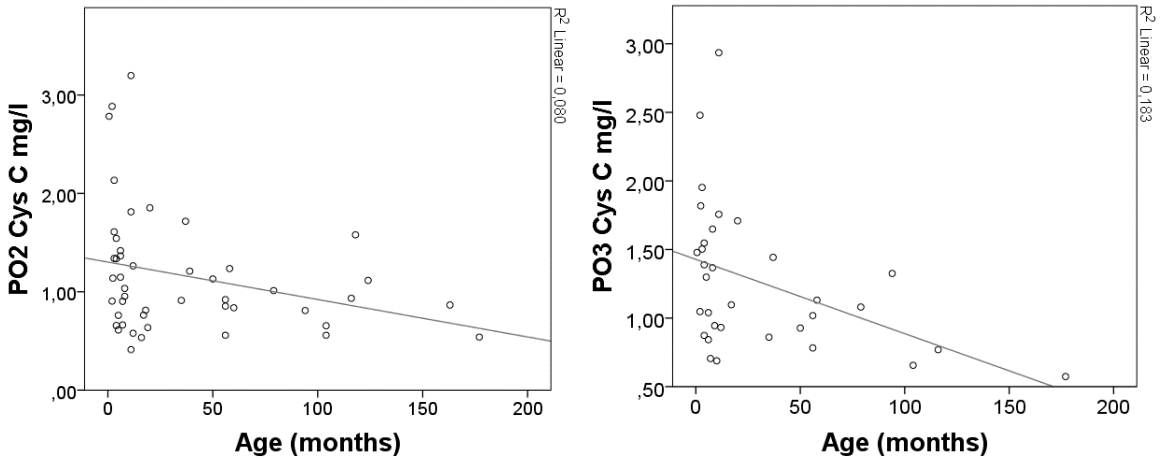

Figure 7.2. Age correlation to PO2 and PO3 Cystatin C levels. Abbreviations: Cys C: Cystatin C; PO: post-operative.

Cyanotic status presented a negative correlation to Cystatin C at PO2 (r_s= -0.420, p=0.003). Variations were statistically significant (p=0.004) (*Figure 7.3*).

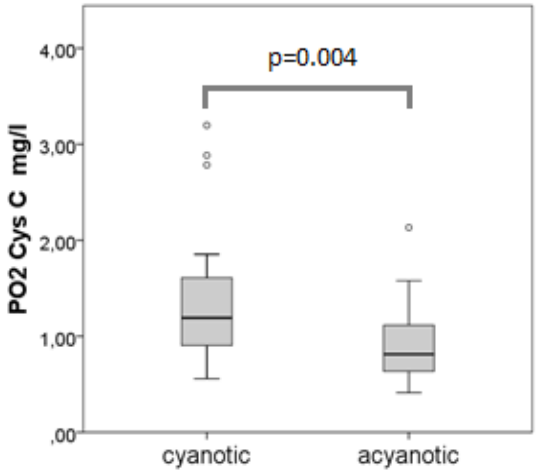

Figure 7.3. PO2 Cystatin C levels according to cyanotic status. Abbreviations: Cys C: Cystatin C; CPB: cardiopulmonary bypass; PO: post-operative.

In addition CPB duration had a positive correlation to Cystatin C at PO1 (r_s=0.407, p=0.003) and PO2 (r_s=0.543, p<0.0001); aortic clamp duration correlated to PO2 Cystatin C (r_s=0.467, p=0.001) (*Figure 7.4*).

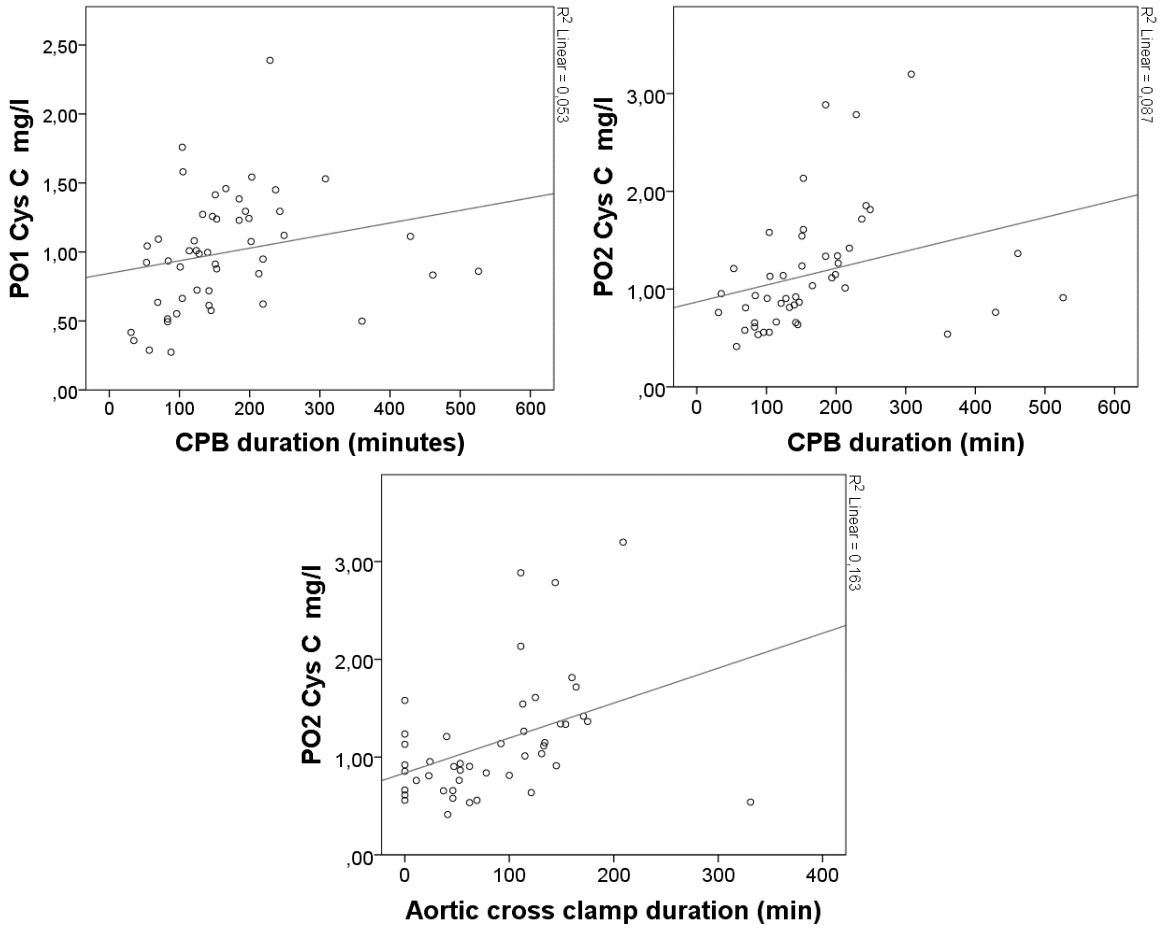

Figure 7.4. Correlation of PO1 and PO2 Cystatin C to CPB duration and PO2 Cystatin C to aortic cross clamp duration. Abbreviations: Cys C: Cystatin C; CPB: cardiopulmonary bypass; PO: post-operative.

There was a positive correlation between Cystatin C values and corresponding cTnI measurements at PO2 (r_s=0.581, p<0.0001) and PO3 (r_s=0.545, p<0.0001) (*Figure 7.5*).

The correlation of Cystatin C to creatinine at post-operative time points was explored, finding that they presented a strong correlation at all corresponding post-operative time points (PO1: r_s=0.420, p=0.003; PO2: r_s=0.535, p<0.0001; PO3: r_s=0.672, p<0.0001) (*Figure 7.6*).

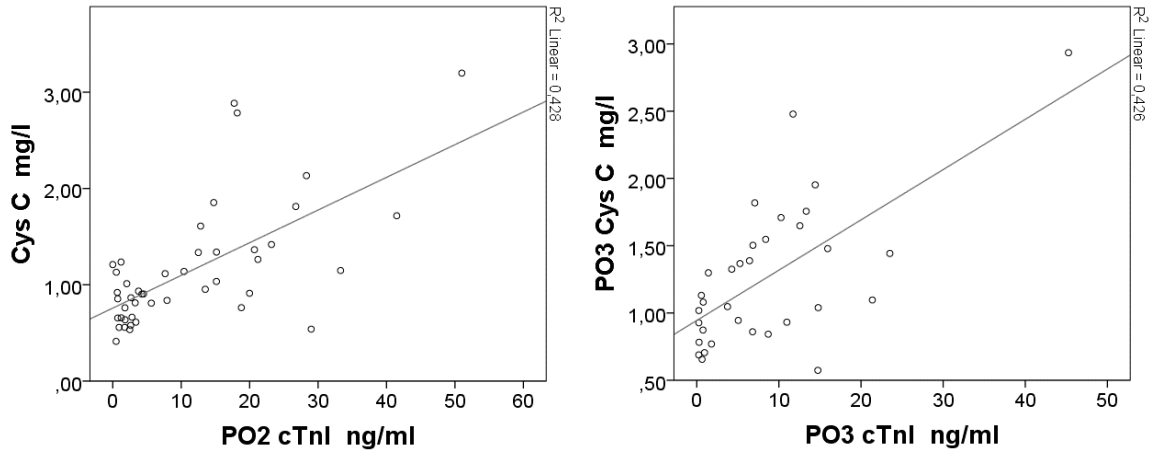

Figure 7.5. Correlation of Cystatin C to cTnI values at PO2 and PO3 time points. Abbreviations: cTnI: cardiac troponin I; Cys C: Cystatin C; PO:post-operative.

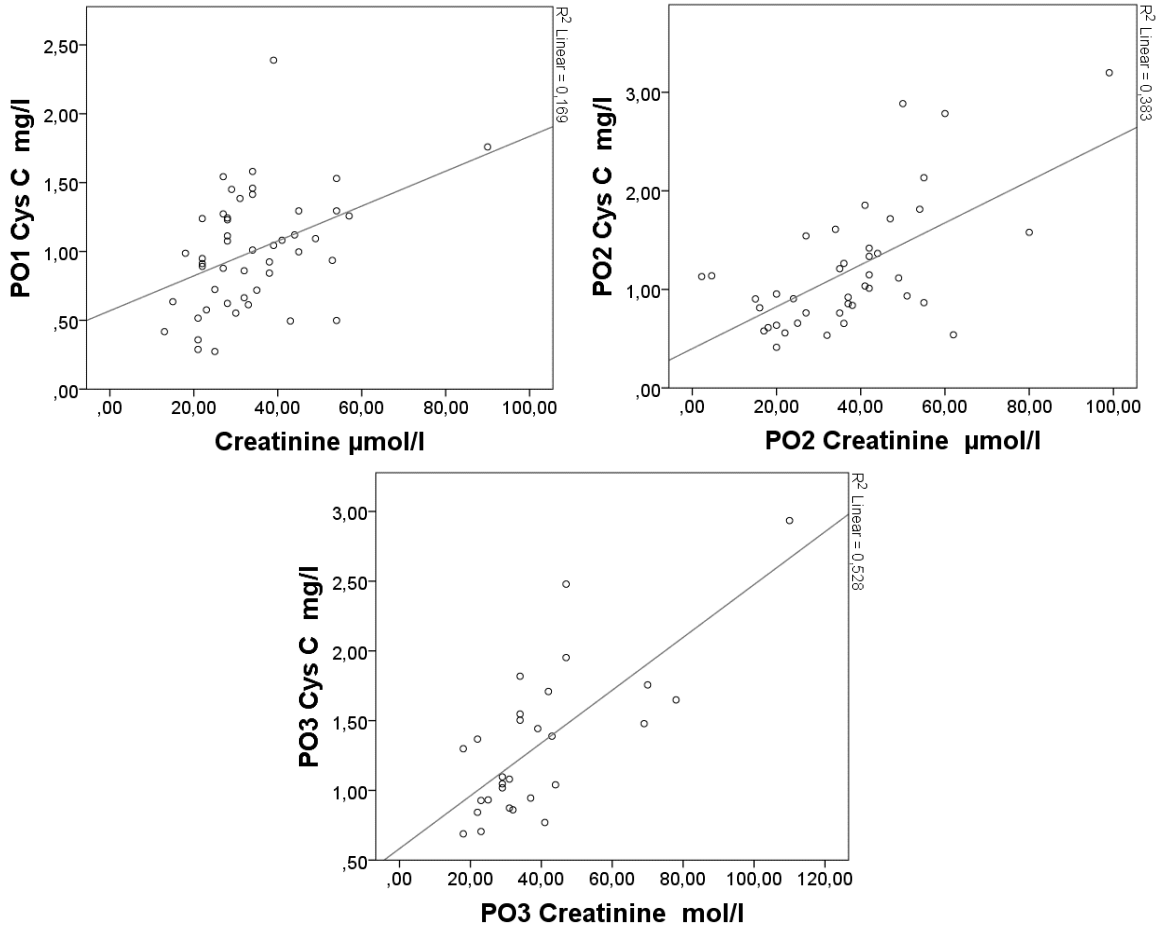

Figure 7.6. Correlation of Cystatin C to creatinine values at PO1, PO2 and PO3 time points. Abbreviations: Cys C: Cystatin C; PO: post-operative.

7.5.2 RIPC analysis

Two cases were excluded from analysis for RIPC as parents asked for the intervention to be stopped before its completion due to discomfort.

Cystatic C values according to RIPC and control group allocation are shown in *Figure 7.7*.

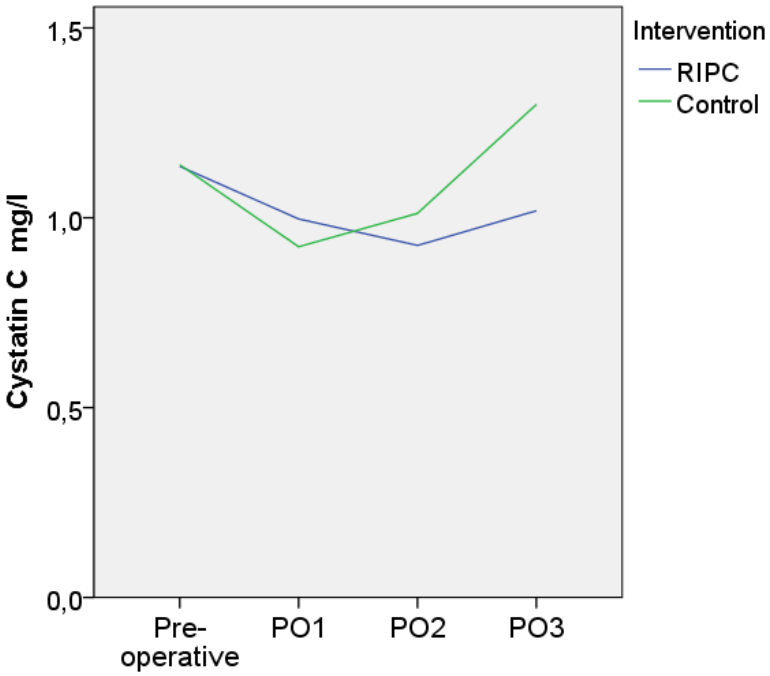

Figure 7.7. Plasma Cystatin C levels variation for time points according to group allocation. Values expressed in medians. Abbreviations: VCAM-1: vascular cell adhesion molecule 1; PO: post-operative; RIPC: remote ischaemic preconditioning.

Descriptive data according to group allocation for each time point is summarised in *Table 7.2*.

Variable	Intervention	N	Mean – SD	Median – IQ range	P value
Baseline	RIPC	22	1.06 ± 0.32		0.581
	Control	19	1.13 ± 0.45		
PO1	RIPC	25	1.03 ± 0.45		0.750
	Control	23	0.99 ± 0.36		
PO2	RIPC	24		0.93 (0.71-1.30)	0.699
	Control	21		1.01 (0.81-1.36)	
PO3	RIPC	15		1.02 (0.86-2.48)	0.551
	Control	17		1.30 (0.95-1.50)	

Table 7.2. Descriptive statistics for plasma Cystatin C values according to group allocation for each time point. Data is expressed in mean – SD or median – IQ range as appropriate. Abbreviations: IQ: interquartile; PO: post-operative; RIPC: remote ischaemic preconditioning, SD: standard deviation.

There was no significant difference in Cystatin C values between RIPC and control groups at any time point (*Figure 7.8*).

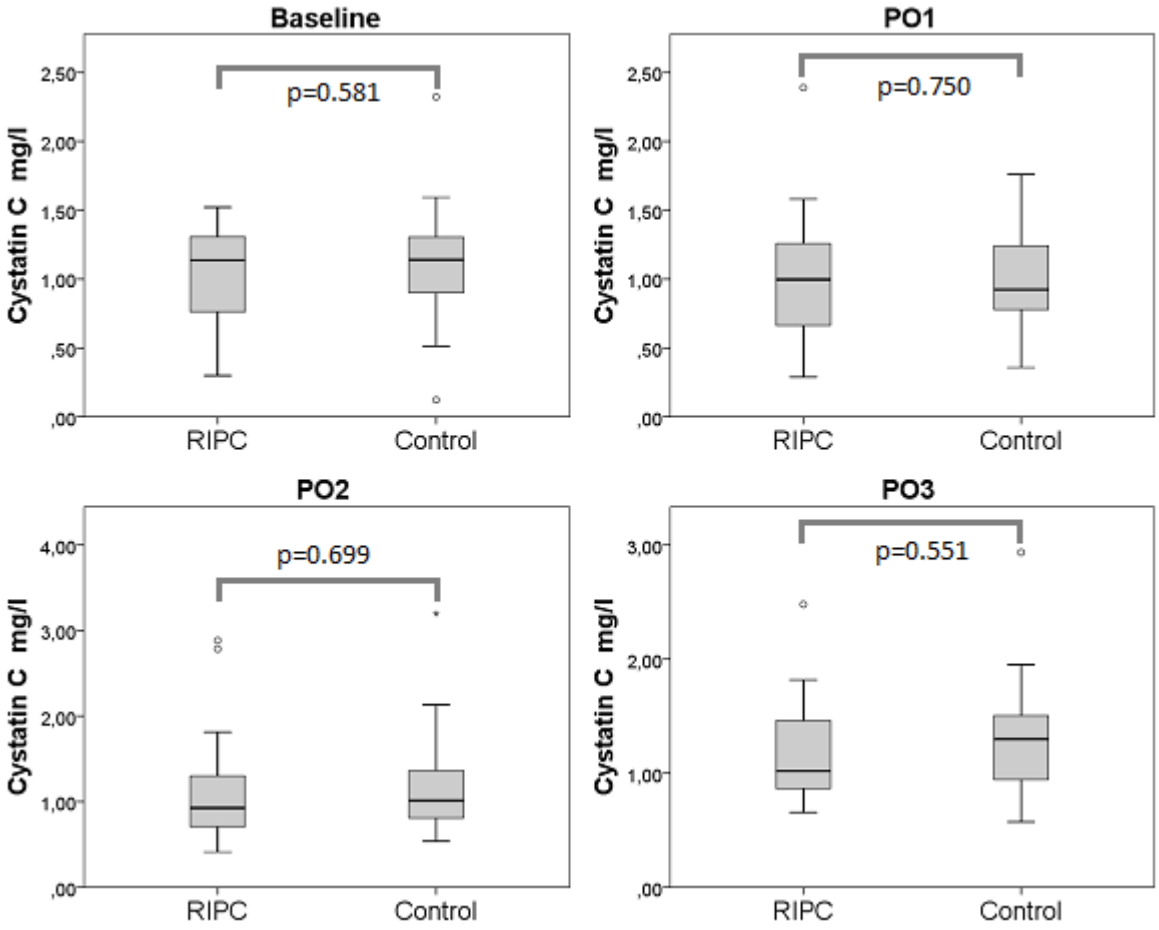

Figure 7.8. Plasma Cystatin C levels according to group allocation for each time point. Abbreviations: PO: post-operative; RIPC: remote ischaemic preconditioning.

7.6 Summary of key findings

- Cystatin C levels were higher in cyanotic patients.
- Age and weight correlated negatively to Cystatin C at PO2 and PO3 time points.
- CPB duration correlated to Cystatin C at PO2 and PO3 time points.
- Aortic clamp duration correlated to Cystatin C at PO2.
- cTnI values correlated to Cystatin C at corresponding PO2 and PO3 time points.
- Creatinine levels correlated to Cystatin C at all post-operative time points.
- There was no significant difference according to RIPC or control allocation for any time point.

7.7 Discussion

Renal impairment is a common complication of major surgeries such as cardiac interventions and children going through cardiac surgery using CPB are at risk (Skippen and Krahn, 2005). Cystatin C has been identified as an indicator of renal function with the ability to reflect glomerular filtration and predict AKI at early stages (Zappitelli *et al*, 2015).

In our study, Cystatin C levels decreased immediately after surgery to then increase and peak by the second day after surgery. These differences between time points were significantly different during initial repeated measures analysis however they were not significant between pairs after post hoc analysis with appropriate corrections.

Our results showed a correlation of Cystatin C levels to weight and age, with values being lower in older children. Cystatin C patterns according to age seem to be different in adults with the marker increasing with age (Köttgen *et al*, 2008); while in adolescents from 12 to 19 years of age Cystatin C decreased as they grew older (Groesbeck *et al*, 2008). This contradicts claims that age does not affect Cystatin C levels.

Cystatin C was correlated to creatinine values, this correlation became stronger as Cystatin C increased at post-operative time points. This link is to be expected as Cystatin C has been established as a reliable marker of renal function, particularly after cardiac surgery (Zhu *et al*, 2006; Kiessling *et al*, 2014).

In the present study Cystatin C did not only correlate to creatinine but to CPB and aortic clamp duration, as well as to cTnI values, which suggests the processes taking place affect kidneys and myocardium in a similar way. Similar associations were described before after paediatric surgery, defining ischaemia and myocardial injury as independent predictors of Cystatin C levels (Vassalos *et al*, 2011).

RIPC has been widely investigated in relation to renal IRI with a recent large multicentre trial reporting a lower rate of AKI and requirement of renal replacement therapy in patients at risk of renal damage after RIPC (Zarbock *et al*, 2015), previous meta-analysis however did not find significant effects on kidney function (Li *et al*, 2013; Yang *et al*, 2014).

Experience in paediatric patients has not produced positive results with two studies finding no difference in AKI occurrence or in the levels of renal markers (Pedersen *et al*, 2012; Jones *et al*, 2013).

In the present study Cystatin C levels tended to be higher in control patients compared to RIPC two days after surgery, however this was not statistically significant. Two RIPC studies, including a paediatric trial have looked at Cystatin C activity finding no benefit from the intervention (Choi *et al*, 2011; Pedersen *et al*, 2012).

RIPC did not have a significant effect in renal markers explored as part of the clinical surveillance in *Section 3.2.4* including urea, creatinine and electrolyte balance. Cystatin C values obtained corroborate this lack of effect in renal function.

Conclusion

RIPC did not have a significant effect in Cystatin C levels or renal function. Cystatin C presented a link to other renal parameters and to the extent of myocardial injury.

Chapter 8. NO metabolites - cGMP

8.1 Introduction

Respiratory dysfunction is a side effect of cardiac surgery, partially due to surgical factors such as wound pain and pleural effusion, nevertheless CPB is responsible for the majority of detrimental pulmonary effects taking place as IRI is established and inflammatory reactions are triggered leading to interstitial pulmonary oedema and abnormal gas exchange with clinical manifestations ranging from fever and cough to pulmonary injury and respiratory failure requiring prolonged mechanical ventilation and ICU stay (reviewed by Apostolakis *et al*, 2010).

In children presenting with CHD, characteristics of the heart defect can cause a baseline pulmonary pathology including high blood flow and pulmonary hypertension leading to increased pulmonary vascular resistance; after CPB pulmonary endothelial dysfunction occurs, further affecting pulmonary function and having an impact in patient recovery (Schulze-Neick *et al*, 2001).

Nitric Oxide and cyclic guanosine monophosphate

NO is a free radical produced endogenously, it controls vascular tone acting as a potent endothelium relaxing factor, in addition it is a signalling mediator taking part in the regulation of the cardiovascular, nervous and immune systems, it mediates inflammation, inhibits platelets and regulates smooth muscle proliferation (reviewed by Chen *et al*, 2013; Zhao *et al*, 2015).

In the lungs NO is synthesised by eNOS in the endothelium, it regulates blood flow and controls ventilation and perfusion, its activity has been linked to hypoxia, oxidative stress and inflammation (reviewed by Chen *et al*, 2013). NO is oxidised into its major metabolites nitrite and nitrate in a process influenced by particular interactions within organs and body compartments, with circulating nitrite and nitrate representing a constant proportion of the total NO endothelial synthesis (reviewed by Kelm, 1999).

cGMP is a second messenger that interacts with NO as an upstream mediator, it is recognised that this interaction has an important role in the maintenance of vascular tone with alterations in this pathway causing cardiovascular pathologies including systemic and pulmonary hypertension (reviewed by Chen *et al*, 2013).

cGMP is synthesised after enzymatic activity stimulated by NO and natriuretic peptides, it triggers protein kinases and ion channels acting in cardiovascular homeostasis and electrolyte transport (Feil *et al*, 2003). cGMP is hydrolysed and inactivated by phosphodiesterases with phosphodiesterase 5 (PDE-5) predominantly acting in the lung (reviewed by Chen *et al*, 2013).

A low response of eNOS, NO and cGMP during hypoxia results in heightened vascular resistance due to pathological vascular constriction as the pathway fails to produce NO and triggers ROS instead (reviewed by Chen *et al*, 2013). Studies have shown that pulmonary hypertension patients present low levels of NO and lower expression of eNOS (Girgis *et al*, 2005; Malinovschi *et al*, 2011) while PDE-5 has been found to be highly expressed in similar circumstances (Wharton *et al*, 2005).

The NO-cGMP interaction principle has been identified as a potential therapeutic source, inhaled NO is used in clinical practice as therapy for postoperative pulmonary endothelial dysfunction and pulmonary hypertension (reviewed by Checchia *et al*, 2012). In spite of its wide use, no clear recommendations for paediatric patients after cardiac surgery has been made based on the existing evidence as larger clinical trials are required (Bizzarro *et al*, 2014).

PDE-5 inhibitors such as sildenafil have the ability to restore NO-cGMP activity, this has made them the focus of research finding sildenafil to reduce oxidative stress in pulmonary hypertension (Semen *et al*, 2016). Conflicting evidence exists regarding the use of sildenafil before and after cardiac surgery in paediatric patients, finding beneficial results regarding pulmonary hypertension (Palma *et al*, 2011), while further research reported no improvement in pulmonary endothelial function and a negative impact in ventricular function and oxygenation (Vassalos *et al*, 2011).

cGMP activity has been linked to cardioprotection elicited by cardiac conditioning with favourable results in animal studies in which acidosis during reperfusion was delayed (Inserte

et al, 2011); furthermore cGMP has been identified as a mediator for late preconditioning (Kodani *et al*, 2002).

8.2 Aims

To describe the activity of cGMP and NO metabolites before and after CPB and to analyse the effects of RIPC in paediatric patients going through CHD corrective surgery.

8.3 Materials and Methods

Blood samples were obtained from the right atrium, pre-bypass samples were drawn after heparin infusion and post-bypass samples were obtained before protamine administration, both drugs of standard use during the bypass process. Samples were spun in a centrifuge for 10 minutes at 2000 g to obtain plasma which was aliquoted and stored at -70°C.

8.3.1 Nitrc oxide metabolites quantitation

NO metabolites, nitrite and nitrate were quantified using a commercially available colorimetric Nitric oxide assay kit by Abcam® (catalogue number ab65328) following the manufacturer´s instructions.

This assay measures total nitrite/nitrate concentration by initially converting nitrate into nitrite through the action of nitrate reductase; then Griess reagents are used to convert nitrite into a deep purple azo compound which is then measured.

Nitrite and nitrate measurements were done separately for duplicate pre and post-bypass EDTA plasma samples obtained from the right atrium.

Nitrite or nitrate standards were reconstituted into a 100mM solution using 1.1 ml assay buffer, a 1mM solution was then prepared in order to generate a standard curve range of dilutions producing: 0, 2, 4, 6, 8 and 10 mmol/well concentrations.

EDTA plasma samples did not go through any treatment or dilution before the assay; 85 µl of sample or standards were added to each well and blank wells contained 115 µl assay buffer.

5 µl of enzyme cofactor were added to the standard and sample wells and 5 µl nitrate reductase were added to standard and sample wells for nitrate measurement only.

Plates were covered with an adhesive strip and incubated for 1 hour at room temperature, during incubation nitrate converts to nitrite if nitrate reductase was added to the reaction.

5 µl of enhancer were then added to standard and sample wells and plates were incubated for 10 minutes at room temperature. A Griess reaction was then produced by adding 50 µl Griess reagent R1 and R2 to standard and sample wells, where nitrite reacts with sulphanilamide producing a diazonium salt which then reacts with N-(1-napthyl) ethylenediamine producing an azo dye the intensity of which can be measured (reviewed by Coneski and Schoenfisch, 2012).

The optical density (OD) of each well was swiftly measured using a microplate reader and Dynex technologies revelation 4.25 software using a test filter of 550.

The reader generated OD measurements after subtracting blank values which represent the background reading. The best fir for the standard curve in this assay was obtained through linear regression (*Figure 8.1*).

The standard curve used for nitrate quantitation failed on initial analysis, however an adequate curve was produced on a later attempt. Due to limited availability of plasma samples it was not possible to repeat the assay, hence the valid OD measurements obtained from the initial analysis were used in order to generate nitrate concentration values by comparing them to the valid standard curve using linear regression analysis. As nitrate values were required to be comparable to nitrite measurements a similar linear regression model was used for its analysis.

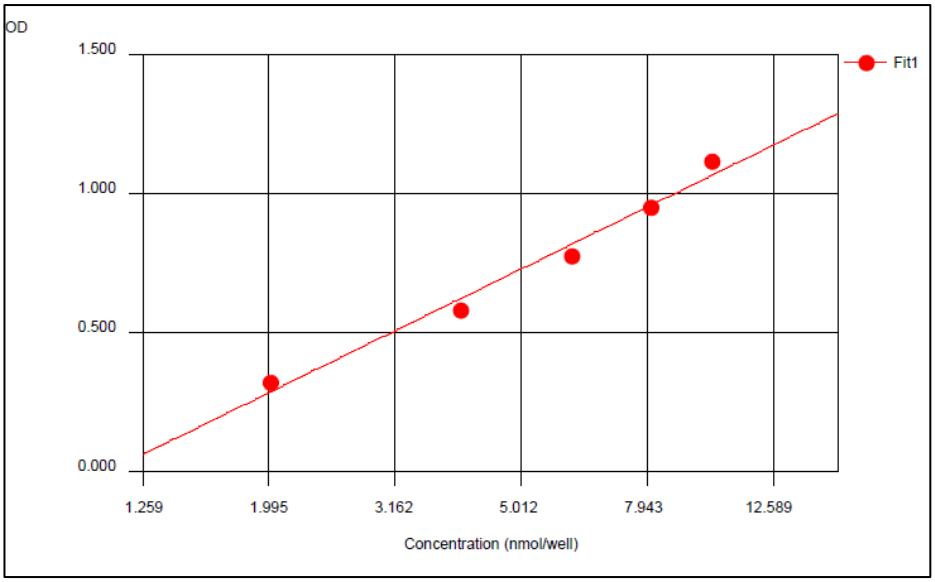

Figure 8.1. Standard curve for nitrite assay. A linear regression equation was used in order to produce the best fit curve for the standards.

All samples were analysed in duplicate, OD values per well were then averaged before being fitted to the appropriate standard curve.

Final concentration was calculated using the formula:

$$nitrate\ or\ nitrite\ concentration = \left(\frac{A}{B}\right) * D$$

Where A= amount of nitrate or nitrite in the sample well (nmol).
B= sample volume added to the well (85 µl)
D= dilution factor (1)

Obtaining a final concentration on nmol/µl.

As nitrate measurements include the nitrate present in the original sample which converted to nitrite plus the nitrite originally present in the sample, final nitrate concentration was obtained after subtracting the nitrite concentration from the overall nitrate measurement.

8.3.2 cGMP quantitation

cGMP was quantified in pre and post-bypass plasma samples obtained from the right atrium using a competitive enzyme immunoassay by R&D Systems (catalogue number KGE003) following the manufacturer´s instructions.

Competitive assays are based on the use of a fixed amount of labelled antigen, in this case cGMP, which will compete for binding sites in a limited amount of a determined antibody with the cGMP present in the sample, hence the proportion of labelled antigen that binds to the antibody is indirectly proportional to the analyte's concentration in the sample (reviewed by Wild, 2013)

150 µl of calibrator diluent were added to the non-specific binding (NSB) wells, and 100 µl were added to the zero standard (B_0) wells of a 96 well microplate pre-coated with a goat anti-rabbit polyclonal antibody.

The standard was reconstituted in deionised water producing a stock solution of 5000 pmol/ml which was diluted in calibrator diluent in order to create a dilution series of 500, 167, 56, 18.5, 6.2 and 2.1 pmol/ml.

Samples were diluted using a 1/20 factor (25 µl sample in 475 µl reagent diluent). 100 µl of diluted samples were added to each well followed by the addition of 50 µl cGMP conjugate producing a slight violet colouration; this fixed amount of horseradish peroxidase conjugated to cGMP competes with the cGMP present in the plasma specimens.

50 µl of primary antibody solution were then added excluding the NSB wells; this rabbit polyclonal antibody provides the binding sites that the conjugate and sample cGMP compete for. Wells with the primary antibody turned to a slight turquoise colour.

Plates were covered with an adhesive strip and incubated for 3 hours on a horizontal orbital microplate shaker at high speed and room temperature. During incubation the polyclonal antibody binds to the goat anti-rabbit antibody coated to the microplate.

The microplates were then washed using a squirt bottle and the buffered surfactant washing buffer provided in the kit for 4 times in order to remove excess conjugate and unbound sample, followed by decanting of wash buffer residue and blotting the plates against clean paper towels.

In order to determine the bound enzyme activity, 200 µl of substrate solution provided in the kit were added to each well. Plates were incubated for 30 minutes away from direct light at room temperature, producing a blue colouration of the wells.

Colour development was then stopped adding 50 µl of stop solution to each well, this changed colouration from blue to yellow.

The optical density (OD) of each well was immediately determined using a microplate reader and Dynex technologies revelation 4.25 software with a test filter of 450 nm and wavelength correction of 550 nm.

The reader generated OD measurements after subtracting blank values which represent background reading. Using a 4 Parameter Logistic regression model the software produces a sigmoid curve with mean OD values for duplicate standard measurements which represent known cGMP concentrations; as it is a competitive assay the intensity of the colour is inversely proportional to cGMP concentration in the sample, hence lower OD values are linked to higher concentrations (*Figure 8.2*).

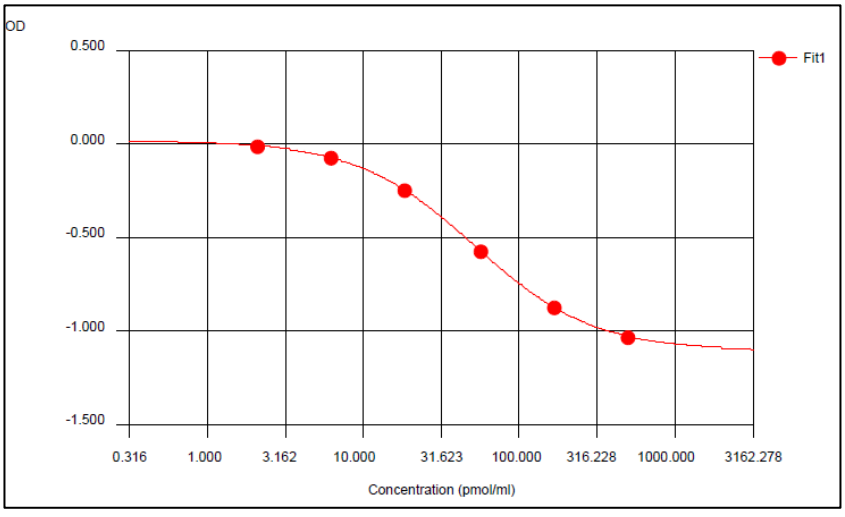

Figure 8.2. cGMP assay standard curve. The competitive basis of the assay produces an inverse sigmoid curve where lower OD measurements represent higher cGMP concentrations.

8.4 Statistical analysis

The signed ranked Wilcoxon test for paired samples was used in order to evaluate differences between time points.

Correlation analysis to demographic characteristics including weight, age, gender and cyanosis status, as well as to post-operative cTnI, CPB and aortic clamp duration, and length of intubation were tested using the Pearson test for normally distributed data while the Spearman correlation test was used for non-parametric data.

Independent samples t-test or Mann Whitney test were used to compare variation according to RIPC or control allocation for each time point according to data distribution.

Statistical analysis was performed using SPSS Software version 23 at a significance level of 5% (2 tailed).

8.5 Results

General analysis

8.5.1 NO metabolites

Descriptive data for nitrite and nitrate levels before and after bypass is presented in *Table 8.1*.

Marker	Time point	N	Descriptive
Nitrate	Pre-bypass	40	14.62(9.70-17.55)
	Post-bypass	40	12.613(8.79-15.94)
Nitrite	Pre-bypass	40	3.62(2.88-5.64)
	Post-bypass	40	8.25±3.77

Table 8.1. Descriptive statistics for plasma nitrate and nitrite levels according to time point. Data expressed in mean – SD or median – IQ range as appropriate. Abbreviations: IQ: interquartile; SD: standard deviation.

Thirty nine patients presented complete measures for both time points, variation was not significant between nitrate time points (p=0.054), while nitrite pre and post-bypass measurements were significantly different (p<0.0001) (*Figure 8.3*).

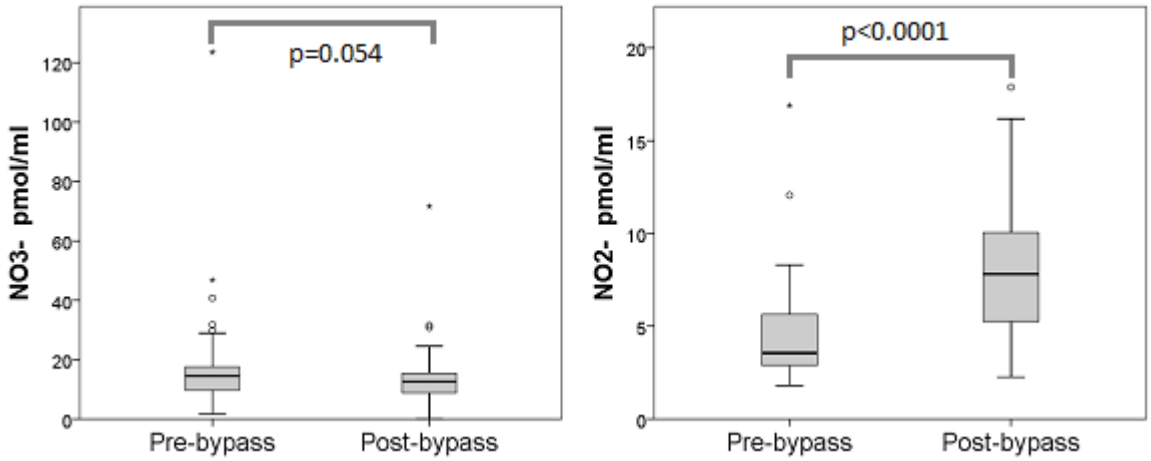

Figure 8.3. Plasma nitrate and nitrite levels according to time point. Abbreviations: NO2-: nitrite; NO3-: nitrate.

Nitrate values before bypass correlated negatively to age (r_s= -0.378, p=0.016) and weight (r_s= -0.466, p=0.002) (*Figure 8.4*).

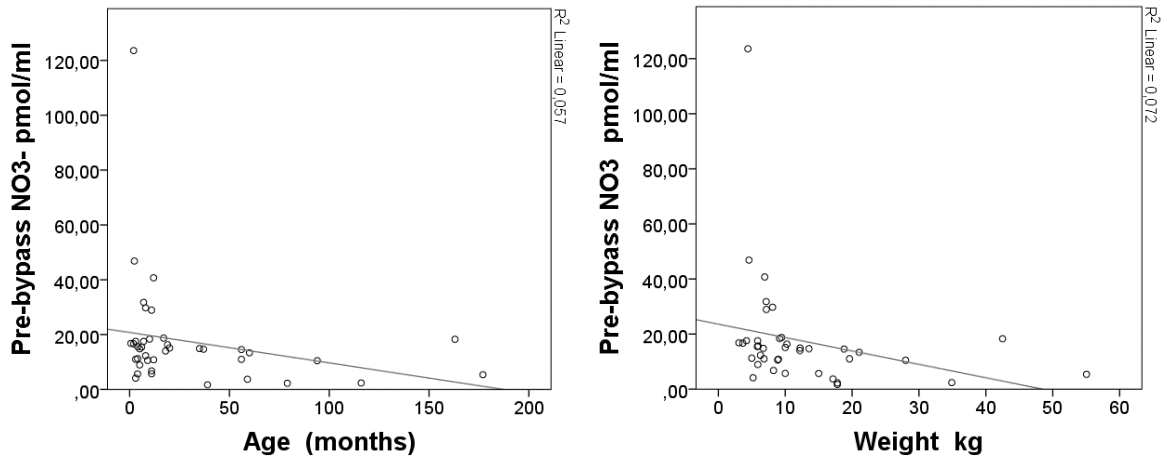

Figure 8.4. Correlation of pre-bypass nitrate levels to age and weight. Abbreviations: NO3-: nitrate.

Nitrite levels after bypass correlated negatively to cyanotic status (r= -0.528, p<0.0001), analysis of means between cyanotic and non-cyanotic status resulted in a significant difference (10.03±3.42 vs 6.08±3.02, p<0.0001) (*Figure 8.5*).

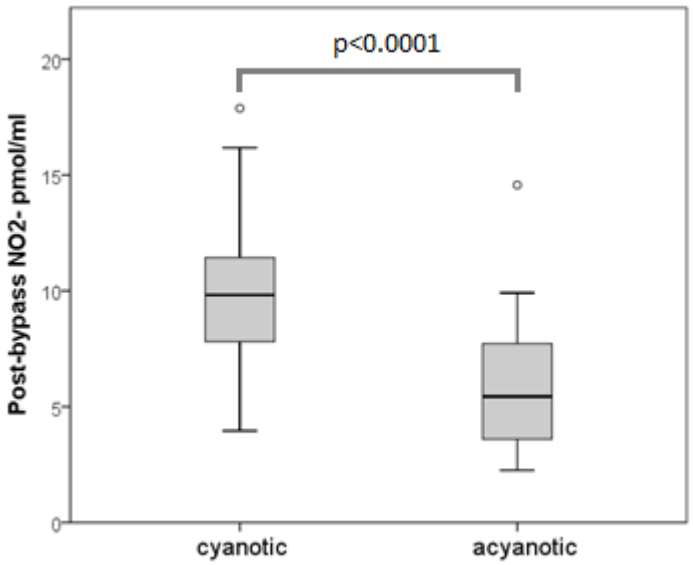

Figure 8.5. Plasma nitrite levels according to cyanosis status after CPB. Abbreviations: NO2-: nitrite; CPB: cardiopulmonary bypass.

In addition post-bypass nitrite levels were positively correlated to CPB duration (r=0.346, p=0.029) and to length of intubation (r_s=0.448, p=0.004) (*Figure 8.6*).

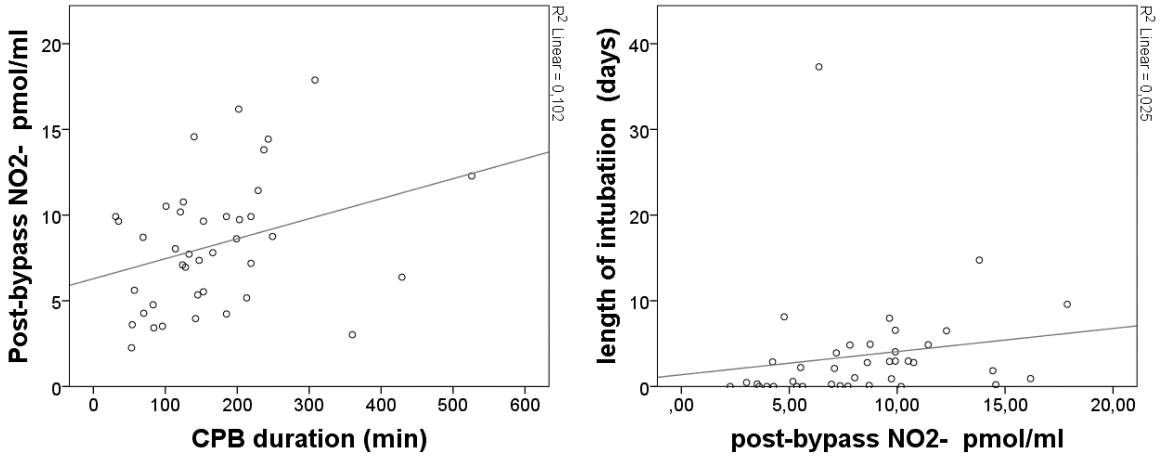

Figure 8.6. Correlation of post-bypass nitrite levels to CPB duration and length of intubation. Abbreviations: NO2-: nitrite; CPB: cardiopulmonary bypass.

Post-bypass nitrite levels also showed a positive correlation to post-operative cTnI measurements (PO1: r_s=0.426, p=0.006) and PO2 (r=0.349, p=0.029) (*Figure 8.7*).

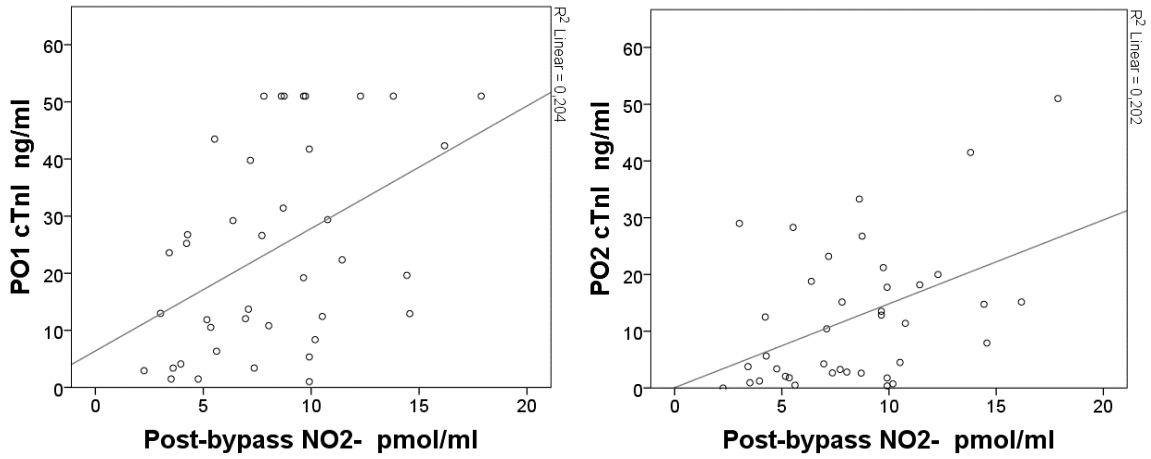

Figure 8.7. Correlation of post-bypass plasma nitrite levels to post-operative cTnI values. Abbreviations: cTnI: cardiac troponin I; NO2-: nitrite.

8.5.2 cGMP

Descriptive statistics for cGMP plasma levels according to time point are shown in *Table 8.2*.

Time point	N	Median – IQ range
Pre-bypass	42	361.34 (327.95-411.70)
Post-bypass	41	341.23 (301.74-372.28)

Table 8.2. Descriptive statistics for plasma cGMP levels according to time point. Data expressed in median and IQ range. Abbreviations: IQ: interquartile.

Forty one patients presented complete measures for both time points, variation was significant between pre and post-bypass measures (p=0.012) (*Figure 8.8*). cGMP levels did not present a significant correlation to any of the analysed variables.

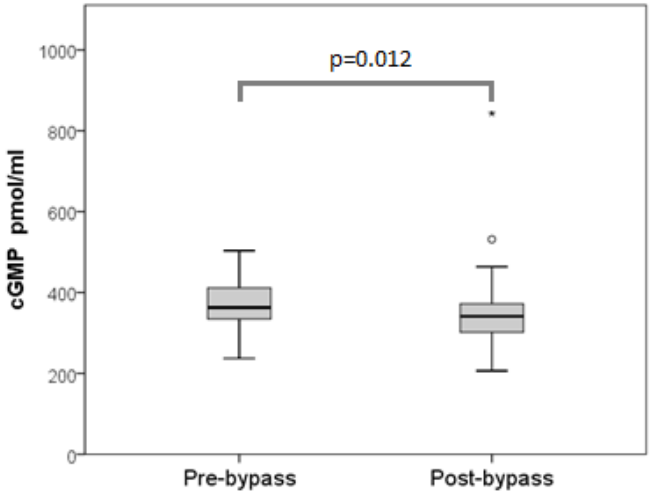

Figure 8.8. Plasma cGMP levels according to time point. Abbreviations: cGMP: cyclic guanosine monophosphate.

RIPC analysis

Two cases were excluded from analysis for RIPC as parents asked for the intervention to be stopped before its completion due to discomfort.

8.5.3 NO metabolites – RIPC

Descriptive data for NO metabolite values according to RIPC and control group for both time points is shown in *Table 8.3*.

Variable	Time point	Group	N	Mean – SD	Median – IQ range	P value
Nitrate	Pre-bypass	RIPC	20		14.04 (7.85-16.75)	0.687
		Control	19		14.56 (10.47-18.72)	
	Post-bypass	RIPC	20		11.81 (5.495-17.004)	0.411
		Control	19		13.60 (9.23-16.42)	
Nitrite	Pre-bypass	RIPC	20		4.92 (3.20-6.11)	0.149
		Control	19		3.29 (2.75-5.26)	
	Post-bypass	RIPC	20	8.47 ± 3.79		0.524
		Control	19	7.70 ± 3.63		

Table 8.3. Descriptive statistics for plasma nitrate and nitrite values according to group allocation for each time point. Data is expressed in mean – SD or median – IQ range as appropriate. Abbreviations: IQ: interquartile; RIPC: remote ischaemic preconditioning; SD: standard deviation.

Nitrate and nitrite levels were not significantly different according to group allocation for any time point (*Figure 8.9*).

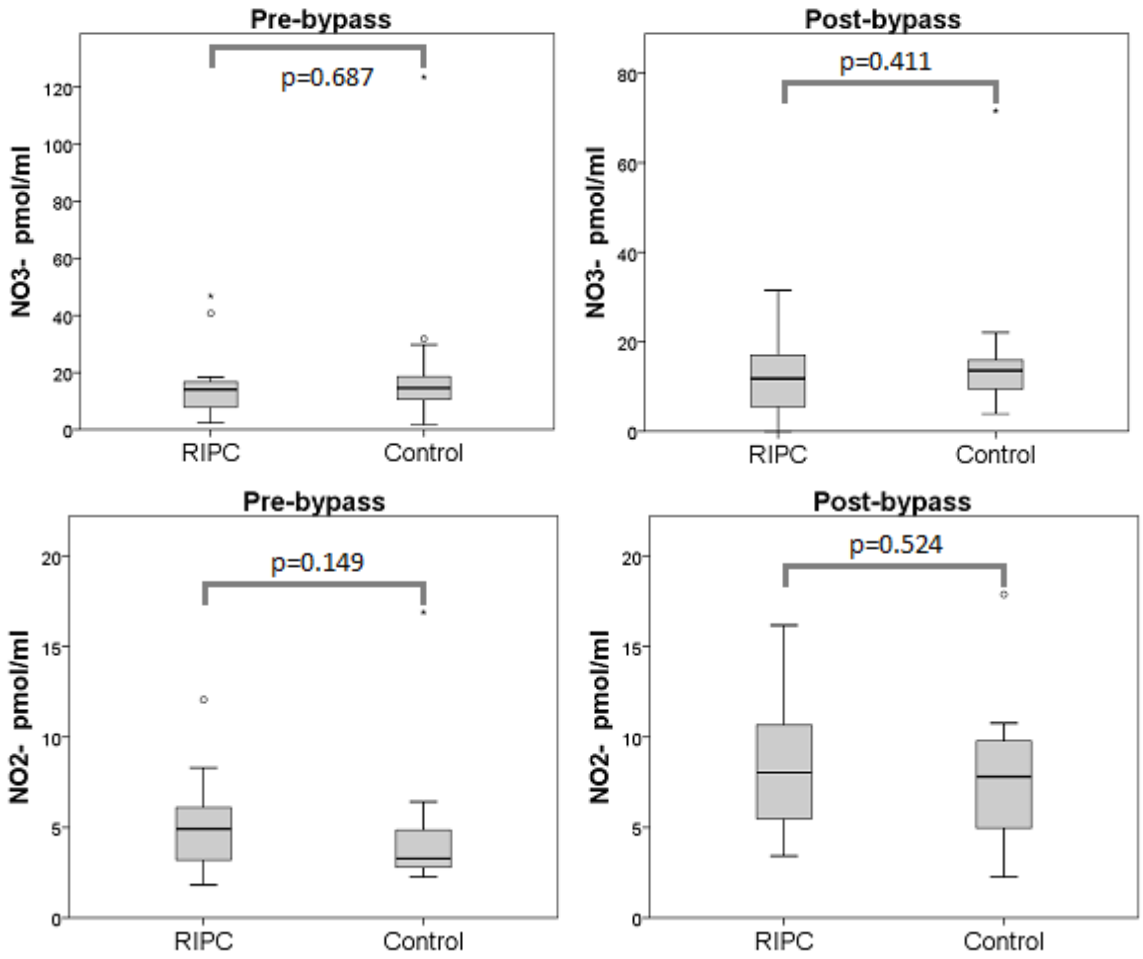

Figure 8.9. Plasma nitrate and nitrite levels according to group allocation for each time point. Abbreviations: NO2-: nitrite; NO3-: nitrate; RIPC: remote ischaemic preconditioning.

8.5.4 cGMP – RIPC

Descriptive information on cGMP according to group allocation for each time point is shown in *Table 8.4*.

Time point	Intervention	N	Mean – SD	Median – IQ range	P value
Pre-bypass	RIPC	21	359.19 ± 50.94		0.500
	Control	20	371.79 ± 66.78		
Post-bypass	RIPC	20		335.01 (301.16-351.57)	0.341
	Control	20		346.12 (303.15-402.98)	

Table 8.4. Descriptive statistics for plasma cGMP values according to group allocation for each time point. Data is expressed in mean – SD or median – IQ range as appropriate. Abbreviations: cGMP: cyclic guanosine monophosphate; IQ: interquartile; RIPC: remote ischaemic preconditioning.

RIPC or control allocation did not result in significant variation of cGMP levels before or after bypass (*Figure 8.10*).

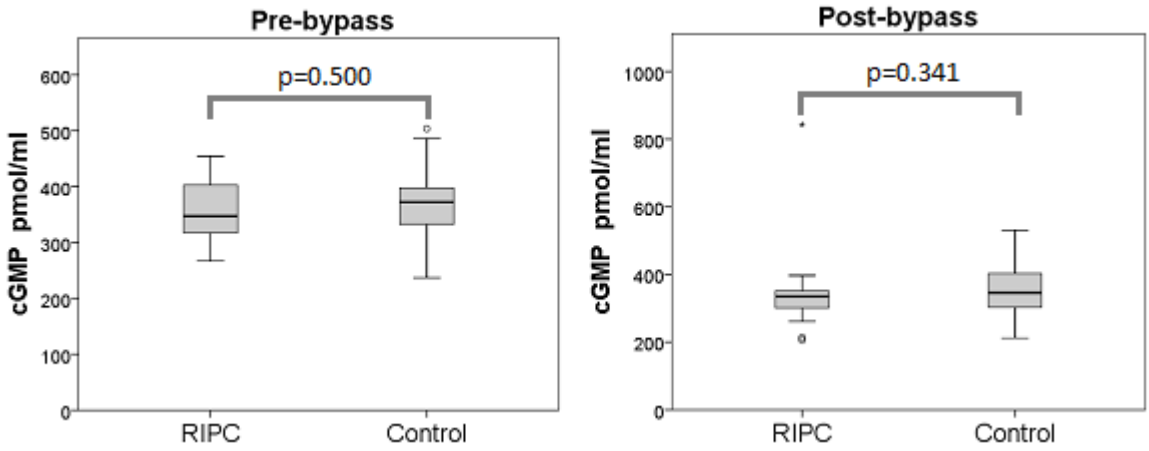

Figure 8.10. Plasma cGMP levels according to group allocation for each time point. Abbreviations: cGMP: cyclic guanosine monophosphate; remote ischaemic preconditioning.

8.6 Summary of key findings

- Baseline nitrate correlated negatively to weight and age.
- Nitrite increased after CPB.
- Post-bypass nitrite was higher in cyanotic patients.
- Post-bypass nitrite correlated to CPB duration, length of intubation and cTnI levels.
- There was no significant difference in any of the parameters according to allocation to RIPC or control groups.

8.7 Limitations

The standard curve used for nitrate quantitation failed on initial analysis, valid OD measurements obtained from the initial analysis were used in order to generate nitrate concentration values by comparing them to a valid standard curve obtained later using linear regression analysis. As nitrate values were required to be comparable to nitrite measurements a similar linear regression model was used for its analysis.

Nitrate and nitrite levels were very low compared to the standard curve values, hence these values should be interpreted with caution.

8.8 Discussion

Lungs are some of the organs most affected by the ischaemic and inflammatory process of CPB. Having an impact in patient outcome and recovery, conservation of pulmonary function is crucial.

Although NO is majorly involved in inflammatory and vascular responses, its activity along with that of cGMP are involved in the molecular and subcellular processes contributing to pulmonary hypertension and lung dysfunction (Malinovschi *et al*, 2011; Bubb *et al*, 2014).

This study aimed to analyse the activity of these markers in CHD patients before and after the inflammatory process of bypass and to assess the effects of RIPC in their activity.

Nitrate levels before bypass correlated to weight and age, reducing its levels as age increased; population studies have found an influence of age in NO metabolite levels in serum, however

in adults NO metabolite levels increased with age, peaking at 50-59 years (Ghasemi *et al*, 2008). Different patterns have also been reported according to gender, with levels increasing in males as they age while females presented a reduction until the menopausal stage (Kawakatsu *et al*, 2002).

Our results showed overall extremely low values of NO metabolites present in plasma, nitrate levels tended to be reduced after CPB, however this did not reach statistical significance, while nitrite increased significantly after CPB.

NO is a key mediator during inflammatory responses to CPB and during the development of IRI (reviewed by Zhou *et al*, 2015), however the quantitation of its metabolites in blood samples has not shown differences linked to CPB exposure during surgery in previous research (Viaro *et al*, 2008).

It has been suggested that the inflammatory response would not be fully developed until several hours after CPB with the possibility that differences could have become apparent later (Palomero *et al*, 2009).

In spite of the low values, nitrite presented a correlation to CPB duration, as well as to intubation length and cTnI levels. This suggests an overall influence of CPB on nitrite activity and a link between nitrite, the extent of injury inflicted to the myocardium and even patient outcome.

Nitrite levels showed a relationship to cyanotic status, presenting higher values in cyanotic patients after CPB; an analysis of NO metabolites in cyanotic adult CHD patients concluded values were significantly higher in cyanotic patients compared to healthy controls, the primary metabolite however, was nitrate (Han *et al*, 2007).

Analysis in paediatric patients found increased levels of nitrate and nitrite in cyanotic patients compared to acyanotic CHDs (Buchoorn *et al*, 2001); interestingly the mechanisms for this difference seem to be based on a downregulation of eNOS activity while iNOS is enhanced (Ferreiro *et al*, 2001).

cGMP tended to be lower after bypass without being statistically significant, it did not correlate to any variables. cGMP – NO activity has the potential to reduce oxidative stress, this is supported by the use of PDE-5 inhibitors which increase cGMP (Semen *et al*, 2016). The actual

therapeutic efficacy and safety of PDE-5 inhibition has not been established (Palma *et al*, 2011; Vassalos *et al*, 2011).

RIPC did not have any significant effect in the levels of NO metabolites or cGMP. NO activity had previously been analysed through measures of iNOS and eNOS expression in the myocardial tissue without finding significant results in an adult preconditioning trial (Wagner *et al*, 2010), blood quantitation of NO metabolites has not been done before in the RIPC context in clinical trials.

Animal studies have confirmed the participation of NO during the RIPC protective process mainly through iNOS activation, resulting in cardiac injury reduction (Wang *et al*, 2001; Xiao *et al*, 2001; Li *et al*, 2004). In a similar way, cGMP has delayed reperfusion acidosis contributing to cardioprotection after conditioning and has been particularly identified as a mediator of late preconditioning (Kodani *et al*, 2002; Inserte *et al*, 2011).

NO presents dual roles within IRI development and cardiac protection, a balance of its activity is one of the factors leading to the final injury in the heart and function of other organs including lungs.

Conclusion

The results obtained in this study linked nitrite levels to myocardial injury and suggested a link to patient outcome. RIPC did not result in a significant variation of NO metabolites or cGMP.

Chapter 9. Asymmetric dimethylarginine

9.1 Introduction

Asymmetric dimethylarginine (ADMA) is an intracellular amino acid with the ability to inhibit NO synthesis, produced after post-translation methylation of arginine by the enzyme protein arginine methyltransferase (PRMT), ADMA is released into the cytosol as a product of normal protein turnover; ADMA is present in tissues, plasma and urine, it has been linked to endothelial dysfunction and hence identified as a potential marker for cardiovascular disease (reviewed by Blackwell, 2010).

PRMTs are located in the cellular nucleus and have a role in gene transcription and RNA processing, presenting two types, PRMT I has ADMA as its major product, PRMT II forms symmetric dimethylargininde (SDMA) while monomethyl-L-arginine (MMA) can be produced by both types of enzymes. PRMT I is expressed in the heart, endothelium and smooth muscle and can be upregulated by light density lipoprotein (LDL) and shear stress (reviewed by Alpoim *et al*, 2015).

ADMA is metabolised by dimethylarginine dimethylaminohydrolase (DDAH) into citrulline and dimethyl-amine with only 10% of human ADMA production being excreted in urine (Achan *et al*, 2003) while SDMA is completely excreted in urine (reviewed by Blackwell, 2010). Oxidative stress, high homocysteine or glucose levels and inflammatory mediators such as TNF-α are thought to intervene with DDAH function resulting in high ADMA levels (reviewed by Alpoim *et al*, 2015).

ADMA interferes with NO synthesis from arginine by competing with NOS, with increased ADMA significantly lowering endothelial NOS activity and NO production (Faraci *et al*, 1995; Segarra *et al*, 2001). While SDMA does not affect NO production, both ADMA and SDMA compete for NOS transport adding to the overall inhibitory effect (Closs *et al*, 1997).

ADMA has been shown to cause a dose dependant rise in blood pressure after its arterial administration in guinea pigs, in addition healthy volunteers receiving ADMA presented a dose dependent decrease in blood flow, an effect counteracted by L-arginine administration (Vallance *et al*, 1992). A similar study in human volunteers determined that ADMA increased blood

pressure and reduced heart rate and cardiac output (Achan *et al*, 2003). The circulatory effects of ADMA involve kidney function as well, affecting renal perfusion and sodium excretion without altering the renin-angiotensin system (Kielstein *et al*, 2004).

The endothelium is able to absorb large amounts of ADMA, causing circulating concentrations to be much lower than cellular levels, hence variations in plasma could have an important intracellular effect in NO production contributing to cardiovascular disease. Effects of ADMA have related it to several pathological processes including diabetes, hypercolesterolaemia, chronic kidney disease, hypertension, atherosclerosis and heart failure (reviewed by Blackwell, 2010; Alpoim *et al*, 2015). ADMA has been found to be a good outcome predictor after myocardial infarction and in intensive care patients (O´Dwyer *et al*, 2006; Zeller *et al*, 2008).

The importance of NO activity during the establishment of IRI has been clearly characterised, in that context the intervention of ADMA in NO synthesis could play a role in the physiopathology of IRI. ADMA has been linked to renal, lung and gastric injury after ischaemia and reperfusion finding that NO activity and oxidative stress were related to accumulation of the amino acid (Nakayama *et al*, 2014; Wu *et al*, 2011; Magierowski *et al*, 2014).

A mouse model of cardiac IRI found increased tissue ADMA, low tissue NO levels and reduced DDAH activity in a process mediated by ICAM-1 and low eNOS activity; in addition DDAH overexpression and administration of L-arginine resulted in an important injury reduction (Stühlinger *et al*, 2007). The involvement of ADMA in IRI was further explored in rat hearts finding that its levels were consistently increased after ischaemia and reperfusion in cardiac tissue and serum (Burma *et al*, 2014; Coskun *et al*, 2014).

9.2 Aims

This study aimed to describe ADMA and SDMA values before and after surgery using CPB in paediatric patients requiring CHD correction and to evaluate the effects of RIPC in ADMA activity.

9.3 Materials and Methods

Baseline samples were taken after anaesthesia induction before surgery. An early postoperative sample was drawn at PICU arrival of the patient (PO1) followed by an early morning sample on the following day (day 2 – PO2) and the day after (day 3 – PO3). Samples were spun within 4 hours of extraction in a centrifuge for 10 minutes at 2000 g to obtain plasma which was aliquoted and stored at -70°C.

ADMA-SDMA quantitation

In order to detect ADMA and SDMA through chromatography reagents were prepared as follows:

Borate buffer 50mM

1.6 g boric acid were dissolved in 500 ml deionised water and pH was adjusted to 8.5 using potassium hydroxide (2M).

Borate buffer 200nM

2.6 g boric acid were dissolved in 200ml deionised water and pH was adjusted to 8.5 using potassium hydroxide (2M).

Ammonia-methanol solution

50 ml ammonia were mixed with 250 ml methanol and 200 ml deionised water.

Derivatising agent

10 mg phthaldialdehyde were dissolved in 200 µl of methanol, this was added to 1.8 ml 200mM borate buffer followed by the addition of 10 µl mercaptopropionic acid under the protection of a fume hood. A dilution of 1:5 in 200 mM borate buffer was performed prior to use.

Mobile phase

2.05 g of sodium acetate were dissolved in 454 ml of deionised water, pH was adjusted to 6.3 using acetic acid and 46 ml of acetonitrile were added followed by filtering and degassing of the mixture.

Sample preparation

200 µl standards or plasma samples were put into glass tubes and mixed with 80 µl internal standard and 20 µl borate buffer.

Solid phase extraction

Solid phase extraction cartridges were primed with 2ml methanol followed by 2 ml 50mM borate buffer. Samples and standards were then added onto columns followed by the addition of 1 ml borate buffer. Columns were washed with 3ml deionised water and 3 ml methanol. Samples were then eluted into fresh glass tubes with 3 ml ammonia-methanol solution.

Samples were dried in a heating block at 80°C for approximately 30 minutes.

Cartridges were reutilised after washing them with 3ml methanol and 2ml borate buffer.

Derivatisation

Once the samples were completely dry, 100 µl deionised water were added to each tube and mixed thoroughly. 100 µl derivatising agent dilution were added and mixed. The samples were then covered and left to stand in the dark for 15 minutes to ensure the reaction was completed before being transferred to Macrovials Target DP Clear (Thermo Scientific).

Chromatography

Samples were loaded into an Alliance 2475 instrument, mobile phase was pumped through the column at a rate of 1.5 ml/minute, excitation and emission wavelengths were 340nm and 455 nm, EUFS was 32000.

9.4 Statistical analysis

A repeated measures ANOVA was used in order to test for differences between time points for the normally distributed ADMA values applying post hoc tests using the Bonferroni correction. SDMA data was not normally distributed, hence repeated samples Friedman analysis of variance was performed in order to test for differences between time points with post-hoc comparisons using Wilcoxon signed rank tests with Bonferroni correction for multiple testing.
Correlation analysis of post-operative measurements to demographic characteristics including weight, age, gender and cyanosis status, as well as to post-operative cTnI and CPB and aortic clamp duration were tested using the Pearson test (r) for normally distributed data or Spearman´s rank correlation test (r_s) for non-parametric data.

Independent samples t-test or Mann-Whitney tests were carried out in order to determine differences between RIPC or control groups for each time point according to data distribution.

Statistical analysis was performed using SPSS Software version 23 at a significance level of 5% (2 tailed).

9.5 Results

9.5.1 Perioperative analysis

ADMA levels did not correlate to gender, age, weight, cyanotic status, CPB or aortic clamp duration times. PO2 ADMA presented a positive correlation to cTnI levels at the corresponding time point (r_s=0.505, p=0.001) (*Figure 9.1*).

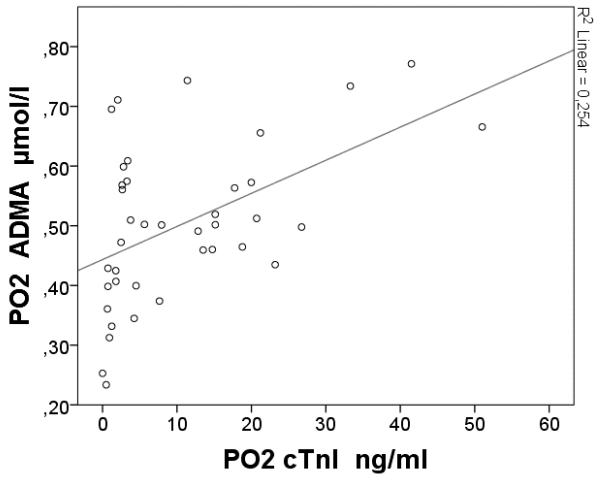

Figure 9.1. Correlation of PO2 ADMA to PO2 cTnI. Abbreviations: ADMA: asymmetric dimethylarginine; cTnI: cardiac troponin I; PO2: post-operative 2.

SDMA presented a negative correlation to cyanosis status at all time points (Baseline: r_s= -0.368, p=0.018; PO1: r_s= -0.479, p=0.001; PO2: r_s= -0.481, p=0.001; PO3: r_s= -0.526, p=0.005), SDMA was significantly higher in cyanotic patients at all time points, PO3 levels according to cyanosis are shown in *Figure 9.2*.

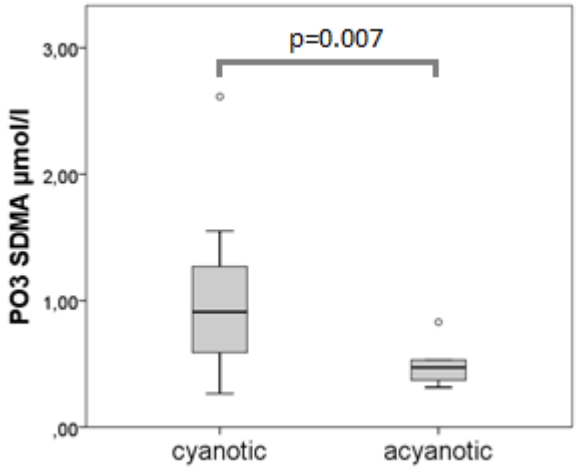

Figure 9.2. PO3 SDMA according to cyanosis status. Abbreviations: PO3: post-operative 3; SDMA: symmetric dimethylarginine.

A negative correlation of SDMA was seen to age at baseline (r_s= -0.480, p=0.001), PO1 (r_s= -0.510, p<0.0001) and PO3 (r_s= -0.484, p=0.011); and to weight at all time points (Baseline: r_s= -0.584, p<0.0001; PO1: r_s= -0.517, p<0.0001; PO2: r_s= -0.390, p=0.012; PO3: r_s= -0.658, p<0.0001).

A positive correlation to CPB duration at PO1 (r_s=0.356, p=0.019) PO2 (r_s=0.573; <0.0001), PO3 (r_s=0.547, p=0.003), and to aortic cross clamp duration for PO1 (r_s=0.404, p=0.008), PO2 (r_s=0.506, p=0.001) and PO3 (r_s=0.661, p<0.0001) was present.

In addition SDMA levels showed a positive correlation to cTnI levels at corresponding time points PO1 (r_s=0.471, p=0.001), PO2 (r_s=0.772, p<0.0001) and PO3 (r_s=0.828, p<0.0001) (*Figure 9.3*).

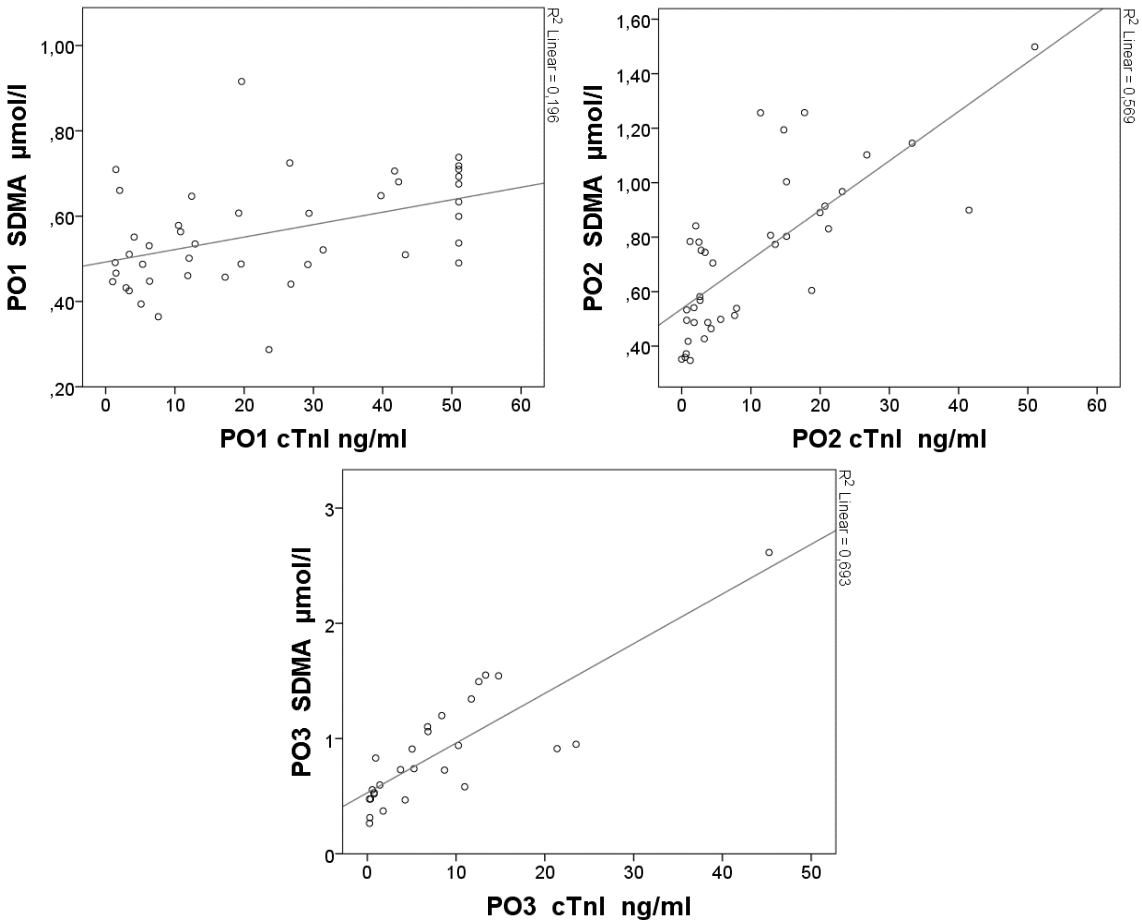

Figure 9.3. Correlation of SDMA to cTnI at post-operative time points. Abbreviations: cTnI: cardiac troponin I; PO: post-operative; SDMA: symmetric dimethylarginine.

Samples of forty five patients were used for analysis, descriptive statistics for ADMA are shown in *Table 9.1*.

Time point	N	Mean – SD
Baseline	41	0.663±0.146
PO1	43	0.626±0.171
PO2	41	0.509±0.137
PO3	27	0.492±0.152

Table 9.1. Descriptive statistics for plasma ADMA values according to time point. Data expressed in mean – SD. Abbreviations: PO: post-operative; SD: standard deviation.

Twenty five patients presented complete measures for all time points. A repeated measures ANOVA was used, Maucchly´s test of Sphericity indicated that the assumption of sphericity had not been violated, $X^2(2)=4.720$, $p=0.451$. There was a significant difference between time points ($p<0.0001$), post hoc tests using the Bonferroni correction determined that ADMA levels varied significantly between baseline and PO2 ($p<0.0001$), and baseline and PO3 ($p<0.0001$). *Figure 9.4* presents the variation between time points.

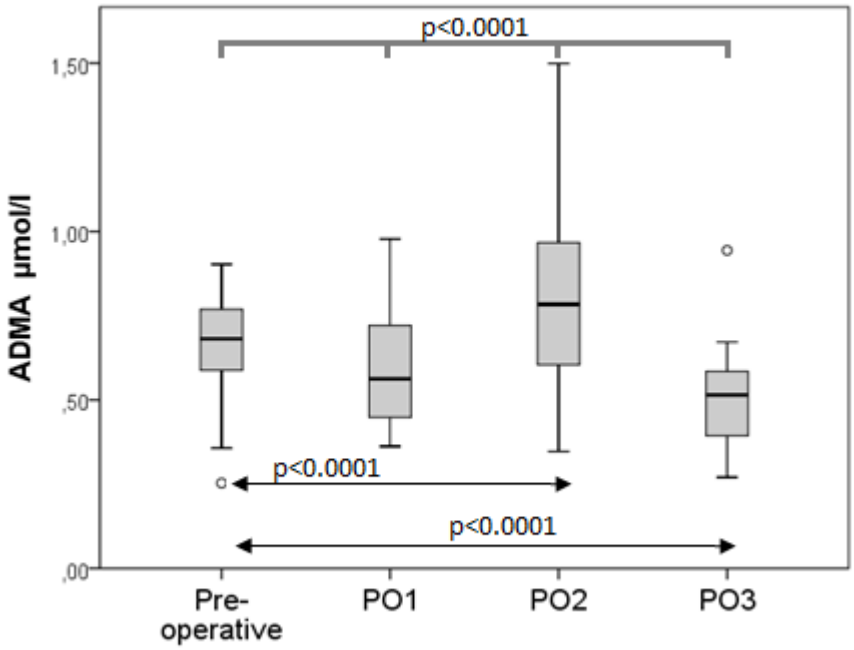

Figure 9.4. ADMA variation according to time points. Abbreviations: ADMA: asymmetric dimethylarginine; cTnI: PO: post-operative.

Table 9.2 presents descriptive statistics for SDMA according to time points.

Time point	N	Median – IQ range
Baseline	41	0.51(0.43-0.62)
PO1	43	0.53(0.47-0.66)
PO2	41	0.75(0.50-0.90)
PO3	27	0.74(0.52-1.10)

Table 9.2. Descriptive statistics for plasma SDMA values according to time point. Data expressed in mean – SD. Abbreviations: PO: post-operative; SD: standard deviation.

Twenty five patients presented complete measures which were analysed using the Friedman repeated measures test which determined there was a statistical significant variation between time points in SDMA levels ($X2(2)=31.512$, $p<0.0001$). Post hoc analysis with, Wilcoxon signed rank test was performed applying Bonferroni correction, resulting in a significance level at $p<0.0083$. Variation was significant between baseline – PO2 ($p<0.0001$), baseline – PO3 ($p<0.0001$), PO1 – PO2 ($p<0.0001$) and PO1 – PO3 ($p=0.001$). *Figure 9.5* shows the variation of SDMA according to time points.

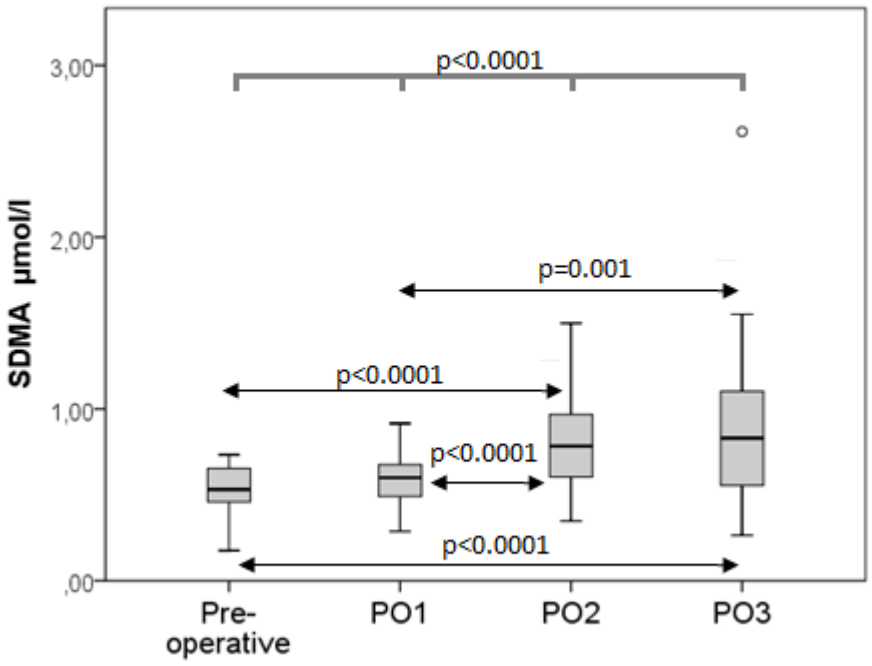

Figure 9.5. SDMA variation according to time points. Abbreviations: cTnI: PO: post-operative; SDMA: symmetric dimethylarginine.

9.5.2 RIPC analysis

Two cases were excluded from analysis for RIPC as parents asked for the intervention to be stopped before its completion due to discomfort.

Figure 9.6 shows the variation of ADMA according to RIPC or control allocation across time points.

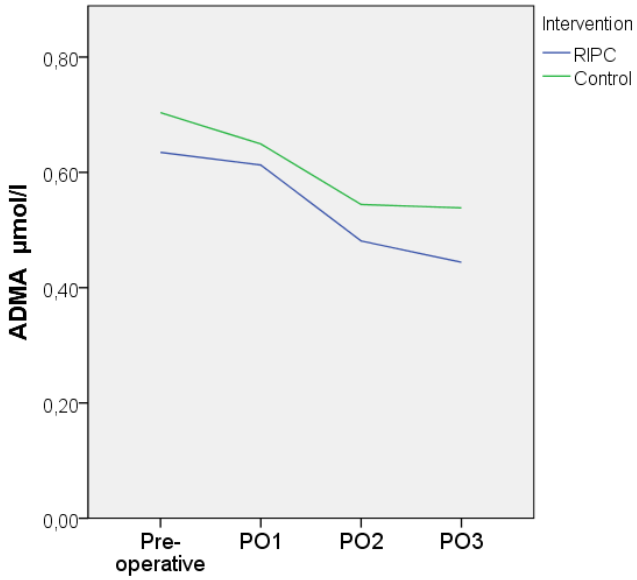

Figure 9.6. Variation of ADMA values according to group allocation. Mean values are represented. Abbreviations: ADMA: asymmetric dimethylarginine; PO: post-operative; RIPC: remote ischaemic preconditioning.

Forty three patients were included in the analysis of ADMA according to group allocation to RIPC or control. *Table 9.3* summarises descriptive information.

Time point	Intervention	N	Mean - SD	P value
Baseline	RIPC	20	0.635±0.112	0.143
	Control	19	0.704±0.171	
PO1	RIPC	22	0.613±0.149	0.522
	Control	19	0.649±0.203	
PO2	RIPC	20	0.481±0.137	0.163
	Control	19	0.544±0.140	
PO3	RIPC	11	0.444±0.105	0.116
	Control	15	0.539±0.169	

Table 9.3. Descriptive statistics for plasma ADMA values according to group allocation for each time point. Data is expressed in mean – SD. Abbreviations: ADMA: asymmetric dimethylarginine; PO: post-operative; RIPC: remote ischaemic preconditioning, SD: standard deviation.

There was no significant difference between RIPC and control ADMA levels for any time point (*Figure 9.7*).

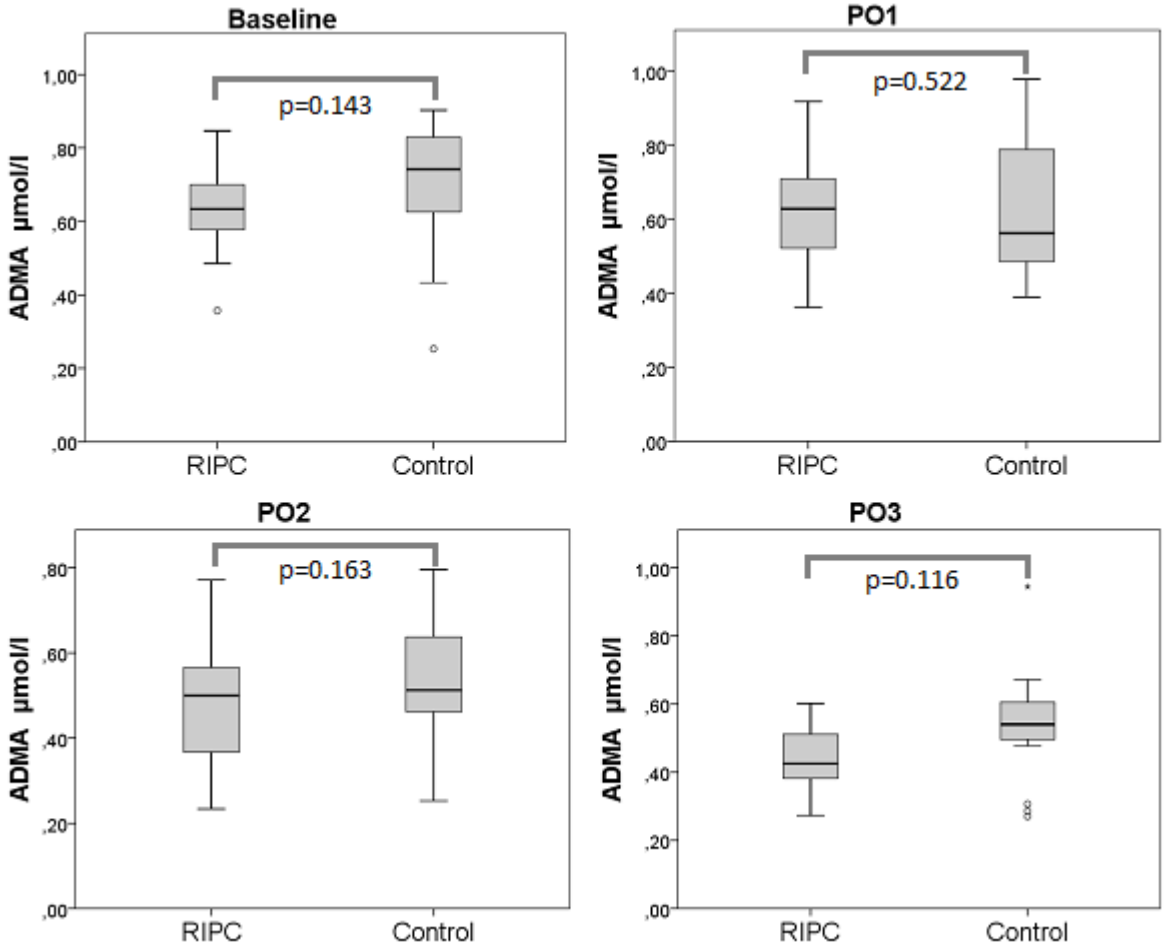

Figure 9.7. Plasma ADMA according to group allocation for each time point. Abbreviations: ADMA: asymmetric dimethylarginine; PO: post-operative; RIPC: remote ischaemic preconditioning.

Figure 9.8 shows the variation of SDMA according to RIPC or control allocation across time points.

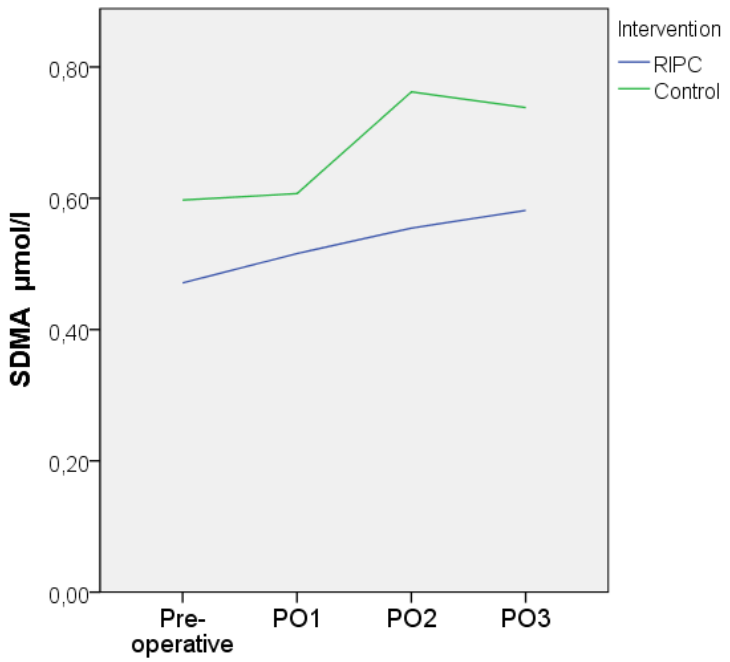

Figure 9.8. Variation of SDMA values according to group allocation. Mean values are represented. Abbreviations: PO: post-operative; RIPC: remote ischaemic preconditioning; SDMA: symmetric dimethylarginine.

SDMA values were compared between RIPC and control groups for every time point, *Table 9.4* shows descriptive data for SDMA.

Time point	Intervention	N	Mean - SD	Median – IQ range	P value
Baseline	RIPC	20	0.495±0.086		0.100
	Control	19	0.555±0.133		
PO1	RIPC	22	0.524±0.105		0.078
	Control	19	0.586±0.115		
PO2	RIPC	20		0.55(0.48-0.86)	0.158
	Control	19		0.76(0.53-1.00)	
PO3	RIPC	11		0.58(0.37-1.34)	0.721
	Control	15		0.74(0.53-1.06)	

Table 9.4. Descriptive statistics for plasma SDMA values according to group allocation for each time point. Data is expressed in mean – SD or median – IQ range as appropriate. Abbreviations: PO: post-operative; RIPC: remote ischaemic preconditioning, SD: standard deviation; SDMA: symmetric dimethylarginine.

Differences in SDMA values between control and RIPC groups were not statistically significant (Figure 9.9).

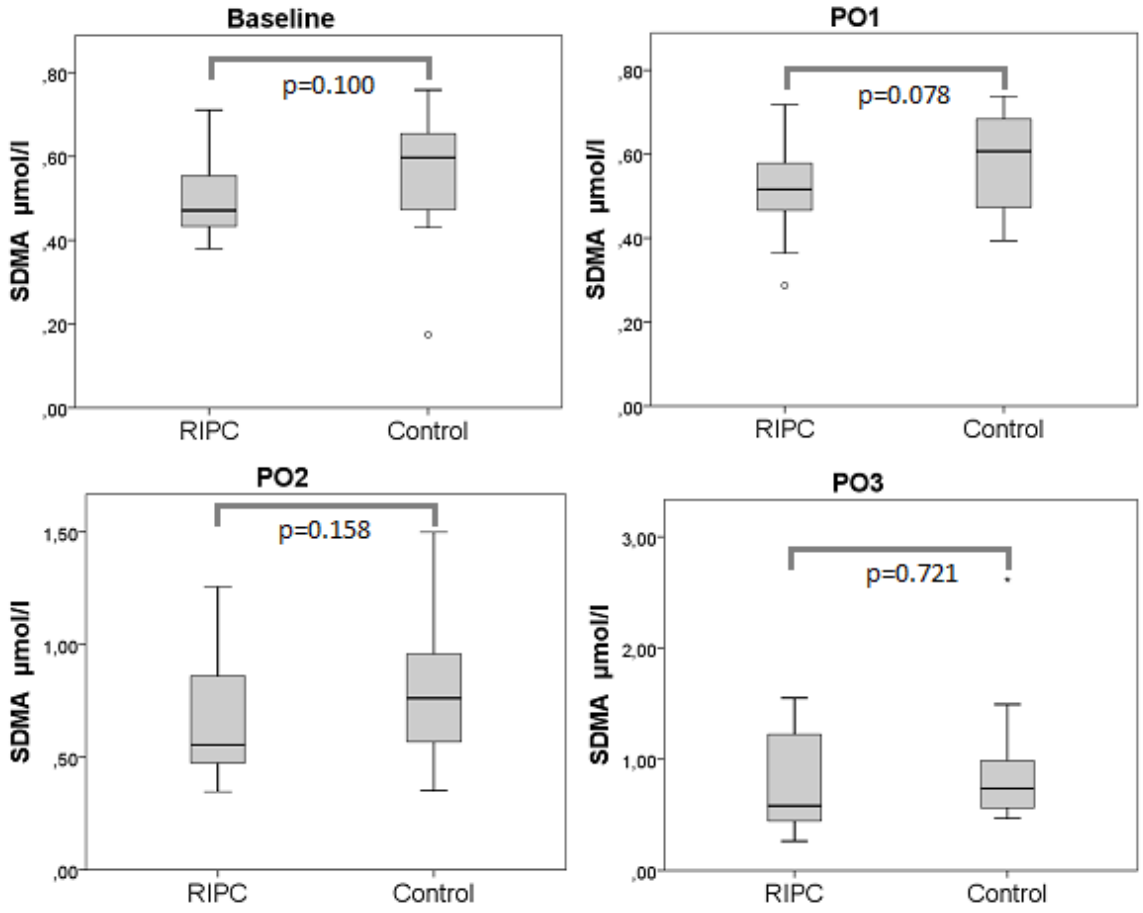

Figure 9.9. Plasma SDMA according to group allocation for each time point. Abbreviations: PO: post-operative; RIPC: remote ischaemic preconditioning. SDMA: symmetric dimethylarginine.

9.6 Summary of key findings

- ADMA correlated to cTnI at PO2.
- SDMA correlated negatively to weight and age after surgery.
- SDMA correlated to cTnI, CPB and aortic clamp duration after surgery.
- SDMA was higher in cyanotic patients.
- ADMA and SDMA varied across time points.
- ADMA and SDMA were not significantly different according to RIPC or control allocation.

9.7 Discussion

ADMA has gained attention as a potential marker for renal and cardiac disease (Alpoim *et al*, 2015). As ADMA interferes with NO activity, it is reasonable for it to be involved in cardiovascular processes and in this case in the development of IRI.

The chromatography method used for this study allowed for the simultaneous quantitation of the arginine methylated derivatives ADMA, SDMA and homoarginine (Blackwell *et al*, 2009). ADMA and SDMA were analysed showing that ADMA presented measurable values at baseline which were lowered immediately after surgery to then increase and peak the day after surgery, and reduce its values below baseline levels by the second day after surgery. This pattern was similar to that observed in cell adhesion molecules (see S*ection 12.3.4*).

At its highest point (PO2), ADMA correlated to cTnI suggesting a link to the degree of injury suffered by the myocardium. Previous research in a mice model or IRI reported increased ADMA and low NO levels in tissue in relation to ICAM-1 activity (Stühlinger *et al*, 2007), increased levels were also found in serum consistently after IRI in rats (Burma *et al*, 2014; Coskun *et al*, 2014). Analysis in paediatric CHD revealed that patients presenting pulmonary hypertension had higher levels of ADMA than other CHD patients (Sanli *et al*, 2012).

SDMA profile is less known, it interferes to a degree with NO activity by reducing L-arginine availability; in addition it is influenced by ROS, which potentially could involve it in numerous vascular processes (reviewed by Mangoni, 2009). SDMA has been better studied regarding renal function, with one meta-analysis describing it as a potentially suitable marker of glomerular filtration (Kielstein *et al*, 2006).

In the current study SDMA levels presented an increasing trend along post-operative times, they decreased according to age and weight, and were correlated to cTnI levels, CPB and aortic clamp duration, revealing an involvement not only in inflammatory responses but in the injury process.

In addition, SDMA values were influenced by cyanosis at all time points including baseline, reaching significantly higher values than in acyanotic patients. Higher values of L-arginine and its derivatives ADMA and SDMA have been documented in patients presenting chronic hypoxia (chronic obstructive pulmonary disease) compared with acutely hypoxic patients (acute lung injury) (Molnar *et al*, 2013).

A correlation of SDMA plasma levels to echocardiographic E/A ratio and NTproBNP in chronic heart failure patients was found (Tang *et al*, 2008); although our patients have a different pathophysiological background, these variables could be further explored.

Our study revealed RIPC had no influence in the activity of ADMA or SDMA. Levels of these markers have not been previously evaluated in cardiac RIPC clinical trials. One renal study found that the percent change of ADMA was significantly lower in the RIPC group compared to control linked to AKI prevention (Igarashi *et al*, 2013).

ADMA has potential as a cardiovascular marker, a large meta-analysis considering long term outcome concluded there is an association of ADMA circulating values and CVD, however it emphasised the need to clarify this association (Willeit *et al*, 2015).

In spite of the focus being placed on ADMA, the present results showed a correlation of SDMA to injury and inflammatory variables that ADMA did not have. This is the first analysis of methylated arginine derivatives in the context of cardiac IRI in paediatric patients and we have found links that motivate further investigation.

Conclusion

ADMA and SDMA showed links to the inflammatory and injury process taking place after cardiac surgery using CPB. RIPC did not significantly affect the activity of arginine derivatives.

Gene expression analysis

Chapter 10. Heat shock proteins

10.1 Introduction

Heat shock proteins were first observed as transcriptional activity presented in a characteristic chromosomal puffing pattern after heat exposure of the salivary glands of the fruit fly, *Drosophila busckii* (Ritossa, 1962). Twelve years later HSPs were discovered (Tissières *et al*, 1974) and nowadays are known to be induced by numerous types of stress, having a function to protect against stress.

It has been established that HSPs are highly conserved across eukaryotic and prokaryotic organisms within all cell types and play a role in basic cellular mechanisms as molecular chaperones, proteins that interact with other proteins in order to stabilize them or help them reach its active conformation. Their main function is to assist in protein folding, unfolding and to maintain cellular protein homeostasis (reviewed by Hartl *et al*, 2011; Saibil, 2013).

HSPs are mainly classified according to their molecular weight in kilodaltons (kDa), they include HSP-100, HSP-90, HSP-70, HSP-60, HSP-40 and small HSPs (sHSPs). Most HSPs act on non-native proteins through cycles of ATP binding and hydrolysis in order to promote folding or unfolding while others such as sHSPs protect subunits only until conditions for folding are achieved (reviewed by Saibil, 2013).

10.1.1 Induction and Synthesis

HSPs can be induced after cellular exposure to different types of insults or "stress" other than heat, including hypoxia (Patel *et al*, 1995), ischaemia (Jin *et al*, 2014), as well as viral and bacterial infection (Collins and Hightower, 1982; Varano Della Vergiliana *et al*, 2013).

HSPs transcription is mediated in mammals by the heat shock transcription factor 1 (HSF1), which is present in the cytosol as a monomer and plays a role in metabolism, development and ageing in normal conditions; when acute protein damaging stress takes place, HSF1 is

phosphorylated and translocates to the nucleus where it forms homotrimer complexes that bind to the promoter regions of HSP genes, leading to their transcription; HSF1 activity is transient and stops along with stress relief; the transcriptional activity of HSF1 has been linked to cancer cells in which it seems to be permanently activated (reviewed by Vihervaara and Sistonen, 2014; Dai and Sampson, 2016).

While HSF1 is the main known transcription factor controlling HSP expression, it does not regulate all HSPs; not all HSPs are heat inducible and this could play a part in HSF1 involvement. A strong interaction with HSP-90 and HSP-70 has been reported with a possible suppression activity of HSP-70 on HSF1 expression while the expression of CRYAB or small heat shock protein B5 was not related to HSF1 (Tang *et al*, 2016).

10.1.2 Function

Proteostasis, the control of systems in order to obtain an adequate balance between synthesis, folding, unfolding, degradation and turnover of proteins is a process vital for the appropriate performance of the proteome and cell survival, ensuring proteins are present in their active conformation when required (reviewed by Saibil, 2013).

HSPs are essential for the success of this process, as they mediate it while they contribute to clearing mechanisms including autophagy and lysosomal degradation. HSPs have a role in most cellular functions and are present in all cellular compartments such as cytoplasm, nucleus, endoplasmic reticulum (ER), cytosol and mitochondria (reviewed by Doyle *et al*, 2013).

Chaperones are involved in several stages of the protein quality control network, as polypeptides rise from the ribosome HSPs enable co-translational protein folding. Part of the newly created pool of proteins are not released from the ribosome in their native (folded and operative) conformation, in which case some polypeptides are able to reach their native form by spontaneous folding or through the intervention of chaperones such as HSP-60, HSP-70 and HSP-90, while others fail to achieve it (Willmund *et al*, 2013; reviewed by Preissler and Deuerling, 2012).

Events such as oxidative stress, cellular damage and certain pathological processes can lead to unfolding and inactivation of proteins and it is also the function of HSP-60, HSP-70 and HSP-90 to mediate the reactivation of non-native proteins (reviewed by Mayer, 2010; Priya *et al*, 2013,).

These chaperones are not highly specific to particular protein substrates and due to the disordered structure of misfolded, unfolded, partially folded or aggregated polypeptides, information on their interaction with chaperones is not available, however chaperones seem to be very dynamic and this process linked to ATPases can easily fluctuate and adapt (reviewed by Saibil, 2013).

Degradation of proteins that cannot be refolded and reactivated is part of the homeostatic system taking place and ATP-dependent proteases require the intervention of chaperones to achieve it (reviewed by Sauer and Baker, 2011).

sHSPs, HSP40, HSP70 and HSP90 have the additional property of binding to non-native proteins in order to slow misfolding and aggregation as protein aggregates can be cytotoxic and cause cell death and pathological outcomes (reviewed by Saibil, 2013; Zhang *et al*, 2015).

A role of HSPs in the unfolding of defective proteins and disaggregation has also been described, showing rescue properties within the proteome (reviewed by Priya *et al*, 2013; Mattoo and Goloubinoff, 2014). HSP-100 in primitive organisms and HSP-70 in higher eukaryotes have been shown to be able to disaggregate such compounds acting in association with co-chaperones by extracting tangled polypeptides and releasing non-native forms that can then be refolded, degraded or aggregated again (Glover and Lindquist, 1998; Mattoo *et al*, 2013). The ER plays a primordial role in protein production and quality control, if it becomes overwhelmed by misfolded polypeptides apoptosis can be triggered by the unfolded protein response (reviewed by Korennykh and Walter, 2012).

The functions described so far take place within the cell, in addition extracellular presence of HSPs has been established in physiological and pathological conditions, with cell signalling and immunological processes as their known function (reviewed by Binder, 2014).

Immunity

HSPs work as mediators within the innate and adaptive immune system, playing a role in antigen presentation, expression of innate receptors, cytokine activity, tumour surveillance and autoimmunity. HSPs have been linked to both inflammatory and anti-inflammatory processes and particular responses seem to depend on the nature of the stimulus and on the type of cell that the HSP interacts with, as well as the presence of other mediators; hence pathogenic derived HSPs will have different activity to self-stress proteins, which seem to regulate and resolve inflammation (reviewed by Pockley *et al*, 2008).

Important immunological components require the protein folding properties of HSPs, including most TLRs and integrins (Wu *et al*, 2012). As the function of these receptors and mediators are crucial to appropriate immune activity, a lack of NF-κB mediated activation and cytokine release expected after TLR activity has been observed, as well as the occurrence of immunosuppression in mice models which was linked to a lack of HSP gp96 due to inappropriate lymphopoiesis (Staron *et al*, 2010).

The protein binding properties of HSPs including cytosolic HSP-70, HSP-90 and ER gp96 or HSP-90B1 have been proven to act in peptides derived from antigenic proteins within the cell including those resulting from viruses and bacteria antigens (Ag) from infected cells (Basta *et al*, 2005; Rapp and Kaufmann, 2004), transfected cells (Binder *et al*, 2007), alloantigens from mismatched major histocompatibility complex (MHC) cells (Arnold *et al*, 1995) and from tumour cells (Ishii *et al*, 1999), mediating immune responses.

HSPs transport these antigenic peptides to the cell surface where MHC class I molecules can present them to lymphocytes leading to the activation of $CD8^+$ T cells. As an alternative, Ags being chaperoned by HSPs can be released to the extracellular space where the complex is taken by antigen presenting cells (APC), MHC class I molecules on the surface of APCs then present the complex to T lymphocytes completing cross-presentation and eliciting a stronger T cell response as a complex compared to cross-presentation of the Ag alone (reviewed by Tsan and Gao, 2009).

These processes are important for tumour immunosurveillance, which relies on cross-presentation of Ag, furthermore HSPs seem to aid co-stimulation through APC activity and by facilitating tumour rejection (reviewed by Binder, 2014). Possible therapeutic options based on this principle, using tumour derived HSPs have already been tested in melanoma patients and are very promising (Testori *et al*, 2008).

HSPs have the ability to activate certain specific immunoreceptors, HSP-60 and HSP-70 use receptors such as TLRs, CD14 and CD40, potentiating immune response (reviewed by Pockley *et al*, 2008). Initial studies reported significant *in vitro* induction of cytokine production by HSPs however these findings were later attributed to contamination of the HSP preparations used; when experiments were performed with highly purified preparations the cytokine induction properties were lost (reviewed by Tsan and Gao, 2009). On the other hand *in vivo* studies, in which such contamination is unlikely, have found cytokine induction mediated by HSPs (Chen *et al*, 2002).

Self-reactive HSPs, with no pathologic components can also interact with APCs in the absence of inflammatory activity, be transported to the cell surface and interact with low affinity T lymphocytes which can recognise a wide range of endogenous antigens and trigger anti-inflammatory activity instead of the adaptive response to pathological Ags (reviewed by Pockley *et al*, 2008).

A link has been established between HSPs and autoimmune pathologies such as rheumatoid arthritis (RA), where synovial tissue concentration of HSPs was higher in affected joints only (Huang *et al*, 2009). Further studies found that the release of IL-4 and IL-10 by HSP-60 stimulated T cells had beneficial effects in RA patients, as the stimulated T cells of the Th2 class showed anti-inflammatory effects, while the pathologically stimulated Th1 variety related to detrimental effects (van Roon *et al*, 1997; de Kleer *et al*, 2003).

Heat Shock Protein Families

Classified according to their molecular weight HSPs vary in distribution and functionality, this account will focus on the HSPs most relevant to cardiac ischaemia, IRI, RIPC and the research performed.

10.1.3 Heme oxygenase-1 (HSP-32)

Heme oxygenases (HO) are highly conserved enzymes with the function of catalysing the degradation of heme, a hemoprotein iron prosthetic group containing iron which has the ability to catalyse the production of free radicals, a function that is strictly controlled while heme is bound to hemoproteins; however in its free form it can cause the release of significant amounts of ROS under oxidative stress stimulation (reviewed by Igarashi and Watanabe-Matsui, 2014). Heme degradation uses nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor and its products include carbon monoxide (CO), iron (Fe) and biliverdin which is then converted into bilirubin by biliverdin reductase (reviewed by Agarwal and Bolisetty, 2013).

With two isoforms expressed in humans, the constitutive HO-2 and the stress induced HO-1, HOs play a key role in controlling free heme mediated oxidative stress with HO-1 providing a rapid response increasing heme catabolism rate and thus preventing apoptosis while HO-2 provides with the basal control of heme, it is not induced and offers limited protection in stress situations (reviewed by Gozzelino *et al*, 2010).

First described in a rat model in 1968 by Tenhunen *et al*, HO-1 weighs 32 kDa, it is expressed in all tissues at low levels in normal conditions and can be highly inducible. Located in the ER, mitochondria, nucleus and specialised domains within the plasma membrane of the cell, it has been suggested that specific subcellular locations correspond to different aspects of HO-1 function, with heme degradation taking place at the ER, mitochondria and cellular membrane while gene transcription would occur at the nucleus (reviewed by Dunn *et al*, 2014).

HO-1 expression is fundamentally controlled at the level of transcription, however mRNA stabilization takes place during acidosis and hypoxia while its enzymatic function can be altered

during hyperoxia. The interaction of several transcription factors and cytokines control HO-1 expression including mitogen activated protein kinases (MAPKS), nuclear factor E2-related factor 2, the transcriptional repressor Bach1, PKC, PKA, Akt, NK-κB and HIF-1 among others (Czibic *et al*, 2008; reviewed by Gozzelino *et al*, 2010; Kim *et al*, 2011).

Cytoprotection

HO-1 provides cellular protection and part of the mechanisms have been attributed to the effects of the end products of heme degradation. Biliverdin and its subsequent metabolite bilirubin can go into a cycle in which bilirubin acting as an antioxidant can be oxidised into biliverdin and then recycled back into bilirubin in an amplification series (Barañano *et al*, 2002). In addition bilirubin has been shown to prevent IRI (Adin *et al*, 2005).

Fe on the other hand can contribute to the production of free radicals when released after heme degradation, however it can be neutralised by several pathways including the fast expression of the Fe sequestering molecule FtH which prevents TNF induced apoptosis, hence Fe could promote cellular protection through the secondary upregulation of FtH (reviewed by Gozzelino *et al*, 2010).

The last heme degradation product, CO acts as a signalling molecule presenting anti-apoptotic and anti-inflammatory features with the ability to amplify HO-1 response (reviewed by Dunn *et al*, 2014). With similar functions to NO, CO acts as a neurotransmitter, it is a vascular smooth muscle relaxant, controls blood pressure and inhibits platelet activation and aggregation (reviewed by Olas, 2014). CO has been proven to prevent ischaemia (Fujita *et al*, 2001), IRI (Zhao *et al*, 2014) and to aid angiogenesis (Deshane *et al*, 2007).

A combination of the effects of all these metabolites has an impact in apoptosis; FtH stimulus and hence the control of labile Fe inhibit the transcription of proinflammatory genes, CO triggers a transient oxidative reaction through cytochrome c oxidase and leads to the suppression of caspase activation while independently HO-1 mediates the suppression of the mitochondrial intrinsic apoptotic pathway (reviewed by Gozzelino *et al*, 2010).

In addition to the action of these metabolites, evidence of independent cytoprotection towards oxidative stress exerted by HO-1 has been described involving the induction of nuclear transcription factors linked to a nuclear localisation of the enzyme (Lin *et al*, 2007).

Cardiac Involvement

HO-1 effects play an important role in cardiovascular disease and response to ischaemia and IRI, providing antioxidant protection, anti-inflammatory activity, preventing apoptosis and detrimental vascular proliferation and remodelling, exerting antithrombotic activity and having a vascular relaxant effect (reviewed by Fredenburgh *et al*, 2015).

HO-1 is thought to be involved in the heart´s susceptibility to ischaemia as a polymorphism in its gene promoter region has been linked to higher incidence of coronary artery disease (Kaneda *et al*, 2002) with reduced bilirubin levels in serum adding to this vulnerability; HO-1 activity reduces acute inflammatory responses, oxidative stress, ventricular dysfunction, fibrillation and overall cellular death and infarct size, in addition reducing ventricular remodelling after ischaemia and reperfusion (reviewed by Czibic *et al*, 2014).

HO-1 seems to have an important role in IRI as demonstrated in an initial model of targeted heterozygous disruption of the gene, resulting in reduced ventricular recovery, increased creatine kinase release and infarct size, becoming more vulnerable to IRI (Yoshida *et al*, 2001). Further HO-1 overexpression studies showed an important reduction in apoptosis rate, lower inflammatory infiltration, oxidative damage, infarct size and better ventricular function (Yet *et al*, 2001; Vulapalli *et al*, 2002).

Vascular lesions are inhibited by HO-1 through anti-proliferative effects in vessels as shown in a HO-1 overexpression model of femoral artery injury which favoured vascular relaxation and reduced arterial remodelling, while absence of HO-1 resulted in augmented proliferation and increased formation of vascular lesions (Duckers *et al*, 2001). Furthermore, a recent placental endothelial study showed that IRI and vascular smooth muscle growth can be restricted by the HO-1/bilirubin pathway through an increase in CO release (Onda *et al*, 2015).

Strong protective activity through maintainance of endothelial homeostasis and resistance to injury has been associated to HO-1 and its anti-apoptosis and anti-inflammatory effects (Mylroie *et al*, 2015).

HO-1 expression in patients going through coronary artery bypass procedures was not affected by cardioplegia or reperfusion and was not different between groups in a RIPC study (Czibic *et al*, 2008).

HO-1 has been identified as a promising target for gene and drug induced modulation in order to avoid ischaemia and IRI producing favourable results (Tang *et al*, 2005; Lakkisto *et al*, 2009). Its antioxidant properties have been repeatedly proven, having a protective effect against hypoxia, limiting cardiac damage after ischaemia through the conservation of mitochondrial membrane potential (Issan *et al*, 2014). Enhanced HO-1 expression has been proven after RIPC in several organs accompanied by protective effects against IRI (Jancsó *et al*, 2007; Zeynalov *et al*, 2009; Peng *et al*, 2011).

The benefits of HO-1 seem to require a careful balance of its activity and of the products of heme degradation as damaging effects involving the metabolites CO and bilirubin have been reported, with CO causing an increased expression of NOS contributing to IRI of the myocardium (Meyer *et al*, 2015). Bilirubin has been associated to mitochondrial protection, however causing ER stress and low metabolic and proliferative activity in hepatic cells (Müllebner *et al*, 2015).

10.1.4 HIF-1

HIF-1 is a transcription factor key to oxygen metabolism, it has a function in adaptation to reduced oxygen availability, thus playing an important role in RIPC; HIF-1 is made up of two subunits: HIF-1β, which is constitutively expressed and HIF-1α which is upregulated as a response to hypoxia (reviewed by Semenza, 2012). HIF-1 however does not act by itself, it requires the intervention of the oxygen sensors prolyl hydroxylases (PHDs) and factor inhibiting HIF, with the latter leading to the activation of HIF-1α subunit which would then form an heterodimer with HIF-1β in order to regulate the transcription of numerous target genes involved

in the adaptive response to hypoxia, characterising the HIF-PHD pathway as an important component of RIPC cardioprotection (reviewed by Martin-Puig *et al*, 2015).

Based on its influence in the expression of secondary genes such as IL-10, HIF-1 has classically been linked to the late phase of protection (Cai *et al*, 2013); however its deactivation has been shown to influence early phase efficacy as well, relating to aspects of endothelial cells and showing a connection to both phases of the process (Sarkar *et al*, 2012).

The mechanisms through which HIF-1α would contribute to early cardioprotection are not clear, however PKC seems to have a role on modulating its activity; HIF-1α could influence ROS activity and affect adenosine induced effects by upregulating the enzyme CD73, which facilitates the generation of extracellular adenosine, as well as adenosine A2B receptor (reviewed by Ong and Hausenloy, 2012). However, HIF-1α influence in early RIPC remains unclear as its inhibition did not alter RIPC effects in a rat model (Kalakech *et al*, 2013).

The activity of HIF-1α as a transcription factor has been linked to its influence in several downstream target genes which have been identified as mediators of late RIPC including erythropoietin, HO-1, adiponectin and iNOS (reviewed by Ong and Hausenloy, 2012), it has been related to a reduction of mitochondrial oxidative stress preventing MPTP opening (Ong *et al*, 2014).

A human study involving 32 patients undergoing CPB demonstrated a protective effect, obtaining lower troponin and influencing the circulating levels of inflammatory mediators in relation to RIPC, which was also linked to higher concentrations of HIF-1α before CPB (Albrecht el al, 2013).

10.1.5 HSP-60

The HSP-60 family, also known as chaperonins are highly conserved molecules across species subdivided into two groups: Group I are present in bacteria (GroEL) and eukaryotic myochondria (HSP-60) whereas Group II are localised in archae thermosome subunits and eukaryotic cytoplasm. This account and research will focus on the mitochondrial HSP-60

(HSPD1), one of the most abundant chaperones within the mitochondria, that has also been identified in other subcellular locations as well as in extracellular spaces and the blood stream (reviewed by Gammazza *et al*, 2012).

HSP-60 is heat inducible and although it is controlled by HSF-1, it is not the only transcription factor influencing its expression (Tang *et al*, 2016); under stress conditions cytoplasmic HSP-60 can be swiftly imported into the mitochondria by HSP-70 in order to aid cellular survival (Itoh *et al*, 2002).

Structure

Classically, chaperonins present a common structure formed by twin heptameric rings joined to form a cylindrical structure, within each ring a cavity is used to bind non-native or unfolded proteins in a self- contained manner in order to prevent their aggregation, these proteins would then be folded to their native form in an ATP dependent process (reviewed by Boshoff, 2015).

Chaperonins require the activity of co-chaperonins in order to work, with a single heptameric ring of 10 kDa subunits co-chaperonins bind to chaperonins in order to create a lid structure that allows the chamber to expand and to fold proteins when ATP is bound (Saibil *et al*, 1996). The complex forms a cage type structure that allows one molecule in at a time preventing any disruptions to folding as well as avoiding aggregation or rebinding, it is also thought to accelerate the folding process while controlling energy usage (reviewed by Hartl *et al*, 2011).

Most of what is known of chaperonin structure and mechanisms comes from the well-studied chaperonin GroEL and its co-chaperonin GroES in *Eschericha coli*, however the human equivalent HSP-60 presents a single ring structure and interacts differently with its co-chaperonin HSP-10, presenting different affinity and stability in its structure while conserving functionality and the need for ATP hydrolysis (Nisemblat *et al*, 2015).

HSP-10 (HSPE) is very highly conserved, presenting a similar structure across species; it is mainly localised in the mitochondria but is also present in the cytosol, cell surface, extracellular space and blood; in addition to its co-chaperonin function it limits immune reactions thus aiding autoimmunity and facilitating pregnancy while assisting tumour formation by supressing apoptosis (reviewed by Jia *et al*, 2011).

Function

HSP-60 provides transport, intervenes in the early stages of folding and refolding of proteins and under stress conditions prevents denaturation, however when proteins are beyond repair it can aid denaturation as well (reviewed by Saibil *et al*, 2013).

HSP-60 can accumulate in the cytosol causing dual apoptotic effects; when the accumulation is caused by mitochondrial release the chaperonin interacts with caspase-3 resulting in the induction of apoptosis. However when cytosolic accumulation is not caused by mitochondrial release HSP-60 does not interact with caspase-3 and has an anti-apoptotic effect (Chandra *et al*, 2007).

HSP-60 has been found to form cytosolic complexes with the apoptotic molecules bcl-2-like protein 4 (Bax), bcl-2 homologous antagonist/killer (Bak) and B-cell lymphoma-extra large (Bcl-XL) in normal conditions, after apoptosis induction the HSP-60/Bax complex separates, Bax reaches the mitochondria and cytochrome-C is released triggering apoptosis through the caspase cascade, hence the initial cytosolic complex is thought to inhibit apoptosis and HSP-60 to have an important regulatory role (Gupta and Knowlton, 2005).

Immunity

In immunity HSP-60 can elicit pro and anti-inflammatory responses subject to its concentration as moderate levels of the chaperonin result in anti-inflammatory actions through TLR2 signalling to CD4+, CD25+ regulatory T cells while a high HSP-60 concentration leads to TLR4 activation in dendritic cells and macrophages producing maturation of dendritic cells, antigen presentation and release of inflammatory cytokines. In addition HSP-60 is involved in the resolution of inflammation through specific regulatory T cells mediated by suppressive cytokines while B cells participate at the innate and adaptive response with the ability to mediate inflammatory responses through antibodies, antigen presentation and anti-inflammatory effects via IL-10 production (reviewed by Quintana and Cohen, 2011).

The highly conserved nature of HSP-60 suggests that bacteria contain similar forms which are potent triggers for antibodies in the human organism. Autoantibodies to HSP-60 have been

characterised and linked to diabetes and arthritis among other autoimmune disorders, entailing potential for therapy development (Imatoh *et al*, 2009; Zonneveld-Huijssoon *et al*, 2012).

Cardiac Involvement

Apoptotic control within cardiac myocytes has been demonstrated as HSP-60 reduction resulted in a marked increase in apoptosis, after hypoxia apoptosis was triggered seemingly due to a redistribution of HSP-60 from the cytosol to the plasma membrane; in addition, HSP-60 levels decreased during reoxygenation, showing a picture of the possible activity of HSP-60 during cardiac IRI (Knowlton and Gupta, 2003).

HSP-60 presents dual potential according to its location and concentration; extracellular HSP-60 could have a detrimental effect as presented in an IRI mouse model showing HSP-60 associated apoptosis mediated by TLR2 and TLR4 (Heiserman *et al*, 2015). Furthermore, it induced inflammation and cytokine production after cardiac ischaemia in rat hearts (Tian *et al*, 2013).

The extracellular transport of HSP-60 could be partly mediated by exosomes, lipid vesicles transporting molecules between cells, as reported in cardiomyocytes under stress circumstances such as IRI and oxidative stress where exosome production increased along with their extracellular release of HSP-60 (Malik *et al*, 2013).

10.1.6 HSP-70

The ubiquitous and highly conserved HSP-70 is the most abundant chaperone, with 13 human members, HSP-70 is present in several cellular compartments, it coordinates unfolding, disaggregation, refolding and degradation of proteins while cooperating with a range of cofactors fulfilling several different functions including translocation through organelle membranes (reviewed by Saibil *et al*, 2013).

HSP-70 interacts with most proteins in aggregated, unfolded or misfolded forms and does not seem to interact with folded proteins, however it can regulate the activity and stability of certain native proteins as well (reviewed by Mayer, 2013).

The HSP-70 family includes several members varying slightly in their molecular weight and localisation, HSP-78 and HSP-75 act as chaperones in the ER and mitochondria respectively while the better known HSP-73 (HSC70) and HSP-70 (HSP-72) are located in the nucleus and cytoplasm, they perform similar functions promoting cellular survival with the constitutional HSP-73 maintaining homeostasis in normal circumstances while HSP-70 is an inducible isoform presenting heightened expression during stress (reviewed by Noble *et al*, 2008).

Structure

HSP-70 is formed by a N-terminal nucleotide binding domain (NBD) and a C-terminal substrate binding domain (SBD), the NBD or ATPase domain contains a hydrophilic pocket to which ATP is bound while the SBD includes a substrate binding subdomain β (SBDβ) which recognises target proteins by binding to hydrophobic residues, and an α-helical subdomain (SBDα) which acts as a lid for SBDβ (reviewed by Mayer and Kityk, 2015).

SBD arrangement is importantly different in its open or closed conformations which affects the interaction with NBD and with substrate proteins. In the open conformation, mediated by ATP binding, SBDα is separated from SBDβ and both subdomains are attached to different sections of the NBD (Kityc *et al*, 2012; Qi *et al*, 2013). This stabilises the SBD, presenting a high rate of substrate association and dissociation reflected on fast binding and release kinetics resulting in a low affinity for polypeptides (Mayer *et al*, 2000).

After ATP is hydrolysed by the NBD, the ADP linked closed conformation takes place with SBD acting as a lid and causing low peptide association and dissociation rates leading to high peptide affinity allowing HSP-70 to limit aggregation (reviewed by Mayer and Kityk, 2015).

Co-chaperones

HSP-70 functions through an ATP dependent process which presents at a very low hydrolysis rate thus limiting its activity; in order to improve hydrolysis rate HSP-70 works with co-chaperones such as J-domain proteins (JDPs) also known as HSP-40 which stimulate HSP-70 ATPase activity up to 1000 fold, or nucleotide exchange factors (NEFs) which promote protein substrate release. JDPs additionally contribute to the process by binding to substrate proteins and preventing its aggregation while it delivers the protein to HSP-70, it targets specific sites

within the chaperone and seems to be an important HSP-70 recruiter. The activity of NEFs promoting the release of substrates allows them to complete folding or to be re-bound by HSP-70 and repeat the process until its appropriate native conformation is achieved (reviewed by Kampinga and Craig, 2010; Saibil *et al*, 2013).

Function

Chaperone

The main function of HSP-70 is to act as a chaperone for aiding protein folding, refolding, transport and degradation of misfolded and unstable proteins. Seemingly the state of the protein substrate that HSP-70 binds to and the activity of its co-chaperones lead to different physiological functions of HSP-70 with a misfolded protein eliciting a different activity than a newly synthesised unfolded polypeptide allowing several different functions by the same chaperone possibly due to the allosteric mechanisms involved (Zhuravleva and Gierasch, 2015; reviewed by Clerico *et al*, 2015).

In normal conditions HSP-70 binds to newly forming proteins after ribosome release, acting as a holdase in order to avoid premature folding when the protein needs to be transported to their destinations in an unfolded state across membranes or until circumstances are adequate for correct folding (Chacinska *et al*, 2009). Holdase activity can also take place in order to avoid aggregation after stress causing partially folded proteins to accumulate (reviewed by Clerico *et al*, 2015). It has been suggested that HSP-70 could also actively unfold incorrectly or alternatively folded proteins when necessary (reviewed by Mattoo and Goloubinoff, 2014).

Degradation is mediated by HSP-70 along with co-chaperones, such as HSP-40 when substrate proteins have failed to reach their appropriate structure (Summers *et al*, 2013). An alternate phosphorylation pattern has been reported to play a role in balancing protein folding and degrading activity for HSP-70 (Muller *et al*, 2013).

Signalling Regulation

HSP-70 is involved in signalling pathways that influence numerous important cellular processes; although the mechanisms by which HSP-70 controls cell signalling have not been

well established they seem to be linked to its chaperone activity as important signalling molecules are assisted by HSPs during their formation (reviewed by Sherman and Gabai, 2015).

Affected pathways include the suppression of kinase cascades influencing events such as inflammatory response by limiting NF-κB (Cao *et al*, 2012), as well as reducing iNOS and NADPH activity and increasing superoxide in neutrophils leading to a limitation in oxidative stress (reviewed by Giffard *et al*, 2008).

HSP-70 acts extracellularly after either being actively secreted or released by dying cells and influences both innate and adaptive immunity; mainly through TLR-2 and TLR-4 activity it can elicit different responses influencing antigen presentation and promoting the release of inflammatory cytokines, having a positive feedback effect on the further release of HSP-70 (Lee *et al*, 2013; reviewed by Borges *et al*, 2012).

HSP-70 furthermore impacts pathways with input in cancer development such as apoptosis and cell cycle through its inhibitor OIS as well as transcription factors including HIF-1, NF-κB and others related to tumour growth and metastasis (Colvin *et al*, 2014).

HSP-70 involvement in tumour formation and cancer beyond cell signalling has been established by its overexpression in certain types of malignant cells and has been found to be a predictor of cancer stage and prognosis (Ciocca *et al*, 1993; Hwang *et al*, 2003).

Anti-apoptotic effects mediated in collaboration with its co-chaperone HSP-40 by stabilising inhibitors and supressing TNF induction, blocking caspase activation, BAX translocation and prevent mitochondrial outer membrane permeability have been reported (Ran *et al*, 2004; reviewed by Sharp *et al*, 2013). In addition, HSP-70 has been deemed essential for the development of cancer in a breast cancer model in which oncogene activation led to 100% penetrance in control animals compared to a rare tumour development in HSP-70 knockout specimens (Meng *et al*, 2011).

The seemingly crucial role of HSP-70 in cancer development makes it a very promising candidate for targeted therapy as its expression is significantly elevated in various cancer types compared to normal cells and it seems to be unessential for survival as HSP-70 knockout mice are viable (reviewed by Sherman and Gabai, 2015).

Cardiac Involvement

HSP-70 is involved in several cardiac processes concerning both beneficial and pathological effects. After ischaemia HSP-70 regulates the innate immune response in the heart as well as its function after reperfusion; as the chaperone is secreted in the myocardium its extracellular activity mediates inflammatory reactions contributing to myocardial dysfunction while cytokine induction takes place as an effect of TLR-4 activation in cardiac tissue and macrophages (reviewed by Liu *et al*, 2012).

On the other hand HSP-70 mediates cardiac tolerance to endotoxins through a supressed TNF-α response in macrophages and inhibition of ICAM, NF-κB and TNF-α within the heart (Zou *et al*, 2008; Su *et al*, 2010).

HSP-70 is found during atherogenesis within the developing plaques, playing an apparent protective role against cellular stress (Dupont *et al*, 2008). Further evidence for cardiac protection aided by apoptosis inhibition has been identified in diabetes studies in which increased cardiac injury was observed and linked to low HSP-70 levels due to insulin dysfunction as insulin is important for the maintenance of adequate levels of constitutive HSP-70 (Chen *et al*, 2006).

HSP-70 enhanced expression after late preconditioning has been linked to myocardial protection after CPB in a pig model (Yan *et al*, 2013). Its exogenous administration in a rat model resulted in reduced levels of cytokines which transcribed in cardiac protection during ischaemia and sepsis conditions (Pasqua *et al*, 2015).

Upregulation of the chaperone has been established in the human heart even after short periods of cardiac ischaemia after CABG surgery (Taggart *et al*, 1997). Longer CPB times were linked to higher levels of HSP-70, and after open heart surgery adult patients presenting higher levels of HSP-70 before surgery showed a link to lower cardiac specific enzymes and injury markers aspartate aminotransferase, lactate dehydrogenase (LDH), CK and CK-MB) (Demidov *et al*, 1999). Children who did not present such pre-operative up-regulation were linked to an important increase in the cardiac injury markers Troponin I, Myoglobin, LDH, CK, CK-MB (Giannessi *et al*, 2003). HSP-70 expression related to improvement in right ventricle

function in human myocardium resected from tetralogy of Fallot paediatric patients (Peng *et al*, 2011), furthermore its protein expression in right atrial tissue was enhanced after RIPC and linked to lower cardiac injury markers, better haemodynamic state and lower inflammatory markers in a paediatric clinical trial (Zhou *et al*, 2010).

Cardiac rhythm dysfunction is a consequence of IRI and the occurrence of atrial fibrillation has been linked to lower HSP-70 expression in atrial cells compared to patients with normal sinus rhythm after cardiac surgery, furthermore a HSP-70 polymorphism causing increased risk of fibrillation after surgery has been reported (Afzal *et al*, 2008; reviewed by de Jong *et al*, 2009).

The cardioprotective effects of HSP-70 against IRI seem to be related to oxidative stress resistance secondary to previous exposure to stress in findings that support the early studies that identified important changes in HSP-70 expression in the myocardium after preconditioning (Kingma, 1999; reviewed by de Jong *et al*, 2009). A RIPC study in knocked out mice showed that late phase RIPC induced the expression of the almost identical subtypes HSP70.1 (HSPA1B) and HSP70.3 (HSPA1A) through the action of *NF-κB,* with HSP70.3 providing cardiac protection while HSP70.1 had detrimental effects over the exposed heart (Tranter *et al*, 2010).

10.1.7 HSP-90

The ATP-dependent heat shock protein 90 (HSP-90) is one of the most abundant chaperones, it is highly conserved across species and has two main isoforms in mammals, cytoplasmic HSP-90α is induced under stress while HSP-90β is expressed constitutively. Further less known analogues are present in other cellular compartments including GRP94 in the ER and TRAP-1 in the mitochondria (reviewed by Li and Buchner, 2013).

Having an essential role in eukaryotic cells, HSP-90 mediates the late stage of protein folding (Nathan *et al*, 1997). HSP-90 works forming complexes with numerous co-chaperones which vary according to cellular compartment, this contributes to a very adaptable system with the capacity to act on a wide variety of clients (reviewed by Jackson, 2013).

HSP-90 assists several different client proteins important for cellular signalling and development including protein kinases, steroid hormone receptors and transcription factors, hence participating in the integration of signalling, transport, and protein degradation (reviewed by Li and Buchner, 2013; Saibil *et al*, 2013). The interaction between HSP-90 and certain important kinases and transcription factors such as p53 and Bcl6 link it to oncogenic processes (reviewed by Khurana and Bhattacharyya, 2015).

It has been suggested that HSP-90 has a role in rescuing mutated proteins with altered properties that could participate in adaptation to different circumstances as part of the mechanisms of evolution (Jarosz and Lindquist, 2010).

Induction

The expression of HSP-90 is enhanced by stress including hypoxia and IRI, it is upregulated in a number of cancers and other pathologies such as chronic obstructive pulmonary disease with its expression levels having potential as an indicator of prognosis (reviewed by Zuehlke *et al*, 2015).

The inducible HSP-90α is mainly controlled by HSF-1 (Tang *et al*, 2016), however other transcription factors are involved including NF-κB (Ammirante *et al*, 2008), STAT1 following interferon-γ stimulation and STAT3 after IL-6 stimulation (reviewed by Taipale *et al*, 2010).

Interestingly HSP-90 has an effect on its regulators as it modulates short term HSF-1 effects in the expression of HSPs (Leach *et al*, 2012), while extracellular HSP-90 can trigger inflammation by interacting with NF-κB and STAT3 (Bohonowych *et al*, 2014).

Structure

HSP-90 is a homodimer of subunits, each subunit is formed by three domains, an N-terminal ATP binding domain (N-domain), a middle domain (M-domain) and a C-terminal dimerization domain (C-domain), these domains are linked by flexible regions; all components allow various dynamic conformations for the chaperone in order to adapt to different client proteins (reviewed by Li and Buchner, 2013; Saibil *et al*, 2013).

The ATP binding domain contains a fold characteristic of a group of split ATPases, a lid is formed which closes over the nucleotide binding pocket during the ATP-bound state and remains open during the ADP-bound state (Ali *et al*, 2006).

The M-domain comprises catalytic residues that participate in the formation of a composite ATPase site, hence it has a role in ATP hydrolysis and it also contributes to the interaction sites for co-chaperones and client proteins (Meyer *et al*, 2004).

Finally, the C-domain allows the dimerization of HSP-90 subunits in a process during which the opening of the C-domain is synchronised with the closing of the N-domain, additionally it contains a docking site for the interaction with certain co-chaperones (Ratzke *et al*, 2010; reviewed by Li and Buchner, 2013).

Cardiac Involvement

The role of HSP-90 in facilitating cell survival through several pathways has been reported to contribute to cardiomyocyte resistance during ischaemia and reperfusion. During ischaemia the expression of HSP-90 was enhanced and linked to eNOS phosphorylation and NO production, which led to a reduction in infarct size after reperfusion (Chen and Meyrick, 2004; Kupatt *et al*, 2004).

The methylated state of HSP-90 seems to have a role in maintaining the integrity of striated and cardiac muscle through mediating stability of muscular bands presenting a potential impact in cardiac function (Voelkel *et al*, 2013).

HSP-90 has been established as an important mediator of cardiac protection, hypoxic preconditioning *in vitro* studies demonstrated increased cardiomyocyte survival after conditioning, related to mitoK_{ATP} channels function; an effect that was lost after HSP-90 inhibition (Jiao *et al*, 2008). Likewise enhanced HSP-90 expression resulted in cardiac protection against serum and glucose deprivation by conserving MMP, reducing toxicity and oxidative stress (Wu *et al*, 2012).

HSP-90 has been reported to mediate the mitochondrial import of protein kinases presenting mitochondrial dependent cardio-protective properties against IRI (Budas *et al*, 2010). In

addition HSP-90 has a role mediating hydrogen sulphide cardiac protection (Yang *et al*, 2011), while other processes including interaction with anti-apoptotic elements conferred decreased ER stress response, promoting cardiac cell survival (Lam *et al*, 2013).

HSP-90 and the pathways involved in these processes have therapeutic potential both in preventing and treating ischaemic responses.

10.2 Aims

To analyse the expression patterns of HSPs and HIF-1 in the myocardium of CHD patients requiring corrective surgery using CPB before and after hyperoxic incubation, and to assess the effects of RIPC on their expression.

10.3 Materials and Methods

Tissue was obtained from patients during surgical intervention, in cases of ventricular tissue resection part of the tissue was immediately processed as a baseline specimen and the rest was exposed to 60% O_2 incubation for 4 hours in order to mimic hyperoxygenation after CPB; atrial samples were only used for baseline analysis due to insufficient amount of tissue.

Timing of tissue resection depended on surgical protocols and due to particular circumstances regarding each case, variability is possible based on the time the tissue was obtained relative to CPB and cross clamping, implying longer exposure to hypoxia and inflammatory responses for some specimes.

mRNA was extracted and used in order to obtain cDNA through a RT-PCR reaction, then Q-PCR analysis was undertaken in order to obtain relative quantification values for each gene. Genes used during the assay are shown in *Table 10.1*.

Details on materials and methods are described on *Chapter 2*.

	Gene name	Gene aliases	Gene Symbol	Assay Design	Amplicon length	Assay ID
HO-1	heme oxygenase 1	HMOX1, HO-1, HSP32	HMOX1	Probe spans exons	82	Hs01110250 _m1
HSP-60	heat shock 60kDa protein 1	CPN60, HSP65, HuCHA60,	HSPD1	Probe spans exons	81	Hs01036753 _g1
HSP-70	heat shock 70kDa protein 1A	HSP70-1A, HSP70I, HSP72, HSPA1	HSPA1A	Both primers and probe map within a single exon	124	Hs00359163 _s1
HSP-90	heat shock protein 90kDa alpha	EL52, HSP86, HSP89A, HSPC1 Hsp89	HSP90AA1	Both primers and probe map within a single exon	133	Hs00743767 _sH
HIF-1	hypoxia inducible factor 1, alpha subunit	HIF-1A HIF-1α MOP1 PASD8	HIF1A	Probe spans exons	76	Hs00153153 _m1

Table 10.1. Genes analysed and assay information. Abbreviations: HIF-1: hypoxia inducible factor 1: HO-1: heme oxygenase-1; HSP: heat shock protein.

10.4 Statistical analysis

The data did not present a normal distribution, hence non-parametric tests were used.

Spearman analysis was used in order to determine correlation between demographic characteristics including patients´ gender, age, weight, cyanosis status, and to cTnI levels.

Expression variation according to tissue type was analysed using Mann Whitney tests, while Wilcoxon signed-rank test for paired measures was used to explore differences before and after tissue incubation.

Mann Whitney tests were used to determine differences according to RIPC or control allocation for each time point at baseline and after incubation. All analyses were performed using SPSS version 23 software, a p value <0.05 (two tailed) was considered significant.

10.5 Results

Fifty patients were recruited for the study, demographic data can be seen in *Table 3.1*.

10.5.1 General analysis

The four HSPs analysed, and HIF-1 were expressed in the myocardial tissue (*Figure 10.1*).

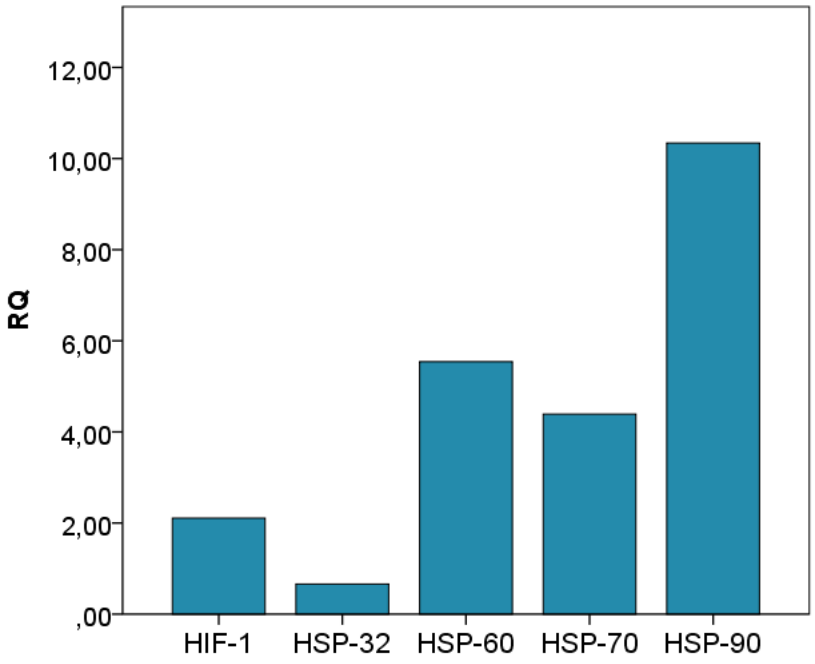

Figure 10.1. mRNA gene expression in myocardium of CHD patients at baseline. Figure shows the median for RQ values of each gene. Abbreviations: HIF-1: hypoxia inducible factor 1: HO-1: heme oxygenase-1; HSP: heat shock protein; RQ: relative quantification.

Weight correlated to HO-1 (r_s=0.393, p=0.029), HSP-70 (r_s=0.433, p=0.015) and HSP-90 (r_s=0.420, p=0.019), while age correlated positively to HSP-70 (r_s=0.525, p=0.002) and HSP-90 expression (r_s=0.494, p=0.005) (*Figure 10.2*). In addition HSP-90 correlated to cyanotic status (r_s=0.422, p=0.18), having a higher expression in acyanotic patients (p=0.020). There was no correlation to cTnI levels.

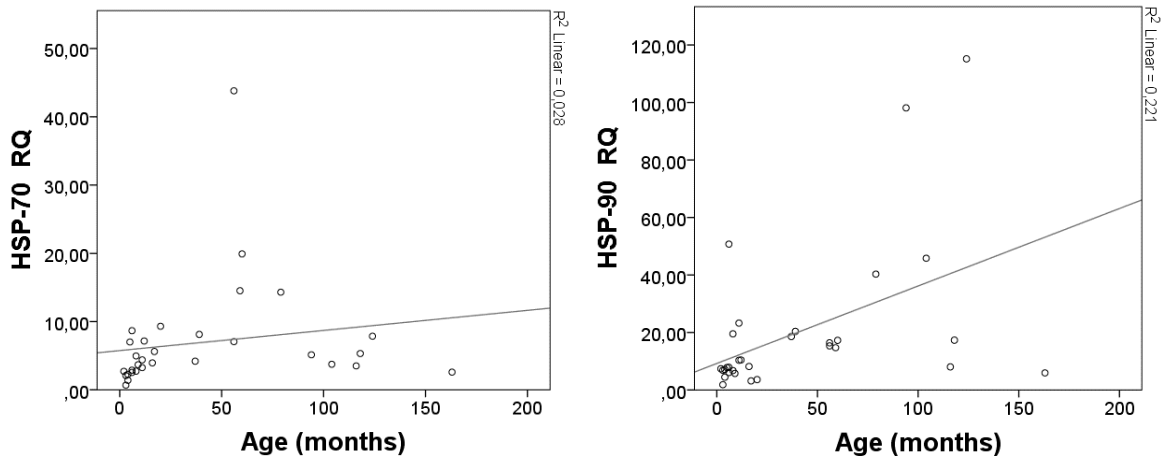

Figure 10.2. Correlation of HSPs to patient demographic and baseline characteristics. Abbreviations: HO-1: heme oxygenase-1; HSP: heat shock protein; RQ: relative quantification.

Expression according to tissue type

Table 10.2 shows descriptive characteristics of gene expression according to ventricular or atrial tissue. HO-1 and HSP-70 expression was significantly higher in atrial tissue while all other analysed genes did not present a significant variation (*Figure 10.3*).

Gene	N	Tissue	Median - IQ range	P value
HO-1	22	Ventricle	0.57(0.26-0.93)	0.010
	9	Atrium	1.64(0.66-3.12)	
HSP-60	22	Ventricle	6.03(3.04-7.57)	0.292
	9	Atrium	2.50(2.28-8.21)	
HSP-70	22	Ventricle	3.71(2.71-5.61)	0.020
	9	Atrium	8.11(5.32-14.30)	
HSP-90	22	Ventricle	7.95(6.76-19.52)	0.716
	9	Atrium	15.33(8.16-17.33)	
HIF-1	22	Ventricle	2.72(1.42-11.19)	0.064
	9	Atrium	1.25(0.56-2.95)	

Table 10.2. Descriptive statistics for mRNA gene expression according to ventricular or atrial tissue in CHD patients. Data is expressed as mean and standard deviations. Abbreviations: HIF-1: hypoxia inducible factor 1: HO-1: heme oxygenase-1; HSP: heat shock protein.

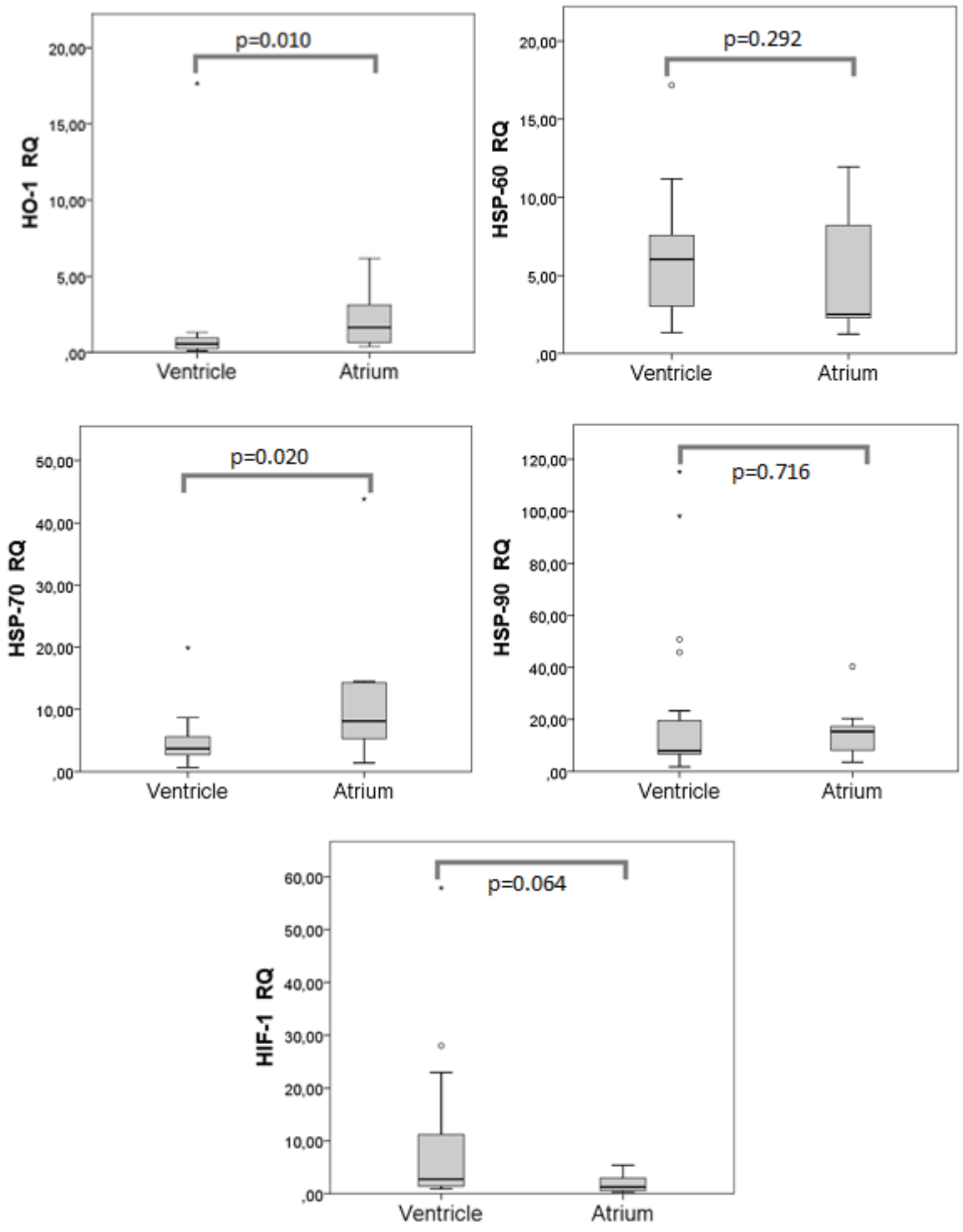

Figure 10.3. mRNA gene expression according to ventricular or atrial tissue in CHD patients. Abbreviations: HIF-1: hypoxia inducible factor 1: HO-1: heme oxygenase-1; HSP: heat shock protein; RQ: relative quantification.

Expression after tissue incubation

Descriptive characteristics of analysed gene expression before and after tissue incubation at 60% O_2 concentration are presented in *Table 10.3*.

Gene	N	Time point	Median – IQ range	P value
HO-1	15	Baseline	0.394(0.25-0.64)	0.001
	15	Incubated	3.72(1.31-6.39)	
HSP-60	15	Baseline	5.54(3.04-6.66)	0.125
	15	Incubated	3.86(2.54-5.36)	
HSP-70	15	Baseline	3.25(2.59-4.39)	0.001
	15	Incubated	19.90(9.99-28.18)	
HSP-90	15	Baseline	7.371 (6.068-10.429)	0.691
	15	Incubated	8.206 (6.816-9.893)	
HIF-1	15	Baseline	1.534 (1.133-3.497)	0.650
	15	Incubated	1.933 (1.237-3.224)	

Table 10.3. Descriptive statistics for mRNA gene expression in the myocardium before and after incubation. Data is expressed as median and IQ range. Abbreviations: HIF-1: hypoxia inducible factor 1: HO-1: heme oxygenase 1; HSP: heat shock protein; IQ: interquartile.

HO-1 and HSP-70 expression was significantly higher after incubation, all other genes did not present significant variation (*Figure 10.4*).

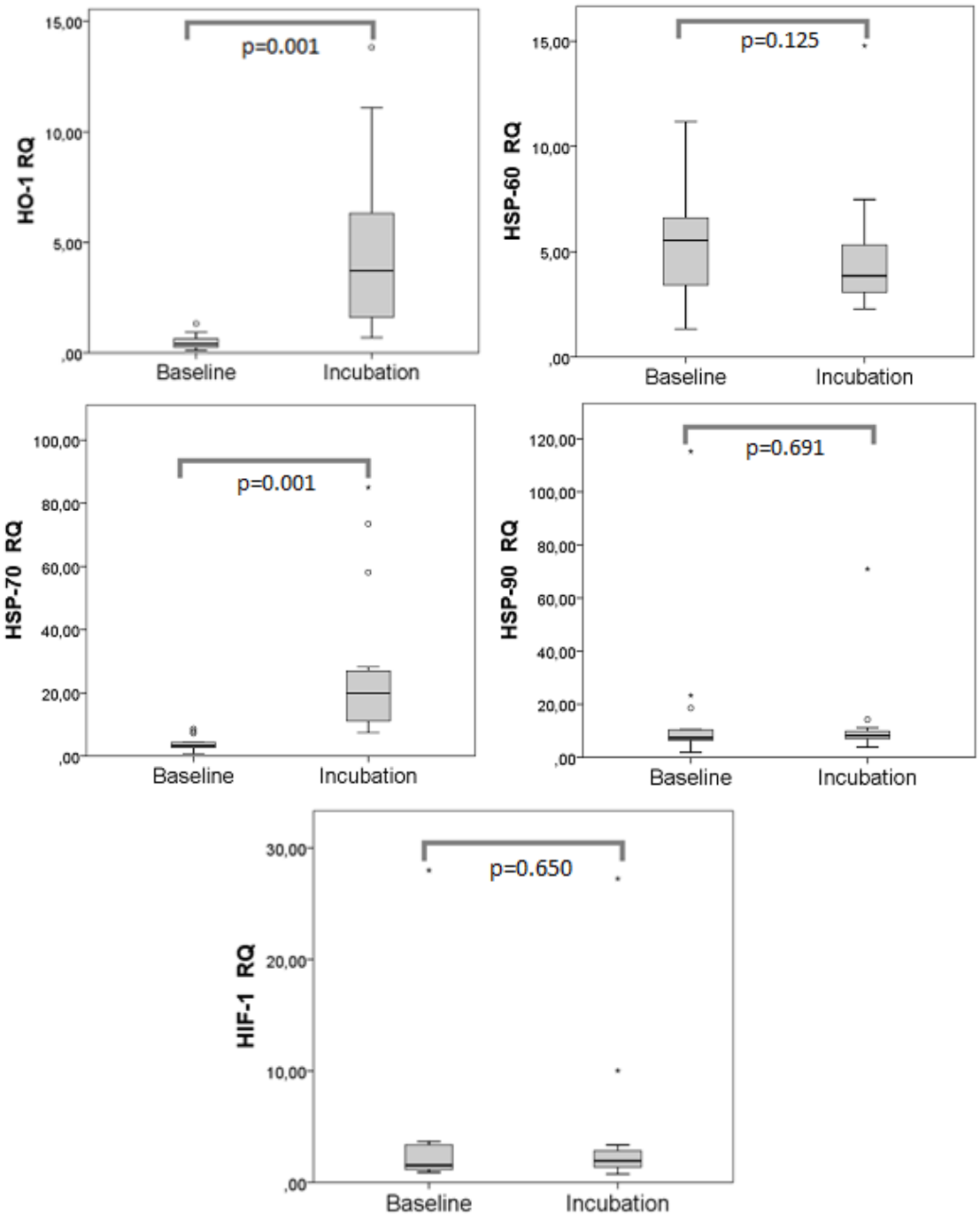

Figure 10.4. mRNA gene expression in the myocardium before and after incubation. Abbreviations: HIF-1: hypoxia inducible factor 1; HO-1: heme oxygenase 1; HSP: heat shock protein; RQ: relative quantification.

10.5.2 RIPC analysis

Forty-eight patients were analysed according to intervention allocation. Descriptive data on gene expression according to patient allocation to RIPC or control is summarised in *Table 10.4*.

Gene	Time point	Intervention	N	Median – IQ range	P value
HO-1	Baseline	RIPC	14	0.45(0.31-0.93)	0.206
		Control	15	0.77(0.39-1.64)	
	Incubation	RIPC	9	3.715(1.916-6.394)	1.000
		Control	6	4.091(1.306-6.248)	
HSP-60	Baseline	RIPC	14	3.95(2.69-6.28)	0.050
		Control	15	6.83(4.74-8.81)	
	Incubation	RIPC	9	3.735(2.539-5.287)	1.000
		Control	6	4.034(3.621-5.354)	
HSP-70	Baseline	RIPC	14	4.29(2.71-7.85)	0.793
		Control	15	4.96(2.74-7.06)	
	Incubation	RIPC	9	20.512(8.803-28.184)	0.864
		Control	6	19.181(13.102-25.703)	
HSP-90	Baseline	RIPC	14	9.23(7.37-17.27)	0.760
		Control	15	16.41(6.07-40.30)	
	Incubation	RIPC	9	8.775(7.223-9.893)	0.689
		Control	6	8.182(6.816-9.820)	
HIF-1	Baseline	RIPC	14	1.79 (1.12-3.08)	0.222
		Control	15	2.95 (1.42-11.19)	
	Incubation	RIPC	9	1.484(1.133-2.451)	0.181
		Control	6	2.338(1.933-3.224)	

Table 10.4. Descriptive statistics for HSP mRNA expression in the myocardium before and after incubation according to group allocation. Data is expressed as mean and standard deviations. Abbreviations: HIF-1: hypoxia inducible factor 1; HO-1: heme oxygenase 1; HSP: heat shock protein; RIPC: remote ischaemic preconditioning.

The expression of HO-1 (*Figure 10.5*), HSP-60 (*Figure 10.6*), HSP-70 (*Figure 10.7*), HSP-90 (*Figure 10.8*) and HIF-1 (*Figure 10.9*) were not differentially expressed according to RIPC or control allocation.

The expression of all genes was not different according to group allocation at baseline when ventricular and atrial specimens were analysed separately.

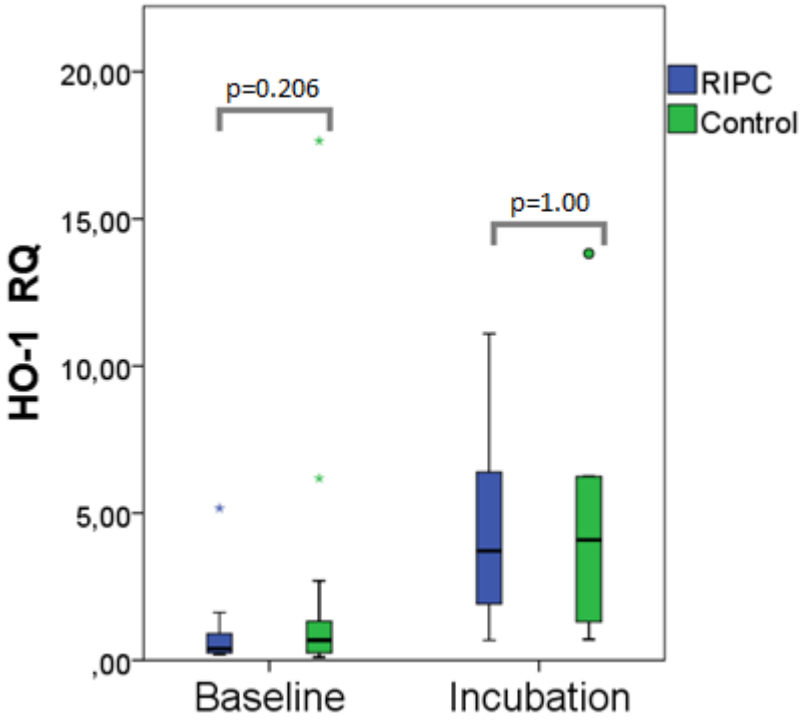

Figure 10.5. HO-1 mRNA myocardial expression before and after incubation according to intervention group allocation. Abbreviations: HO-1: heme oxygenase 1; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

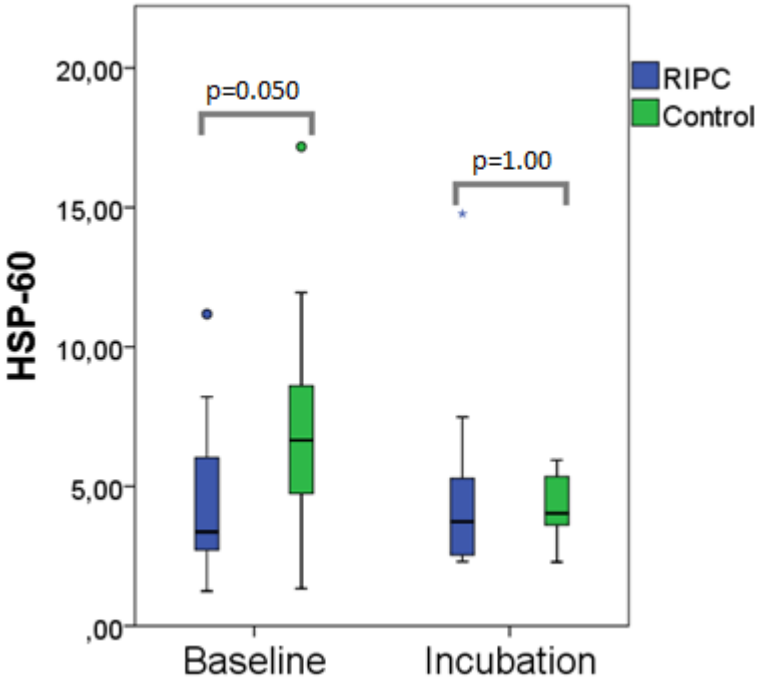

Figure 10.6. HSP-60 mRNA expression in the myocardium before and after incubation according to group allocation. Abbreviations: HSP-60: heat shock protein 60; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

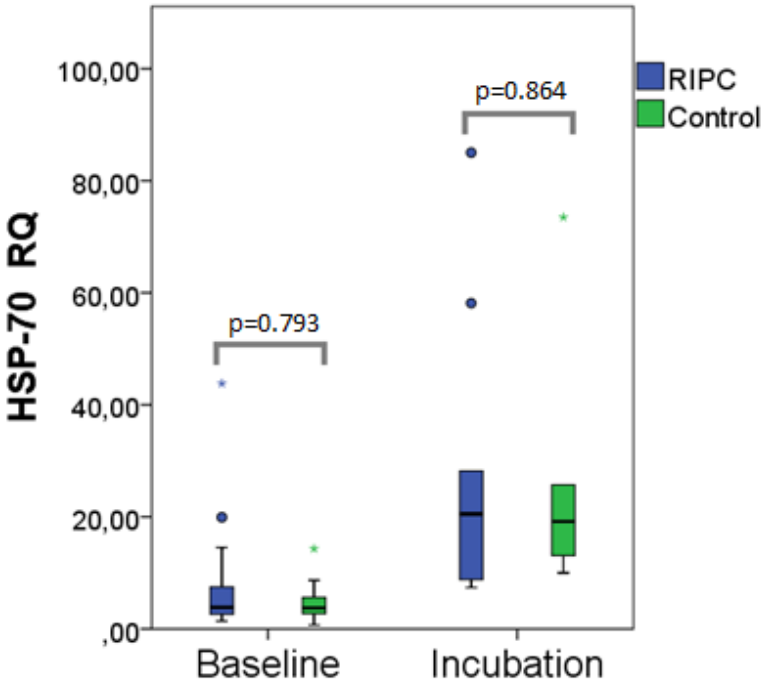

Figure 10.7. HSP-70 mRNA expression in the myocardium before and after incubation according to group allocation. Abbreviations: HSP-60: heat shock protein 70; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

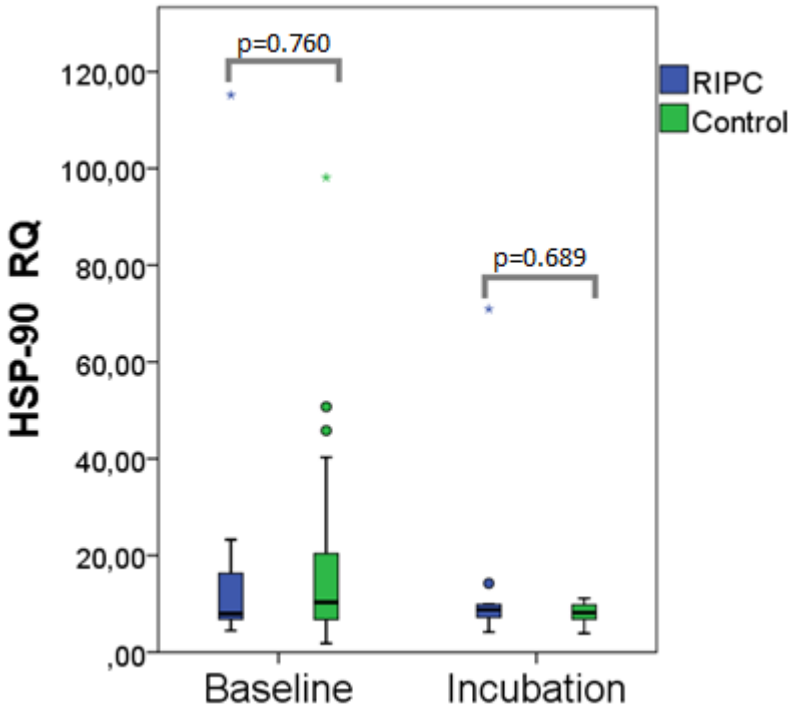

Figure 10.8. HSP-90 mRNA expression in the myocardium before and after incubation according to group allocation. Abbreviations: HSP-90: heat shock protein 60; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

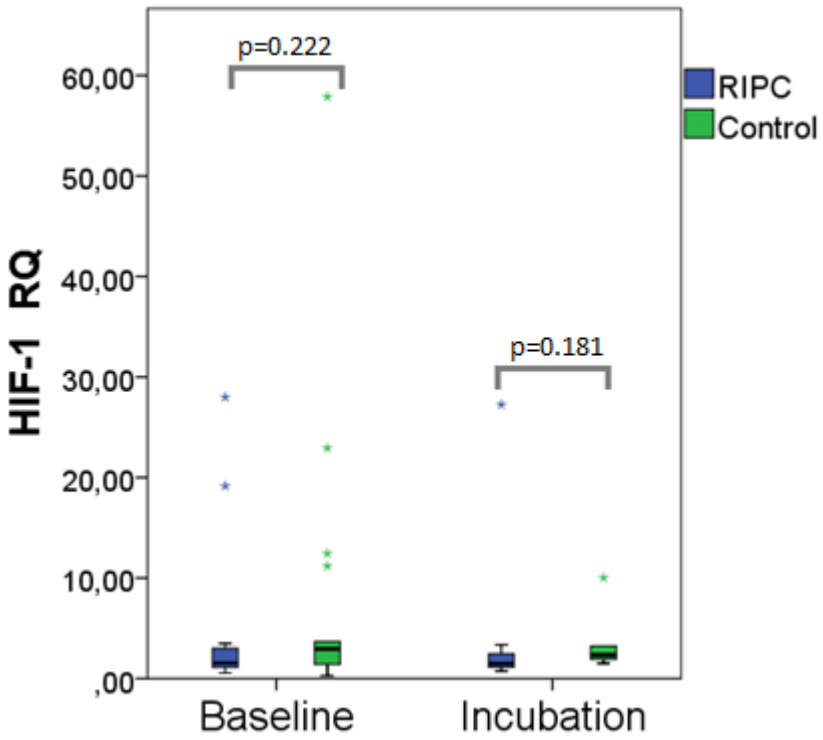

Figure 10.9. HIF-1 mRNA expression in the myocardium before and after incubation according to intervention allocation. Abbreviations: HIF-1: hypoxia inducible factor 1; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

10.6 Summary of key findings

- HSPs were expressed in the myocardium of CHD patients.
- HO-1 expression correlated to weight, while HSP-70 and HSP-90 expression correlated to weight and age.
- HSP-90 presented a higher expression in acyanotic patients.
- HO-1 and HSP-70 presented a higher expression in atrial tissue.
- HO-1 and HSP-70 presented a higher expression after hyperoxic incubation for 4 hours.
- The analysed genes did not show a significant differential expression after RIPC compared to controls.

10.7 Limitations

The number of available atrial tissue specimens was lower to that on the ventricular group. A comparison including additional atrial samples may increase the power to define gene expression differences according to tissue type.

10.8 Discussion

This study demonstrated that the four HSPs analysed and HIF-1 were expressed in the myocardium at baseline conditions, with HSP-90 presenting the highest expression while HO-1 showed the lowest activity. It is to be noted that although baseline tissue was not exposed to any experimental conditions, the stress stimulus of surgery, CPB and ischaemia had already started when tissue was resected which could have influenced expression patterns.

Expression of HSP-70 and HSP-90 increased with weight and age, while HO-1 correlated to weight suggesting that heat shock response in children may be lower than that seen in adult patients, furthermore younger and smaller children such as neonates could be prone to presenting a lower protective activity from these chaperones.

HSP-90 presented a higher expression in acyanotic patients. HSP-90 has been characterised as a protective factor and as RIPC stimulus is based on acute hypoxia, the chronic hypoxia cyanotic patients are exposed to could attenuate the response as pathways may have adapted and the triggering stimulus could have a higher threshold.

Expression of HSPs did not seem to have an effect on cardiac injury represented by cTnI measures.

With the exception of HSP-60, the analysed genes tended to present a higher expression in atrial tissue, with HO-1 and HSP-70 reaching statistical significance. It should be noted that the atrial resection component in the trial was implemented later in the study after an ethics amendment, furthermore atrial tissue was only resected in patients who were not already having ventricular tissue resected, hence the sample size for atrial tissue is smaller than the ventricular tissue available (9:22).

Previous microarray profiling research identified HSP-60, HSP-70 and HSP-90 both in the right atrium and left ventricle (Barth *et al*, 2005).

Once again with the exception of HSP-60, genes tended to have a higher expression after 4 hours of incubation at 60% O_2 concentration with HO-1 and HSP-70 reaching statistical significance. These results are consistent with previous work performed by our laboratory group.

These expression patterns suggest that hyperoxia had an increasing effect in the expression of these genes; considering that after CPB patients are exposed to re-oxygenation at 100% O_2 concentration, it could be inferred that HO-1 and HSP-70 activity would be considerably enhanced.

Consistently, HSP-70 has been found to be induced after hypoxia/re-oxygenation episodes with an inhibitory effect on apoptotic pathways (Terui *et al*, 2004), in addition its enhanced expression has been related to better post-operative right ventricle function in TOF patients, setting it as a potential protective mediator (Peng *et al*, 2011).

A tendency of HSP-60 to lower expression was seen after incubation; HSP-60 reduction after re-oxygenation has been previously described and linked to an increase in apoptosis, setting this reduction as a possible factor in the occurrence of IRI. (Knowlton and Gupta, 2003).

Expression of all genes tended to be higher at baseline in the control group compared to RIPC, however differences were not statistically significant at baseline or after incubation.

RIPC animal studies have linked increased extracellular HSP-60 levels to cardioprotection (Malik *et al*, 2013), however there is no previous experience on tissue expression on RIPC trials.

HSP-70 did not vary significantly in the RIPC group, this is in discordance to previous findings by Zhou *et al*, 2010, who performed the only previous dual protocol including early and late phase RIPC and reported higher HSP-70 expression in the RIPC group along with lower cardiac injury markers. The difference might be explained by the methods of the study as Zhou *et al* assessed HSP-70 protein expression through Western blot, in addition the analysis was performed using atrial tissue only.

HIF-1 did not show significant variation for any of the analysis performed in spite of being an important transcription factor acting during hypoxia and regulating HO-1. HO-1 was altered by hyperoxic incubation and it should be noted that baseline measurements showed a higher expression of HIF-1 compared to HO-1 which could have influenced the variation in HO-1 expression afterwards.

Previous studies linked HIF-1 expression to RIPC finding related oxidative stress reduction (Ong *et al*, 2014), lower inflammatory mediators and cTn levels in relation to HIF-1 in a RIPC clinical trial (Albrecht *et al*, 2013). The present results do not confirm these findings as RIPC had no significant effect on HIF-1 expression.

Conclusion

Hyperoxygenation had an enhancing effect on the expression of HO-1 and HSP-70, there was no significant effect of RIPC on the expression of the analysed genes. The implications of these expression patterns are yet to be elucidated.

Chapter 11. Small HSPs

11.1 Introduction

Small HSPs (sHSPs) have subunit molecular masses between 12-43 kDa, they are formed by a highly conserved α-crystallin domain (ACD) formed by β strands, located in the C-terminal domain, a less or non-conserved N-terminal domain (NTD) and short C-terminal extension (CTE) (reviewed by Haslbeck and Vierling, 2015).

sHSPs form homo or hetero oligomers using ACD as the main building block while NTD and CTE are structurally important for the assembly of large oligomers. CTE carries a conserved segment that attaches to hydrophobic sections of monomers forming tetra or hexamers that can then bind to other similar complexes through NTD contacts resulting in large oligomer formation (reviewed by Haslbeck and Vierling, 2015). These oligomers are reversible complexes, which are important for sHSPs response to high temperatures as parts of its segments become exposed and available to bind to non-native structures (reviewed by Zhang *et al*, 2015).

Ten sHSPs (HspB1 – HspB10) have been identified in humans which are further classified as class I when their expression is ubiquitous to all cell types or class II when their expression is restricted to certain tissue types (Taylor and Benjamin, 2005). *Table 11.1* shows sHSPs, human genome organisation (HUGO) and alternative nomenclature, classification, molecular weight, heat induction status and tissue distribution as well as individual functions in addition to their chaperone activities. Class I sHSPs are mainly heat inducible and function in stress conditions aiding cell survival while Class II types play a role in development, differentiation and specialised tissue functions.

sHSPs are ATP-independent "holdase" chaperones that stop native substrate proteins from establishing irreversible aggregations in order to release them when conditions become adequate for refolding through the action of ATP-dependent chaperones such as HSP-70 (reviewed by Zhang *et al*, 2015).

HUGO name	Aliases	Molecular Weight kDa	Class	Localisation	Heat Induced	Function
HSPB1	HSP-27	22.8	I	Ubiquitous	Yes	Stabilise cytoskeleton, antiapoptotic, antioxidant
HSPB2	MKBP	20.2	II	Cardiac and skeletal muscle	No	Myofibrillar integrity, antiapoptotic
HSPB3	HSPL27	17	II	Cardiac and skeletal muscle	No	Myofibrillar integrity
CRYAA	HSPB4 α-A crystalline	19.9	II	Lens of the eye	No	Genomic stability, lens refractive index
CRYAB	HSPB5 α-B crystalline	20.2	I	Ubiquitous	Yes	Stabilise cytoskeleton, cell cycle, cardio protection, antiapoptotic
HSPB6	HSP-20	16.8	I	Ubiquitous	No	Smooth muscle relaxation, cardioprotection, antiapoptotic
HSPB7	Cardiovascular HSP	18.6	II	Cardiac and skeletal muscle	No	Myofibrillar integrity
HSPB8	HSP-22	21.6	I	Ubiquitous	Yes	Autophagy
HSPB9	None	17.5	II	Testis	No	Testis cancer antigen
ODFI	HSPB10	28.3	II	Testis	No	Elastic cytoskeletal structure

Table 11.1. sHSPs nomenclature, molecular weight, classification, localisation and function. Abbreviations: HUGO: human genome organisation; HSP: heat shock protein. Table modified from Taylor and Benjamin, 2005; Bakthisaran and Tangirala, 2015.

sHSPs play a role in cardiac and neuro protection, angiogenesis, anti-inflammatory and anti-apoptotic reactions as well as participating in cellular functions including protein folding and degradation, cell differentiation, cell cycle and signalling pathways as well as preserving integrity of the cytoskeleton (reviewed by Bakthisaran and Tangirala, 2015).

A role in splicing, the process by which introns are removed and exons are joined together in order to create mRNA, has also been reported with sHPS facilitating splicing recovery after stress through association to splicing speckles in stress situations (Marin-Vinader *et al*, 2006). In addition sHSPs seem to form complexes with these speckles and denatured proteins in order to store them until conditions improve (reviewed by Vos *et al*, 2009).

HSP-27 protein expression in the cardiac tissue of TOF patients was analysed in a RIPC paediatric clinical trial, however no difference was found between treatment groups (Pepe *et al*, 2013).

11.1.1 HSP-20 (HSPB6)

HSP-20 was first described as part of a heterocomplex together with HSP-27 and CRYAB (Kato *et al*, 1994). Weighing 17 kDa it is ubiquitously expressed in most cell types, it is not heat inducible and fulfils different functions including regulation of smooth muscle relaxation, cardiac contractility and inhibition of platelet aggregation and has been linked to cardioprotective activity and to pathological processes such as Alzheimer´s disease and cancer (reviewed by Dreiza *et al*, 2010; Edwards *et al*, 2011).

HSP-20 does not form large oligomers, smaller quaternary structures have been reported conserving chaperone functions for this HSP as well as for HSPL27 and HSP-22 (Bukach *et al*, 2004; den Engelsman *et al*, 2009; Kim *et al*, 2004). HSP-20 tetramers are formed due to broad inter dimer patching made possible by tripeptide motifs located in a NTD section adjacent to the ACD (Weeks *et al*, 2014); this structural mechanism could be extrapolated to other sHSPs that form similar complexes.

HSP-20 interacts with other sHSPs including HSPB1, HSPB5, HSPB8 and the structurally less related HSPB2 suggesting HSP-20 could be universally compatible with other sHSPs (reviewed by Mymrikov *et al*, 2011).

HSP-20 undergoes posttranslational modification through phosphorylation which results in conformational changes that affect its interaction with other proteins including chaperone targets and causing disassociation of oligomers (reviewed by Dreiza *et al*, 2010; Mymrikov *et al*, 2011).

Cardiac Involvement

Cardiac anti-ischaemic, anti-apoptotic and anti-hypertrophic activity has been established for HSP-20 in relation to IRI (reviewed by Edwards *et al*, 2011). These cardioprotective effects are dependent on the phosphorylation of a PKA/PKC site in the NTD section of the chaperone (serine 16), in a process directed by the A-kinase-anchoring protein and tightly regulated by cyclic AMP (cAMP) (Edwards *et al*, 2012). Phosphorylated HSP-20 inhibits cardiac apoptosis (Fan *et al*, 2004), facilitates autophagy activation, a catabolic process of damaged organelle degradation and recycling which is upregulated during IRI, it plays an important role for cellular survival (Qian *et al*, 2009) and prevents hypertrophic responses (Martin *et al*, 2014b).

HSP-20 interacts with important apoptotic signalling molecules, overexpression of HSP-20 resulted in an increased ratio of Bcl-2/Bax, preventing Bax translocation from the cytosol to the mitochondria, avoiding cytochrome c release and caspase 3 activation in an IRI transgenic mouse model linked to reduced apoptosis as confirmed by the measurement of DNA fragmentation, resulting in reduced infarction and better functional recovery of the heart (Fan *et al*, 2005).

Hypertrophic cardiac stimuli such as constant β-adrenergic signalling and aortic constriction are linked to the expression of HSP-20 presenting high levels of the serine 16 phosphorylated variation, which in addition to the benefits already described has been linked to preventing hypertrophic response with mice models of HSP-20 overexpression showing smaller heart size increase along with lower levels of hypertrophic markers including BNP; this seems to be linked to HSP-20 mediated suppression of the apoptotic signalling molecule apoptosis signal-regulating kinase 1 (ASK1) (Fan *et al*, 2006).

Further mechanisms for hypertrophy prevention involve cAMP and phosphodiesterase 4 (PDE4), PDE4 hydrolyses cAMP, a second messenger with inotropic, chronotropic, pro-hypertrophy and apoptotic activities within cardiomyocytes which lead to the activation of PKC

mediated phosphorylation. HSP-20 forms a complex with PDE4, which maintains low surrounding levels of cAMP, thus preventing HSP-20 phosphorylation (reviewed by Edwards *et al*, 2011). Phosphorylation can then only be achieved if PDE4 becomes saturated or if the complex is disrupted leading to the protective effects of phosphorylated HSP-20. This was seen in animal models showing attenuation of action potential prolongation, prevention of cardiac remodelling, improved contractility and reduced perivascular fibrosis (Martin *et al*, 2014b).

A newly identified complex of HSP-20 and protein kinase D1 (PKD1), a kinase activated by chronic adrenergic signalling, hypertension and pressure overload, seems to play a significant role in the development of cardiac hypertrophy as well (Sin *et al*, 2015).

In addition, HSP-20 has been reported to act in myofilament contraction, translocating to the myofilaments after β-adrenergic stimuli and favouring the cycling of Ca^{2+} in the ER, achieving improved cardiac function (reviewed by Martin *et al*, 2014a).

A role of HSP-20 in myocardial angiogenesis has also been established, with the chaperone achieving increased proliferation, migration and tube formation when applied on human umbilical vein endothelial cells while interacting with the vascular endothelial growth factor (VEGF) (Zhang *et al*, 2012).

The importance of HSP-20 for myocardial survival has been further supported by the contribution of its inhibition to IRI mediated by mir-320, a micro RNA (mRNA) capable of downregulating HSP-20 expression, as its activity was linked to an increased susceptibility to IRI (Ren *et al*, 2009).

11.1.2 HSP-22 (HSPB8)

HSP-22 was first identified in human melanoma cells as a H11 protein kinase, it is heat inducible and expressed in all tissues, however higher levels have been found in smooth, skeletal and cardiac muscle, neurological tissue, lungs, kidneys and prostate cells (reviewed by Acunzo *et al*, 2012). HSP-22 structurally differs from other sHSPs as it is enriched in β-strands and lacks the characteristic β2 strand present in other sHSPs (Kazakov *et al*, 2009).

Involved in cellular signalling, recognition and regulation, HSP-22 interacts with glycolytic enzymes and protein kinases and forms hetero-oligomers with HSP-27, HSPB2, HSPB3, HSPB5, HSPB6 and HSPB7 (Fontaine *et al*, 2005; Sun *et al*, 2004), interactions that are influenced by the phosphorylation status of the chaperone binding to HSP-22 (Sun *et al*, 2006).

HSP-22 seems to deter the build-up of insoluble aggregates, either through preventing their aggregation or facilitating degradation of misfolded proteins (reviewed by Mymrikov *et al*, 2011).

HSP-22 can be phosphorylated by PKC, extracellular-signal-regulated kinase (ERK1) and cAMP dependent protein kinases at different sites, with ERK1 and cAMP activity resulting in important structural changes, causing a decrease in its chaperone function although precise mechanism and consequences for HSP-22 phosphorylation are yet to be investigated (reviewed by Acunzo, 2012; Mymrikov *et al*, 2011).

In addition to the chaperone functions described for sHSPs, HSP-22 has been linked to immune processes, apoptosis and cancer pathways as well as to cardiac cell survival and hypertrophy.

Cardiac involvement

HSP-22 has been characterised as one of the genes within a profile of surviving myocardium after chronic repeated ischaemia in the human heart (Depre *et al*, 2004). Cardioprotective effects have been further recognised in HSP-22 overexpression models (Depre *et al*, 2006).

The mechanisms by which HSP-22 aids cardiomyocyte survival have not been completely characterised, however an involvement of apoptotic pathways has been established as HSP-22 activates Akt, which prevents apoptosis through inhibitory phosphorylation of proapoptotic molecules. A second mechanism for survival involves glucose metabolism, assisted by increased translocation of the glucose transporter GLUT to the plasma membrane mediated by the activation of AMP dependent protein kinase as well as a larger content of glycogen within the myocardium (reviewed by Mymrikov *et al*, 2011).

HSP-22 has been reported to potentiate the bone morphogenetic protein (BMP) signalling pathway, BMPs are growth factors important for cardiac development with less known function

in the developed heart which nevertheless is involved in PI3K/Akt activation; a direct link has been established between HSP-22 expression and influence on this pathway which seems to be necessary for myocardial survival, placing HSP-22 as a protein kinase regulator (Sui *et al*, 2009).

HSP-22 overexpression models present with a high iNOS expression (Depre *et al*, 2006), which could be one of the most important aspects of HSP-22 mediated cardioprotection. In a process seemingly dependent on HSP-22 translocation to the cardiac mitochondria, a high expression of HSP-22 augmented the mitochondrial capacity to produce NO, stimulating oxidative phosphorylation in normal conditions and reducing it along with the production of ROS after ischaemia, producing similar results to those expected from RIPC and establishing HSP-22 as an important mediator with therapeutic potential (Laure *et al*, 2012; Rashed *et al*, 2015).

Interestingly, a dual cardiac effect of HSP-22 in apoptosis has been reported as transduction of low doses of adenovirus carrying HSP-22 resulted in cell hypertrophy mediated by the PI3 –Akt pathway while high doses led to apoptosis induction through inhibition of casein kinase 2 (Hase *et al*, 2005). Several studies performed in cancer cell lines have shown the dual pro and anti-apoptotic effect of HSP-22 leading to the assumption that its effects vary according to the cell line and level of expression of the chaperone (reviewed by Acunzo *et al*, 2012; Shemetov *et al*, 2008).

11.1.3 Cardiovascular HSP (HSPB7)

HSPB7 was first identified through bioinformatic algorithms as majorly expressed in cardiovascular and insulin sensitive tissues, finding a link to cardiomyopathies (Krief *et al*, 1999). HSPB7 is mainly expressed in cardiac and skeletal muscle, it has a molecular weight of 18.6 kDa and is not heat inducible (reviewed by Bakthisaran *et al*, 2015). It is located in the cytosol and a high association to myofibrils has been proven as a response to ischaemia (Golenhofen *et al*, 2004).

Basic characteristics seem to differentiate HSPB7 from other sHSPs, it does not seem to form oligomers within cells and has been identified in mono or dimeric forms and its anti-aggregation

properties do not seem to depend on HSP-70 collaboration (Vos *et al*, 2010). HSPB7 presents an association to splicing speckles in non-stress conditions and lacks the property of refolding heat denatured proteins, suggesting that this association to speckles is not related to HSPB7 chaperone activities (Vos *et al*, 2009).

Although a strong link to cardiovascular tissues has been established, the functions and mechanisms of HSPB7 have not been studied in depth. A controlling role in early cardiac morphogenesis in zebrafish embryos has been established resulting in serious morphological defects after HSPB7 depletion (Rosenfeld *et al*, 2013).

HSPB7 has been identified as a risk gene for the development of idiopathic dilated cardiomyopathy in a large study after microarray screening of 2000 genes, implying a strong importance of HSPB7 to the development of this pathology and to cardiac processes in general (Stark *et al*, 2010). Further genome-wide association analysis linked certain variants of the gene to dilated cardiomyopathy and cardiac failure (Cappola *et al*, 2011; Villard *et al*, 2011).

Levels of HSPB7 have been reported to rise and decrease rapidly after cardiac ischaemic events in animal models and human patients, with high levels being linked to increased risk of acute coronary syndrome, presenting potential for its use as an early biomarker (Chiu *et al*, 2012).

In addition to its cardiovascular effects, HSPB7 has been recognised as the most potent sHSP to prevent CAG triple expansion resulting in elongation of the glutamine (polyQ) tract within proteins; this expansion is responsible for neurodegenerative diseases such as Huntington disease, which are characterised by protein misfolding and aggregation contributing to protein toxicity (Vos *et al*, 2010).

11.2 Aims

To analyse the expression patterns of sHSPs in the myocardium of CHD patients requiring corrective surgery using CPB before and after hyperoxic incubation, and to assess the effects of RIPC on their expression.

11.3 Materials and Methods

Tissue was obtained from patients during surgical intervention; ventricular tissue was divided and part of it was immediately processed as a baseline reference, the remaining tissue was incubated at 60% O_2 for 4 hours; atrial tissue was only used for baseline analysis due to insufficient available tissue.

mRNA was extracted from the myocardial tissue and used in order to obtain cDNA through a RT-PCR reaction followed by Q-PCR analysis in order to obtain relative quantification values for each gene. Genes used during the assay are shown in *Table 11.2*. Details on materials and methods are described on *Chapter 2*.

	Gene name	Gene aliases	Gene symbol	Assay Design	Amplicon length	Assay ID
HSP-20	heat shock protein, alpha-crystallin-related, B6	Hsp20	HSPB6	Probe spans exons	96	Hs00328933_m1
HSP-22	heat shock 22kDa protein 8	HSP22, CMT2L, DHMN2, E2IG1, H11, HMN2, HMN2A, PP1629	HSPB8	Probe spans exons	66	Hs00205056_m1
HSP-27	heat shock 27kDa protein family, member 7 (cardiovascular)	RP11-5P18.6, cvHSP	HSPB7	Probe spans exons	79	Hs00205296_m1

Table 11.2. Genes analysed and assays used. Abbreviations: HSP: heat shock protein.

11.4 Statistical analysis

Correlation to gender, age, weight, cyanosis status, and post-operative cTnI levels were explored using Pearson analysis (r) in normally distributed data and Spearman analysis in non-parametric data (r_s).

Expression variation according to tissue type was analysed using the Mann Whitney test. Paired samples t-test or the non-parametric Wilcoxon signed-rank test for paired measures were used to explore differences before and after tissue incubation.

Mann Whitney tests or independent samples t-test were used as appropriate in order to determine differences according to RIPC or control allocation for each time point at baseline and after incubation.

Analysis was performed using SPSS version 23 software, a p value <0.05 (two tailed) was considered significant.

11.5 Results

Fifty patients were recruited for the study, demographic data and its analysis can be seen in *Tables 3.1* and *3.2*.

11.5.1 General analysis

The three sHSPs analysed were expressed in the myocardial tissue (*Figure 11.1*).

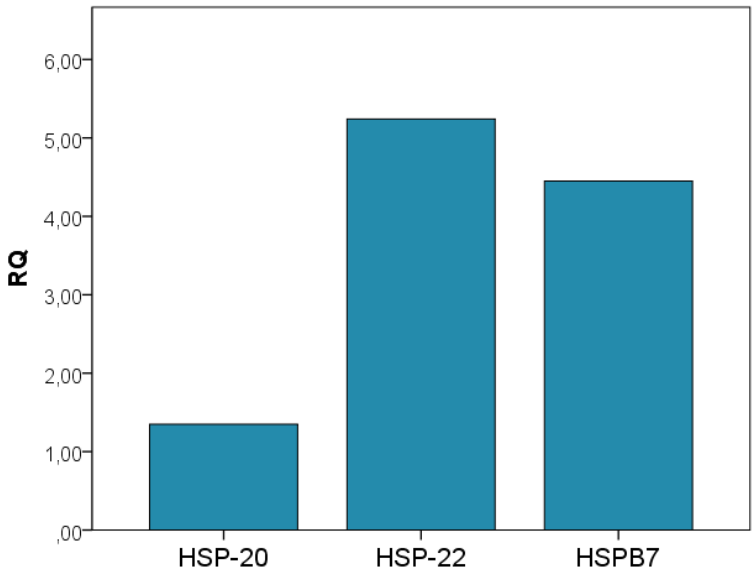

Figure 11.1. sHSP mRNA expression in myocardium of CHD patients. Figure shows the median for RQ values of each gene. Abbreviations: HSP: heat shock protein; RQ: relative quantification.

HSP-20 expression correlated to weight (r_s=0.444, p=0.012) and age (r_s=0.486, p=0.006). HSPB7 expression correlated to PO1 cTnI levels (r_s=0.357,p=0.049) (*Figure 11.2*).

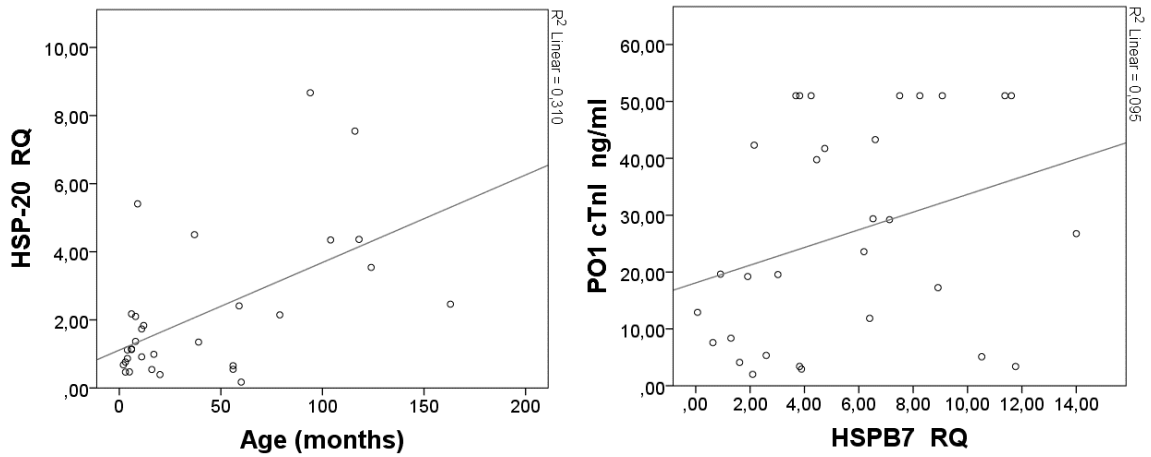

Figure 11.2. Correlation of HSP-20 to age and HSPB7 to cTnI levels. Abbreviations: cTnI: cardiac troponin I; HSP-20: heat shock protein 20; HSPB7: cardiovascular heat shock protein; PO1: post-operative 1.

Expression according to tissue type

Descriptive data for the expression of each gene according to tissue type is shown in *Table 11.3*.

Gene	N	Tissue	Mean – SD	Median – IQ range	P value
HSP-20	22	Ventricle		1.55(0.87-3.54)	0.313
	9	Atrium		1.12(0.55-2.15)	
HSP-22	22	Ventricle	6.391 ± 3.809		0.816
	9	Atrium	6.010 ± 4.810		
HSPB7	22	Ventricle	6.326 ± 3.585		0.057
	9	Atrium	3.514 ± 3.579		

Table 11.3. Descriptive statistics for sHSP mRNA expression according to ventricular or atrial tissue in CHD patients. Data is expressed as mean - SD or median – IQ range as appropriate. Abbreviations: HO-1: heme oxygenase 1; HSP: heat shock protein; HSPB7: cardiovascular HSP; IQ: interquartile; SD: standard deviation.

Expression of the sHSPs analysed did not vary according to atrial or ventricular tissue (*Figure 11.3*).

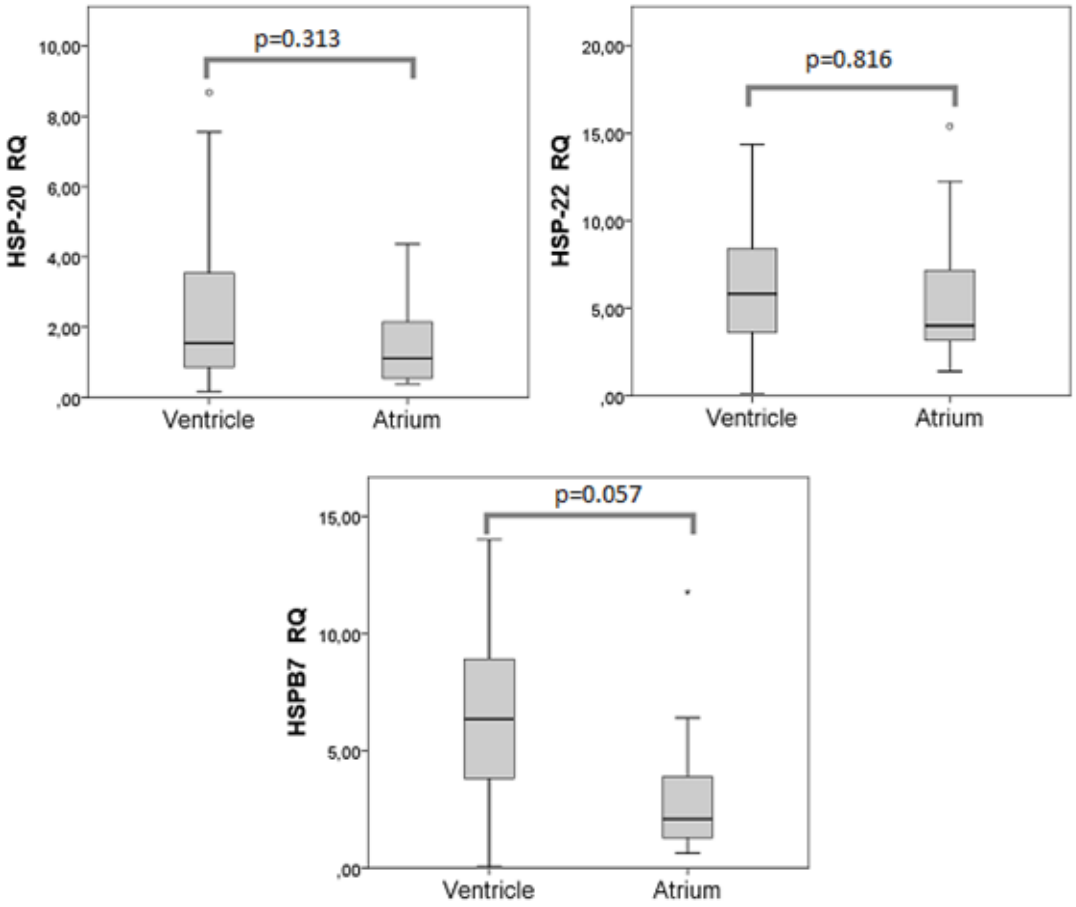

Figure 11.3. sHSP mRNA expression according to ventricular or atrial tissue in CHD patients. Abbreviations: HO-1: heme oxygenase 1; HSP: heat shock protein; HSPB7: cardiovascular HSP; RQ: relative quantification.

Expression after tissue incubation

Descriptive characteristics of sHSPs expression before and after tissue incubation at 60% O_2 concentration are presented in *Table 11.4*.

Gene	N	Time point	Median – IQ range	P value
HSP-20	15	Baseline	1.73(0.87-3.54)	0.061
	15	Incubated	0.95(0.44-1.85)	
HSP-22	15	Baseline	6.411 (3.425-8.389)	0.047
	15	Incubated	2.950 (2.132-6.610)	
HSPB7	15	Baseline	4.746 (3.819-8.914)	0.012
	15	Incubated	2.996 (2.053-4.390)	

Table 11.4. Descriptive statistics for sHSP mRNA expression in the myocardium before and after incubation. Data is expressed as median and IQ range. Abbreviations: HO-1: heme oxygenase 1; HSP: heat shock protein; HSPB7: cardiovascular HSP; IQ: interquartile.

Expression of HSP-22 and HSPB7 was significantly lower after incubation (*Figure 11.4*).

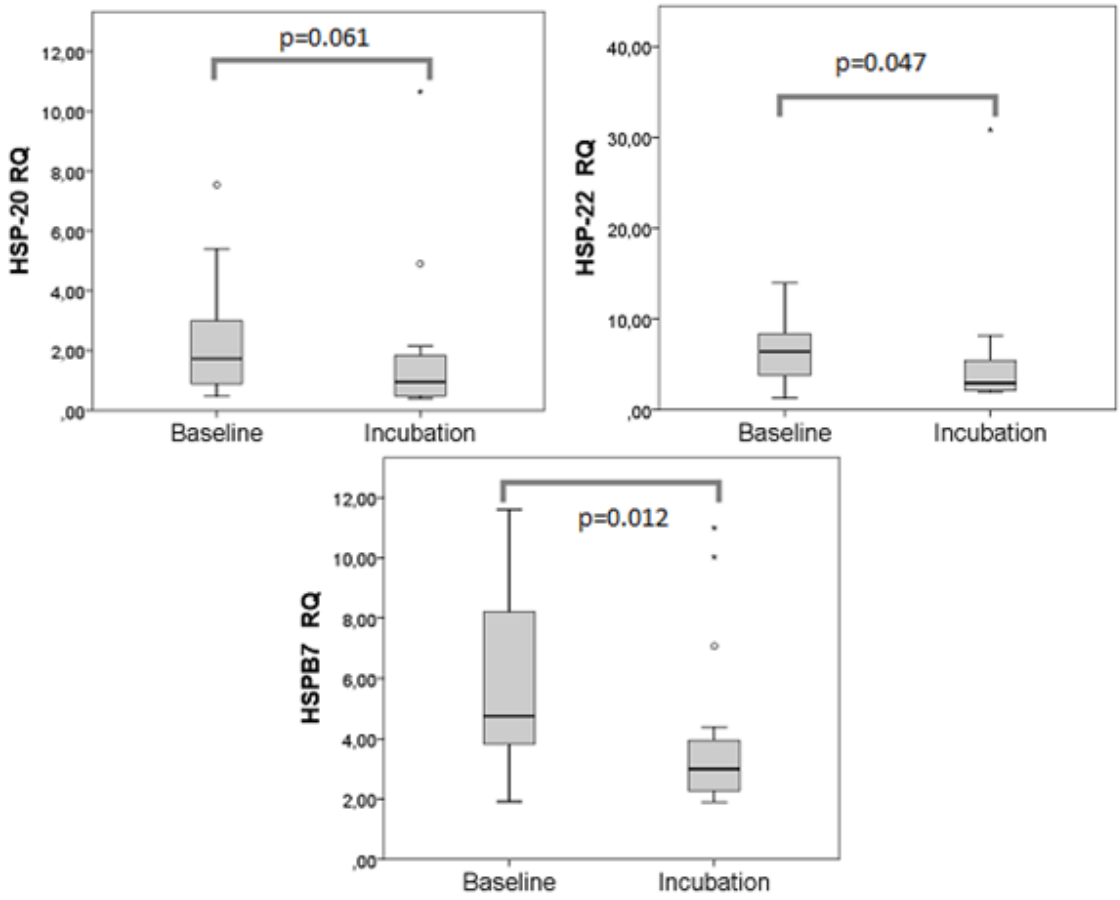

Figure 11.4. sHSP mRNA expression in the myocardium before and after incubation. Abbreviations: HO-1: heme oxygenase 1; HSP: heat shock protein; HSPB7: cardiovascular HSP; RQ: relative quantification.

11.5.2 RIPC analysis

Forty-eight patients were analysed according to intervention allocation. Descriptive data on sHSPs expression according to patient allocation to RIPC or control is summarised in *Table 11.5*.

Gene	Time point	Intervention	N	Mean – SD	Median – IQ range	P value
HSP-20	Baseline	RIPC	14		1.13(0.65-2.46)	0.315
		Control	15		1.73(0.99-4.35)	
	Incubation	RIPC	9		1.577 (0.438-1.845)	0.955
		Control	6		0.945 (0.642-1.120)	
HSP-22	Baseline	RIPC	14	5.375 ± 3.570		0.173
		Control	15	7.484 ±4.448		
	Incubation	RIPC	9		2.830 (2.210-3.770)	0.776
		Control	6		3.613(2.126-7.518)	
HSPB7	Baseline	RIPC	14	5.366 ± 3.9008		0.599
		Control	15	6.116 ± 3.703		
	Incubation	RIPC	9		2.996(2.669-3.288)	0.955
		Control	6		2.780(2.047-4.390)	

Table 11.5. Descriptive statistics for sHSP myocardial mRNA expression before and after incubation according to intervention group allocation. Data is expressed as mean – SD, or median – IQ range as appropriate. Abbreviations: HO-1: heme oxygenase 1; HSP: heat shock protein; HSPB7: cardiovascular HSP; IQ: interquartile; RIPC: remote ischaemic preconditioning; SD: standard deviation.

Variation according to group allocation was not significantly different for HSP-20 (*Figure 11.5*), HSP-22 (*Figure 11.6*) or HSPB7 (*Figure 11.7*).

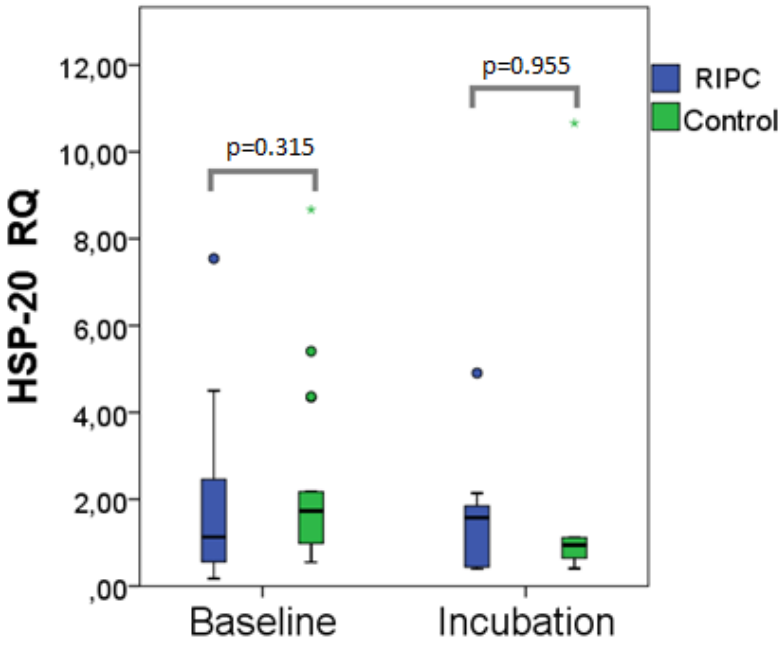

Figure 11.5. HSP-20 mRNA myocardial expression before and after incubation according to intervention group allocation. Abbreviations: HSP-20: heat shock protein 20; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

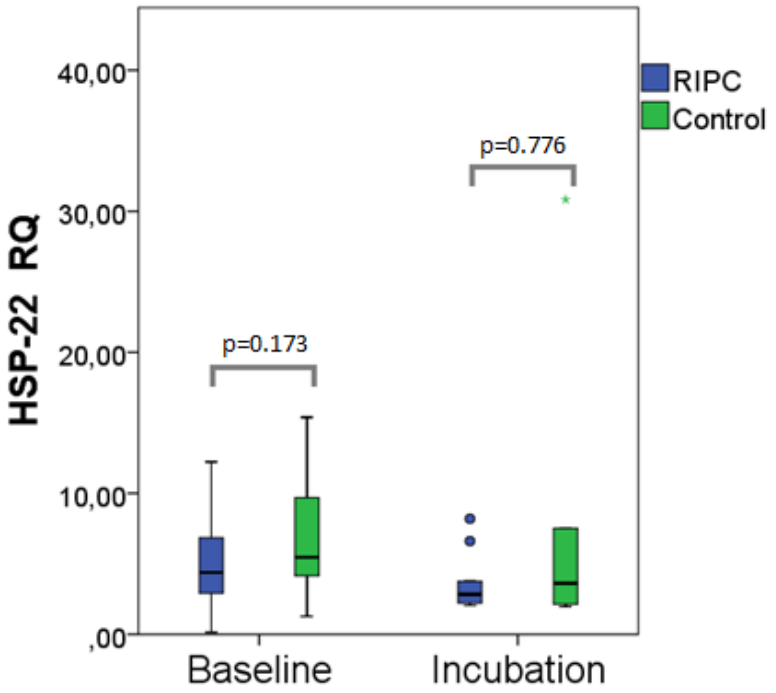

Figure 11.6. HSP-22 mRNA myocardial expression before and after incubation according to intervention group allocation. Abbreviations: HSP-22: heat shock protein 20; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

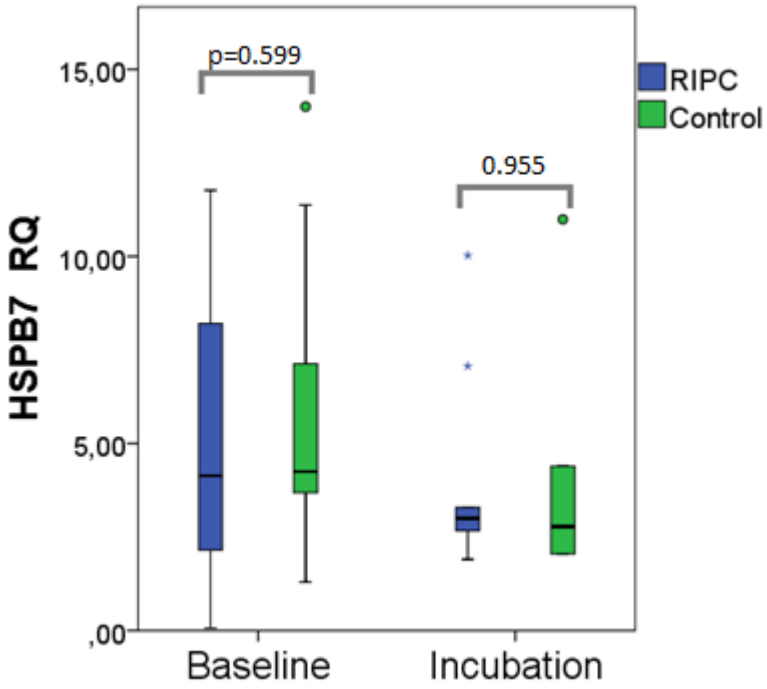

Figure 11.7. HSPB7 mRNA myocardial expression before and after incubation according to intervention group allocation. Abbreviations: HSPB7: cardiovascular heat shock protein; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

11.6 Summary of key findings:

- All analysed sHSPs were expressed in the myocardium of CHD patients.
- HSP-20 expression correlated to weight and age.
- HSPB7 correlated to cTnI release.
- sHSPs did not present a differential expression according to tissue type.
- HSP-22 and HSPB7 presented a lower expression after tissue hyperoxic incubation.
- sHSPs did not vary significantly according to RIPC or control allocation.

11.7 Limitations

The limited availability of atrial tissue may have lowered the power of our analysis to determine differential gene expression between tissue types.

11.8 Discussion

The present study demonstrated that HSP-20, HSP-22 and HSPB7 are expressed in the myocardium of CHD patients without exposure to any experimental conditions. HSP-22 expression was the highest within this group.

HSP-22 expression increased according to age and weight, furthering the concept that the activity of certain HSPs is enhanced as body systems mature.

Expression of cardiovascular HSPB7 at baseline presented a correlation to cTnI levels after surgery, which represent the extent of cardiac injury. As the analysed tissue was resected during the ischaemic phase of CPB, HSPB7 expression would not reflect the complete extent of injury after reperfusion. HSPB7 in plasma has been characterised as an early marker of injury as its levels increase and decrease rapidly after cardiac ischaemic events (Chiu *et al*, 2012). Therefore, an early manifestation of the ischaemic process taking place during CPB could explain the link between HSPB7 expression and later cardiac injury.

The expression of the analysed sHSPs tended to be lower in atrial tissue, however this did not reach statistical significance, these differences could be better explored by increasing the number of atrial specimens for the analysis. Microarray gene profiling determined that HSPB7 was equally present in the right atrium and left ventricle (Barth *et al*, 2005).

Hyperoxic incubation for 4 hours resulted in a tendency to a lower expression of the analysed sHSPs with HSP-22 and HSPB7 presenting significant differences.

HSP-22 studies have mainly found cardioprotective effects regarding its activity, contributing to cardiomyocyte survival and apoptosis inhibition (Depre *et al*, 2006; Mymrikov *et al*, 2011). Based on this, the reduction in HSP-22 expression after hyperoxic incubation could represent part of the mechanisms taking place after high O_2 reperfusion contributing to the establishment of IRI.

HSPB7 has been closely linked to cardiovascular processes and is believed to play a role in the pathogenesis of dilated cardiomyopathy (Rosenfeld *et al*, 2013; Stark *et al*, 2010); however its participation in ischaemic events is less known. HSPB7 has been profiled in array analysis of rat ischaemic myocardium in response to reperfusion and in humans during off pump CABG

surgery (Gene Expression Omnibus – GEO profiles, National Center for Biotechnology Information -NCBI). The results in this study might provide with a first time insight into the expression of HSPB7 during CPB and ischaemia in paediatric patients.

Regardless of the potential benefits involving the activity of sHSPs, the intervention did not result in differential expression of any of the analysed genes according to RIPC or control allocation; expression levels tended to be higher in the control group at baseline, without presenting a marked pattern after incubation.

Pro-survival effects of HSP-20 and a link to cardiac protection have been previously described (Edwards *et al*, 2011), while its inhibition has been identified as a contributing factor to the development of IRI (Ren *et al*, 2009).

HSP-20 presented the lowest baseline expression values among sHSPs; it could be hypothesised that the ischaemic process had inhibited its activity, however this remains to be explored. In addition our results did not demonstrate variation in its activity after incubation or regarding RIPC.

Conclusion

Hyperoxygenation had an inhibitory effect on the expression of HSPB7 and HSP-22. RIPC did not exert effects in the expression of sHSPs in this study.

Chapter 12. Inflammatory pathways

12.1 Introduction

Pro-inflammatory mediators such as cytokines activate TLRs in the cellular membrane which communicate with myeloid differentiation primary response gene 88 (MYD-88) in the intracellular space triggering IKK and MAPK signalling pathways; IKK activates the transcription factor NF-κB which mediates the upregulation of inflammatory genes such as IL-6 and IL-8 further enhancing the inflammatory response (reviewed by Tornatore *et al*, 2012).

12.1.1 TLR pathway

TLRs are a family of highly conserved trans-membrane glycoproteins with a role in innate immunity, they identify and bind to micro-organisms eliciting the production of inflammatory cytokines and upregulating the expression of other stimulating factors; in addition they interact with endogenous ligands resulting in regulation of immune and inflammatory responses (reviewed by Manavalan *et al*, 2011).

The structure of TLRs consists of an extracellular domain responsible for recognising specific ligands, a transmembrane component and an intracellular toll/interleukin-1 receptor domain responsible for initiating intracellular signals (reviewed by Kawai and Akira, 2006).

TLRs use widely conserved motifs in micro-organisms called pathogen associated molecular patterns (PAMPs) in order to recognise pathogens, in addition they are able to recognise stress or damage associated molecular patterns (DAMPs) allowing them to trigger inflammatory reactions (reviewed by Chang, 2010).

Ten types of TLR named from TLR-1 to TLR-10 have been identified in humans, each of them interacting with particular ligands at different cellular locations as shown in *Table 12.1*.

TLR	Localisation	Exogenous ligand	Endogenous ligand
TLR-1	Plasma membrane	Bacteria and mycobacteria lipopeptides, soluble factors	-
TLR-2	Plasma membrane	Bacteria: lipoprotein, lipopeptides, lipoteichoic acid, soluble factors, porins, peptidoglycan, glycolipids Virus: proteins, hemagglutinin Yeast: zymosan	HSP-60, HSP-70, HSP.96, HMGB1, hyaluronic acid
TLR-3	Endolysosome	ssRNA and dsRNA viruses	mRNA
TLR-4	Plasma membrane	Lipopolysaccharide in Gram- bacteria, envelope and fusion viral proteins, *Chlamydia pneumonia* HSP-60, Glycoinositolphospholipids in *Trypanosoma cruzi*, Taxol	HSP-22, HSP-60, HSP-70,HSP-96, HMGB1, β-defensin 2, hyaluronic acid, heparin sulfate, fibrinogen, surfactant protein A
TLR-5	Plasma membrane	Flagellin in Gram+ of Gram- bacteria	-
TLR-6	Plasma membrane	Bacteria: diacyl lipopeptides, phenol-soluble modulin lipoteichoic acid, heat-liable soluble factor. Zymosan in saccharomyces	-
TLR-7	Endolysosome	ssRNA viruses	Endogenous RNA
TLR-8	Endolysosome	ssRNA viruses	Endogenous RNA
TLR-9	Endolysosome	Unmethylated CpG motifs in bacteria and viruses, hemozoin in Plasmodium	Endogenous DNA
TLR-10	Extracellular	Unknown, may interact with TLR-2 an TLR-1	-

Table 12.1. TLRs localisation and ligands. Abbreviations: dsRNA: double stranded RNA; HMGB1: high mobility group box 1; HSP: heat shock protein; mRNA: messenger RNA; ssRNA: single stranded RNA; TLR: toll like receptor. Table modified from Manavalan *et al*, 2011 (published under creative commons CC-BY licence), with information by Chang, 2010.

After PAMP or DAMP recognition, TLR signalling starts with the activation of MYD-88 in a cascade that leads to the upregulation of NF-κB and activating protein 1 (AP-1), two important mediators responsible for gene upregulation of inflammatory and stress pathways (reviewed by Kawai and Akira, 2006; Tornatore *et al*, 2012) (*Figure 12.1*).

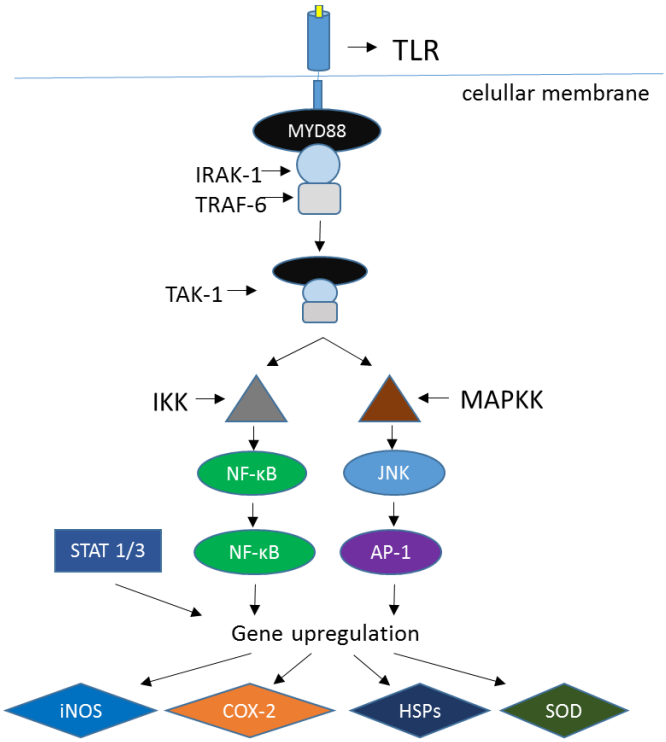

Figure 12.1. TLR – MYD-88 signalling pathway. Abbreviations: AP-1: activating protein 1; COX-2: cyclooxygenase-2; IKK: IκB kinase; iNOS: inducible nitric oxide synthase; IRAK: IL-1R associated kinases; JNK: cJun N-terminal kinase; MAPK: mitogen-activated protein kinases; MYD-88: molecule myeloid differentiation primary response protein 88; NF-κB: nuclear factor κ B; SOD: superoxide dismutase; STAT: Signal transducer and activator of transcription; TAK1: transforming growth factor b activated protein kinase 1; TLR: toll like receptor; TRAF6: tumour necrosis factor receptor associated factor 6. Original figure based on information by Kawai and Akira, 2006; Deguine and Barton, 2014.

The canonical adaptor for all TLRs is MYD-88 which plays a central role within inflammatory pathways, linking TLRs to IL-1R associated kinases (IRAK); IRAK 4 and IRAK1 are phosphorylated and dissociate from MYD-88 leading to the activation of tumour necrosis factor receptor associated factor 6 (TRAF6) which stimulates transforming growth factor β activated protein kinase 1 (TAK1), triggering the IκB kinase (IKK) complex finally leading to NF-κB activation. Simultaneously TAK1 phosphorylates members of the MAP kinase (MAPK) family which activate cJun N-terminal kinase (JNK) and p38 which in turn upregulate AP-1 (reviewed by Kawai and Akira, 2006; Deguine and Barton, 2014).

In addition to MYD-88 signalling, an alternative pathway is used by TLR-3 and TLR-4 through adaptor toll/IL1 receptor domain containing adaptor inducing interferon-β (TRIF) which results in the activation of interferons as well as NF-κB and AP-1 (reviewed by Hyun *et al*, 2013).

Cardiac involvement

TLRs are expressed in circulating blood cells, endothelium and in myocardial cells; during ischaemia and reperfusion TLR action is started by DAMPs creating a positive feedback circuit enhancing inflammation and cardiomyocyte death which triggers the release of more cytokines feeding into the process (reviewed by Arslan *et al*, 2010).

TLR-2 and TLR-4 have been identified as having a role in the pathogenesis of IRI. A mouse model compared the effects of ischaemia and reperfusion between wild type and TLR-2 knockout animals resulting in similar levels of CK, however wild type animals presented a marked left ventricular contractile dysfunction along with higher levels of TNF and IL-1 (Sakata el al, 2007).

Bone marrow transplant experiments between wild type and TLR-2 knock out mice in IRI showed that the final infarct size correlated with the expression of TLR-2 in circulating leukocytes, an effect that was abolished after the administration of a TLR-2 antagonist resulting in reduced infarct size and preserved cardiac function (Arslan *et al*, 2010). TLR-2 inhibition was further proven to be beneficial using a humanised anti-TLR2 antibody in a pig model of IRI (Arslan *et al*, 2012). In addition TLR-2 has been linked to coronary endothelial dysfunction after ischaemia suggesting that TLR-2 activity within the heart, circulation, and the endothelium contribute to the establishment of IRI (Favre *et al*, 2007).

TLR-4 involvement with cardiac IRI was first established in TLR-4 deficient mice presenting reduced myocardial injury (Oyama *et al*, 2004); this was confirmed by blocking TLR-4 activity which resulted in reduced IRI and inflammatory mediators (Shimamoto *et al*, 2006).

Further IRI research related to transplant found that TLR-4 deficient mice presented lower troponin, TNF, IL-1, IL-6, I-CAM and iNOS levels as well as reduced NF-κB nuclear translocation and neutrophil infiltration (Kaczorowski *et al*, 2007). Additional evidence linked TLR-4 activity to the release of I-CAM and V-CAM and to pathologic remodelling of non-

ischaemic myocardium after ischaemia and reperfusion with TLR-4 deficient mice presenting better cardiac function (Zhai *et al*, 2015).

The endogenous ligand high mobility group box 1 (HMGB1) interacts with TLR-4 and seems to be the main DAMP involved in the process (reviewed by Pourrajab *et al*, 2015). Animal models of IRI have shown upregulation of TLR-4 mediated by HMGB1 resulting in apoptosis induction through TLR-4 related cytokine and inflammatory mediators release (Ding *et al*, 2013).

The mechanisms involved seem to include the JUN, NF-κB and AP-1 pathway as reduced activity of these mediators resulted in smaller infarct size (Chong *et al*, 2004), similar results were obtained after modulation of downstream MAPK signalling (Zhao *et al*, 2009).

Previous analysis by our research group compared myocardial expression between cyanotic and acyanotic patients finding a differential expression with higher levels of TLR-2 and TLR-4 in chronic cyanotic patients while MYD-88 expression remained constant. There was no difference when the tissue was exposed to high oxygen levels after excision.

TLRs in preconditioning

TLR-2 and TLR-4 involvement in IRI and cardiac protection have been established, in addition to them TLR-9 has a particular role in mediating cardiac and brain protection against IRI (reviewed by Pourrajab *et al*, 2015).

A study on direct preconditioning found that the protective effects seen in wild type mice were lost in TLR-2 deficient mice, which was not the case in TLR-4 deficient animals suggesting that TLR-2, but not TLR-4, would play a protective role in the preconditioning process (Dong *et al*, 2010).

Further research has shown that the use of a TLR2 agonist provided with preconditioning effects reducing infarct size after ischaemia and reperfusion through a limitation in chemokine release leading to reduced leukocyte recruitment (Mersmann *et al*, 2010). Similarly, stimulation of TLR-2 by administration of its ligands before ischaemia in mice resulted in cardioprotection (Ha *et al*, 2010). In addition, blocking of HMGB1 resulted in a reduction in IRI in a process

dependant on TLR-2 as its inactivation caused loss of the protective effect (Mersmann *et al*, 2013).

This apparently dual influence of TLR-2 in IRI could be due to unbalanced reactions which often become detrimental in the immune system (reviewed by Lepper and Bals, 2012).

The inhibition of TLR-4 has been established as a mechanism of preconditioning resulting in lower levels of cytokines such as TNF-α as shown in an IRI rat model (Yu *et al*, 2010); further investigation found that down regulation of protein and mRNA expression of TLR-4 was linked to reduced infarct size and CK levels after preconditioning (Yang *et al*, 2011).

12.1.2 NF-κB

NF-κB is a transcription factor with an important role in coordinating immunity and inflammation, it responds to redox activity and triggers the expression or inhibition of secondary genes acting as regulators (Tranter *et al*, 2010).

NF-κB is located in the cytoplasm in an inactive state due to the action of its inhibitors IκBα and p100 proteins; after an activating stimulus such as stress, IκBα and/or p100 are phosphorylated by IKKβ or IKKα respectively; this allows NF-κB to reach the nucleus and join its specific DNA binding sites which regulate the transcription of target genes including cytokines and receptors such as IL-2, IL-6, IL-8, TNF-α cyclooxygenase-2 (COX-2), and NOS among others which in turn control the activity of NF-κB, constituting a regulatory circle that amplifies inflammatory responses (reviewed by Sarkar *et al*, 2008).

A higher expression of NF-κB as a response to TNF-α has been linked to apoptosis inhibition while low activity is related to the induction of apoptosis (Van Antwerp *et al*, 1996).

NF-κB was first shown to bind DNA and interact with the immunoglobulin κB or light chain promoter of B lymphocytes (Sen and Baltimore, 1986); however its functions are not limited to B lymphocytes or the light chain, in fact it has been shown to be widely expressed in all cell types and to fulfil numerous direct and regulatory tasks.

NF-κB activity can be described as a signalling system involving 5 homologous proteins or subunits: p65 (RelA), RelB (RelB), c-Rel (Rel), p105/p50 (NFKB1) and p100/p52 (NFKB2).

These units contain a homology domain that allows homo and hetero dimerization, nuclear translocation and association to inhibitory proteins and DNA binding, different combinations result in different transcription factor functionality (reviewed by Hoffman and Baltimore, 2006; Van der Heiden *et al*, 2010).

NF-κB pathways and activity have been linked to oncogenic and metabolic disorders as maintenance of inflammatory responses contribute to pathological development; furthermore NF-κB has been shown to control glycolysis and respiration systems hence regulating energy homeostasis showing a direct link to ageing, cancer and possible therapies (reviewed by Tornatore *et al*, 2012).

In the cardiovascular system NF-κB regulates immunity, inflammation, cell survival, differentiation, proliferation and angiogenesis; according to the type of stimulus it can regulate the transcription of pro-inflammatory genes or that of inhibitors of apoptotic processes (reviewed by Van der Heiden *et al*, 2010). *Table 12.2* shows a summary of the main target genes for NF-κB.

Classification	**Target Genes**
Cytokines	TNF-α, IL-1RA, IL-6, IL-8, IL-10, IL-12.
Cell adhesion	I-CAM1, V-CAM1, Selectins
Enzymes	COX-2, NADPH
Transcription factors	P53, IκBα, p105, p100, HIF-1
Growth factors	IL-2, c-Myc, TRIF
Acute phase proteins	CRP
Immunoreceptors	TLR-2, TLR-9
Antigen presentation	Complement components
Apoptosis	AI, Bcl-2, caspase-11
Angiogenesis	VEGF, MMP-9
Stress response	HSP-32, HSP-70, HSP-90, iNOS, SOD

Table 12.2. NF-κB target genes and their function. Abbreviations: AI: apoptosis inhibitor; Bcl-2: B-cell lymphoma 2; COX-2: cyclo-oxygenase-2; CRP: C reactive protein; I-CAM: intercellular adhesion molecule; IL: interleukin; IL-1RA: IL-1R antagonist; iNOS, inducible NO synthase; MMP-9: Matrix metallopeptidase 9 NADPH: nicotinamide adenine dinucleotide phosphate; p53: SOD, superoxide dismutase; TLR: toll like receptor; TNF-α: tumour necrosis factor α TRIF: TIR (Toll/IL-1-R)-domain-containing adapter-inducing interferon-β; V-CAM: vascular cellular adhesion molecule; VEGF: vascular endothelial growth factor. Table modified from Van der Heiden *et al*, 2010 based on information by Ammirante *et al*, 2008; Tranter *et al*, 2010; NFKB transcription factors www.nf-kb.org.

NF-κB has been linked to cardiac protection when activated early, before ischaemia and reperfusion have been established; some of the genes involved in NF-κB signalling pathways act in the process of late RIPC, including HSPs, TLRs as well as angiogenesis, apoptosis and metabolic mediators including iNOS and COX-2 (Tranter *et al*, 2010). However, the role of NF-κB in the heart has not been completely elucidated as its activity also relates to pathological processes such as heart failure, where its late activation by TNF-α and chronic influence contribute to the pathological process (reviewed by Dhingra *et al*, 2010).

NF-κB up-regulation of HSP-70.1 and HSP-70.3 has been shown to contribute to the protective effect of RIPC in microarray analysis, linked to reduced infarct size in mice models; HSP-70.1 however seemed to have a dichotomous role, contributing to cardioprotection after RIPC while promoting injury when the RIPC stimulus was not present (Tranter *et al*, 2010; Wilhide *et al*, 2011).

NF-κB activation after ischaemia and reperfusion can contribute to the establishment of injury through upregulation of inflammatory mediators (Ling *et al*, 2013). Experiments in animals presenting a subunit NF-κB deletion resulted in lower cytokine activity and apoptosis along with increased Ca^{2+} reuptake by the sarcoplasmic reticulum (Zhang *et al*, 2013). In addition, it has been found to contribute to IRI through regulation of genes inhibiting the protective RISK pathway (Xing *et al*, 2016).

12.2 Preliminary experiments

12.2.1 Aims

To compare NF-κB expression between cyanotic and acyanotic patients and to analyse its expression pattern before and after incubation at hypoxic, normoxic and hyperoxic conditions.

12.2.2 Materials and methods

Patients requiring corrective cardiac surgery were recruited in order to perform two different analysis: a comparison of gene expression between cyanotic and acyanotic patients and a differential gene expression analysis before and after incubation at different O_2 concentrations.

Tissue was resected as part of the surgical procedure during aortic cross clamp, for the cyanosis status experiment samples without previous exposure to any experimental conditions were used.

For the second experiment samples obtained during surgery were divided into an immediate baseline sample after collection in theatre, a second sample processed after transportation to the laboratory in a flask with medium previously equilibrated to 2% O_2, and subsequent samples incubated at hypoxic (2% O_2), normoxic (20% O_2) and hyperoxic (60% O_2) conditions for 4 hours.

mRNA was then extracted from the tissue samples. The stages of work described in this experiment so far were performed by Susan Walker as part of her PhD work within our laboratory group for work in other genes.

As part of this thesis work cDNA was obtained from the mRNA samples through a RT-PCR reaction and Q-PCR was performed in order to obtain RQ values for NF-κB as described on *Chapter 2*.

12.2.3 Statistical analysis

Cyanotic vs acyanotic comparison was performed using an independent sample t-test.

For the second experiment analysis was divided into two sections: a comparison of time points at baseline (theatre collection), laboratory arrival and post-incubation for each O_2 concentration used, and a second comparison between the three O_2 concentrations used for incubation. Friedman repeated measures test was used with post-hoc analysis for significant results ($p<0.05$).

12.2.4 Results

Gene expression analysis according to cyanosis status

Twelve patients were recruited for cyanosis status analysis, *Table 12.3* shows their demographic characteristics.

Patient	Age (months)	Gender	Diagnosis	Cyanosis status
3(1)	13	Female	TOF	Cyanotic
4(1)	14	Male	TOF	Cyanotic
9(1)	14	Male	TOF	Cyanotic
10(1)	11	Male	TOF	Cyanotic
11A	12	Female	TOF	Cyanotic
16A	11	Male	TOF	Cyanotic
23A	13	Female	Fallot variant	Cyanotic
5(1)	7	Male	Subaortic stenosis	Acyanotic
6(1)	148	Female	Subaortic stenosis	Acyanotic
7(1)	32	Male	Subaortic stenosis	Acyanotic
8(1)	1	Male	Truncus arteriosus	Acyanotic
18(a)	11	Female	Subaortic stenosis	Acyanotic

Table 12.3. Demographic characteristics of patients participating in cyanosis status analysis. Abbreviations:TOF: tetralogy of Fallot.

NF-κB expression in cyanotic (n=7) vs acyanotic (n=5) patients was not statistically significantly different (1.46±0.52 vs 0.83±0.66, p= 0.089) (*Figure 12.2*).

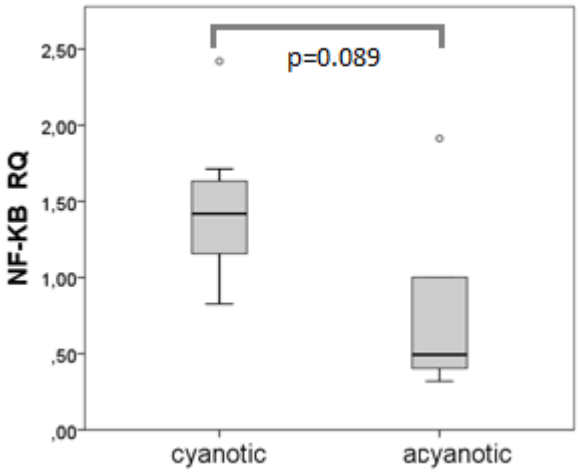

Figure 12.2. NF-κB expression according to cyanosis status. Abbreviations: NF-κB: nuclear factor kappa B; RQ: relative quantification.

Incubation at differential O_2 concentrations

Ten patients were recruited for the experiment, demographic data can be seen on *Table 12.4*.

Patient	Age (months)	Gender	Diagnosis
32	72	Male	TOF
33	10	Male	TOF
34	16	Female	TOF
35	6	Female	TOF
36	77	Female	TOF
37	11	Male	TOF
38	15	Male	Aortic onstruction
39	1	Male	Truncus arteriosus
41	60	Male	TOF
45	14	Female	VSD

Table 12.4. Demographic characteristics of patients participating in O_2 incubation analysis. Abbreviations:TOF: tetralogy of Fallot; VSD: ventricular septal defect.

Eight patients presented complete measurements for all time points, descriptive information is summarised in *Table 12.5*.

Time point	Median – IQ range
Baseline	3.13(1.92-4.16)
Laboratory arrival	0.48(0.23-1.27)
2% O_2 incubation	0.46()0.21-1.08
20% O_2 incubation	0.65(0.28-0.91)
60% O_2 incubation	0.88(0.72-2.48)

Table 12.5. Descriptive statistics for NF-κB gene expression analysis before and after differential O_2 incubation. Abbreviations: NF-κB: nuclear factor kappa B; O_2: oxygen.

Table 12.6 shows X^2 and p value results for repeated measures Friedman test, analysis resulted in non-significant variations for all the variables explored (*Figure 12.3*).

Comparison	X^2, P value	P value
Baseline-laboratory arrival-2% O_2 incubation	5.250	0.072
Baseline-laboratory arrival-20%O_2 incubation	3.250	0.197
Baseline-laboratory arrival -60% O_2 incubation	4.000	0.135

Table 12.6. Results for NF-κB for differences between time points using related samples Friedman tests and post-hoc analysis with Bonferroni correction. Abbreviations: BL: baseline; NF-κB: nuclear factor kappa B; O_2: oxygen; X^2: chi square.

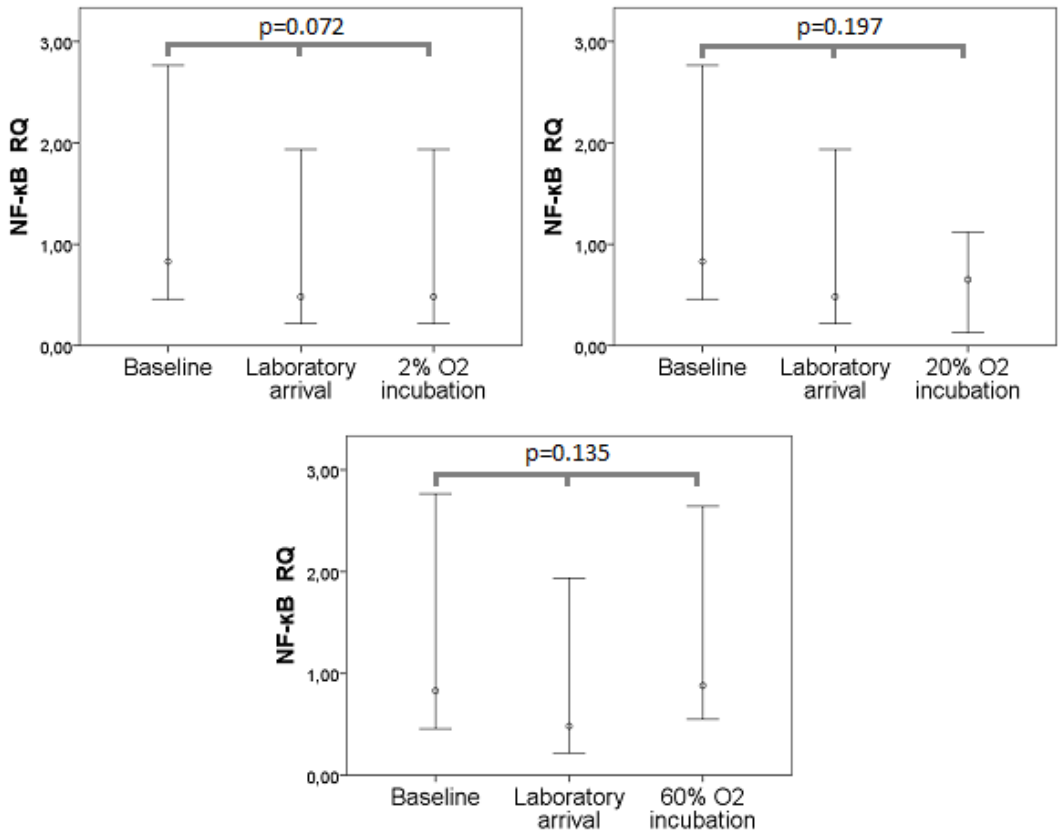

Figure 12.3. NF-κB expression according to time points before and after O_2 incubations. Abbreviations: NF-κB: nuclear factor kappa B; O_2: oxygen; RQ: relative quantification.

All patients (n=10) presented complete data for incubation at 2%, 20% and 60% O_2 concentration, descriptive information is summarised in *Table 12.7*. Repeated measures Friedman test was used in order to determine differences between incubation at different O_2 concentrations, resulting in non-significant variation ($X^2(2)=3.800$, p=0.150) (*Figure 12.4*).

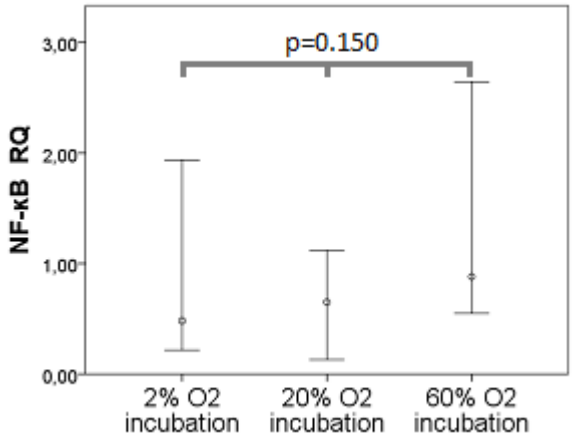

Figure 12.4. NF-κB gene expression analysis according to differential O_2 incubation. Abbreviations: NF-κB: nuclear factor kappa B; O_2: oxygen; RQ: relative quantification.

12.3 RIPC Study

12.3.1 Aims

To analyse the expression patterns of TLRs, MYD-88 and NF-κB in the myocardium of CHD patients requiring corrective surgery using CPB before and after hyperoxic incubation, and to assess the effects of RIPC on their expression.

12.3.2 Materials and Methods

Tissue was obtained from patients during surgical intervention and CPB; in cases that required ventricular tissue resection as part of the procedure tissue was divided in two parts, a section was immediately processed as a baseline reference and the second section was incubated at 60% O_2 for 4 hours. Atrial samples were used as baseline reference only.

mRNA was extracted from the tissue from which cDNA was transcribed and used in order to perform Q-PCR analysis. Genes analysed and assays used are summarised in *Table 12.7*.

	Gene name	Gene aliases	Gene symbol	Assay Design	Amplicon length	Assay ID
TLR-2	toll-like receptor 2	CD282 TIL4	TLR2	Probe spans exons	80	Hs00610101_m1
TLR-4	toll-like receptor 4	CD284 TLR-4	TLR4	Probe spans exons	89	Hs00152939_m1
MYD-88	myeloid differentiation primary response 88	MYD-88D	MYD-88	Probe spans exons	104	Hs00182082_m1
NF-KB	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	EBP-1 NFKB-p105 NFKB p105, p50	NFKB1	Probe spans exons	66	Hs00765730_m1

Table 12.7. Genes analysed and assay information. Abbreviations: MYD-88: Myeloid Differentiation Primary Response 88; NF-κB: nuclear factor – κB; TLR: toll like receptor.

Details on materials and methods are described on *Chapter 2*.

12.3.3 Statistical analysis

Correlations to demographic characteristics including the gender, age, weight and cyanosis status of the patient, and to PO1 cTnI levels were determined using the Pearson test in normally distributed data (r) or Spearman test in non-parametric data (r_s).

Expression variation according to tissue type was analysed using independent sample t-test or the non-parametric Mann Whitney test, while paired samples t-test or Wilcoxon signed-rank test were used for the analysis of paired measures in order to explore differences before and after tissue incubation.

Independent samples t-test or Mann Whitney tests were used to determine differences according to RIPC or control allocation for each time point at baseline and after incubation.

Analysis was performed using SPSS version 23 software, a p value <0.05 (two tailed) was considered significant.

12.3.4 Results

Fifty patients were recruited for the study, demographic data and its analysis can be seen in *Tables 3.1* and *3.2*.

General analysis

All genes analysed were expressed in the myocardial tissue (*Figure 12.5*).

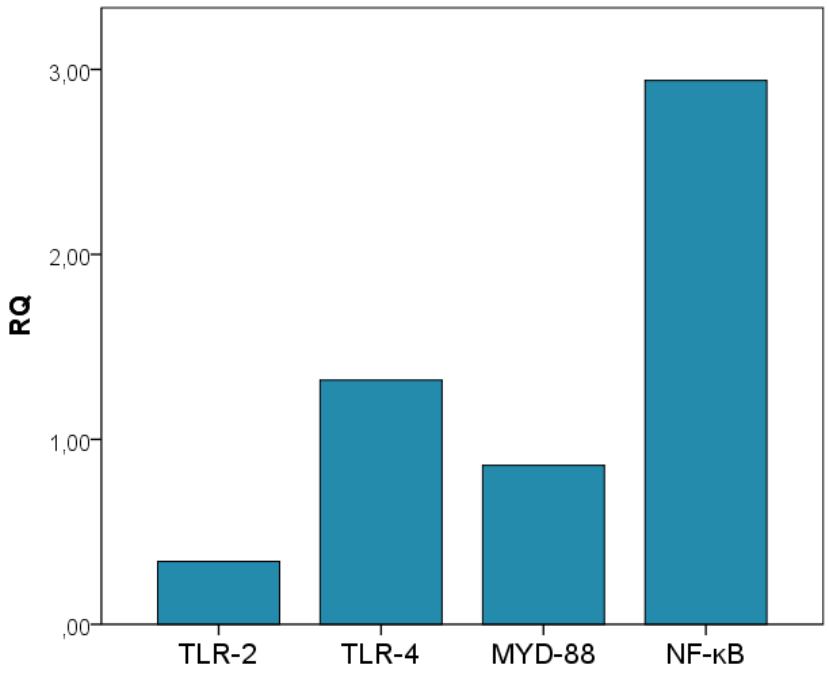

Figure 12.5. TLR pathway mRNA expression in myocardium of CHD patients. Figure shows the median for RQ values of each gene. Abbreviations: MYD-88: Myeloid Differentiation Primary Response 88; NF-κB: nuclear factor – κB; RQ: relative quantification; TLR: toll like receptor.

NF-κB expression correlated negatively to gender (r_s= -0.391, p=0.029), implying that its expression was lower in males; variation according to gender was statistically significant (f: 4.16(2.63-7.85) vs m: 1.94(1.15.3.28), p=0.032) (*Figure 12.6*).

There was no correlation or significant difference in the expression of any of the genes between cyanotic and acyanotic patients.

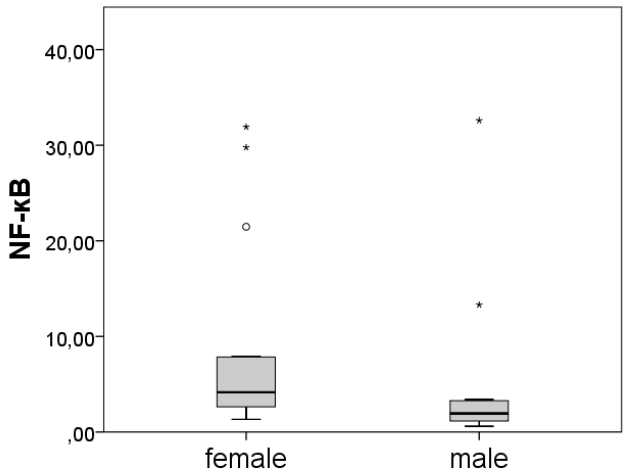

Figure 12.6. NF-κB expression at baseline according to gender. Abbreviation: NF-κB: nuclear factor kappa B.

NF-κB expression also correlated positively to plasma PO1 cTnI levels (r_S=0.577, p=0.001) (*Figure 12.7*).

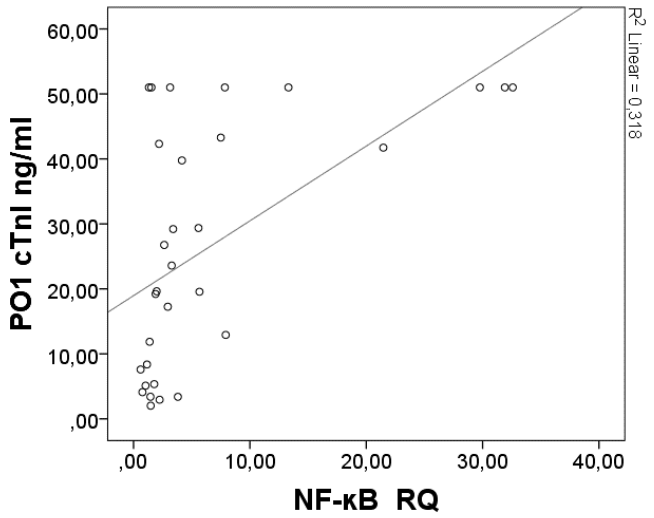

Figure 12.7. PO1 cTnI levels in plasma correlation to NF-κB mRNA expression in the myocardium at baseline. Abbreviations: cTnI: cardiac troponin I; NF-κB: nuclear factor κB; PO1: post-operative 1; RQ: relative quantification.

Expression according to tissue type

Table 12.8 presents descriptive statistics of gene expression according to tissue type.

Gene	N	Tissue	Mean – SD	Median – IQ range	P value
TLR-2	22	Ventricle		0.35 (0.18-0.45)	0.908
	9	Atrium		0.26 (0.21-0.64)	
TLR-4	22	Ventricle		1.91 (1.17-3.04)	0.004
	9	Atrium		0.51 (0.20-0.85)	
MYD-88	21	Ventricle	1.004 ± 0.503		0.580
	9	Atrium	0.885 ± 0.604		
NF-κB	22	Ventricle		3.34(1.89-7.92)	0.018
	9	Atrium		1.47(1.15-2.24)	

Table 12.8. Descriptive statistics for mRNA expression according to ventricular or atrial tissue in CHD patients. Data is expressed as mean – standard deviations, or median – interquartile range as appropriate. Abbreviations: MYD-88: Myeloid Differentiation Primary Response 88; NF-κB: nuclear factor – κB; TLR: toll like receptor.

TLR-4 and NF-κB presented a significantly higher expression in ventricular tissue (*Figure 12.8*)

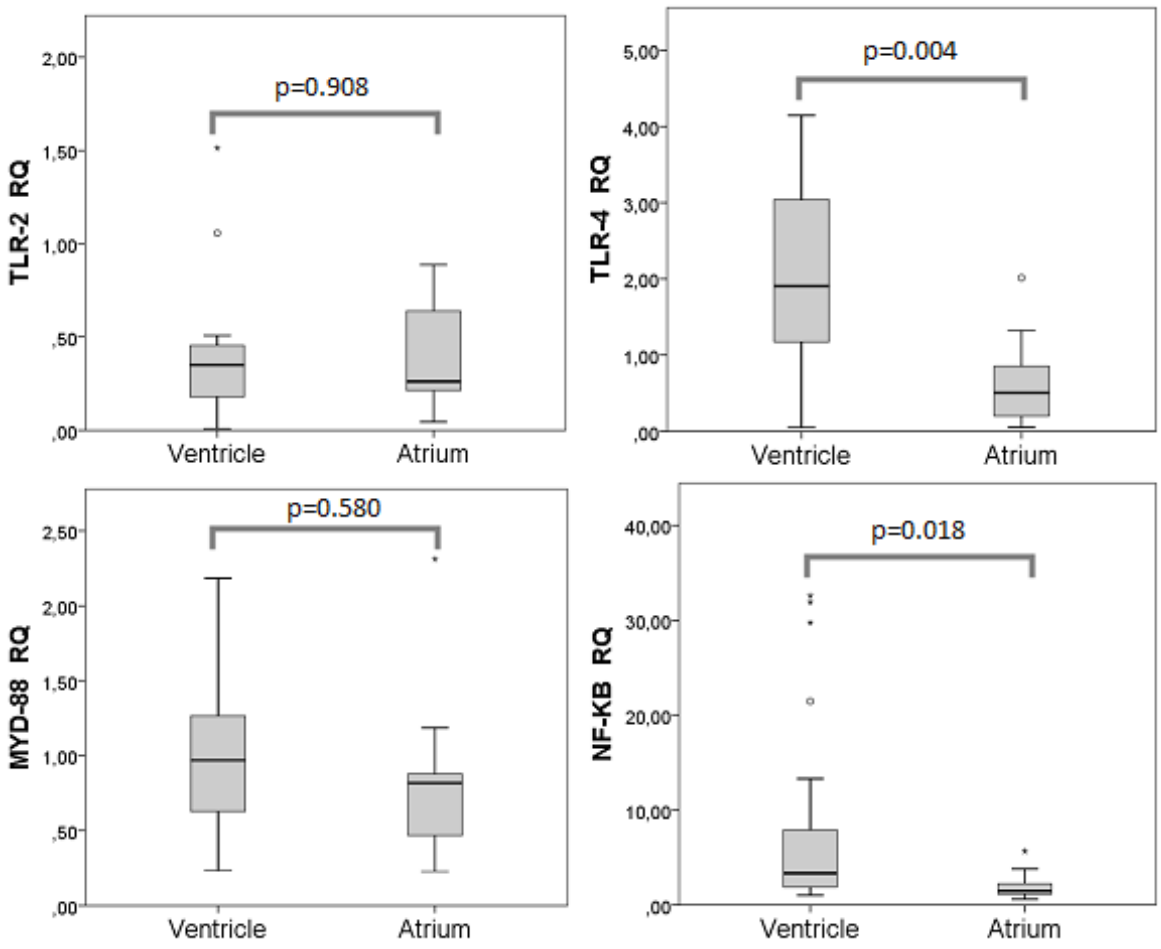

Figure 12.8. TLR pathway mRNA expression according to ventricular or atrial tissue in CHD patients. Abbreviations: MYD-88: Myeloid Differentiation Primary Response 88; NF-κB: nuclear factor – κB; RQ: relative quantification; TLR: toll like receptor.

Expression after tissue incubation

Table 12.9 shows descriptive data of gene expression values at baseline and after 60% O_2 incubation for 4 hours.

MYD-88 presented a significant reduction in expression after incubation, differences in the expression of other genes were not significant (*Figure 12.9*).

Gene	N	Time point	Mean - SD	Median – IQ range	P value
TLR-2	14	Baseline		0.34(0.18-0.38)	0.177
	14	Incubated		0.22(0.16-0.29)	
TLR-4	15	Baseline	1.775 ± 1.086		0.798
	15	Incubated	1.8509 ± 1.068		
MYD-88	14	Baseline	0.855 ± 0.3902		0.005
	14	Incubated	0.521 ± 0.257		
NF-κB	15	Baseline		3.28(1.89-13.31)	0.955
	15	Incubated		7.46(1.51-15.69)	

Table 12.9. Descriptive statistics for mRNA expression in the myocardium before and after incubation. Data is expressed as mean – SD or median – IQ range as appropriate. Abbreviations: IQ: interquartile; MYD-88: Myeloid Differentiation Primary Response 88; NF-κB: nuclear factor – κB; SD: standard deviation; TLR: toll like receptor.

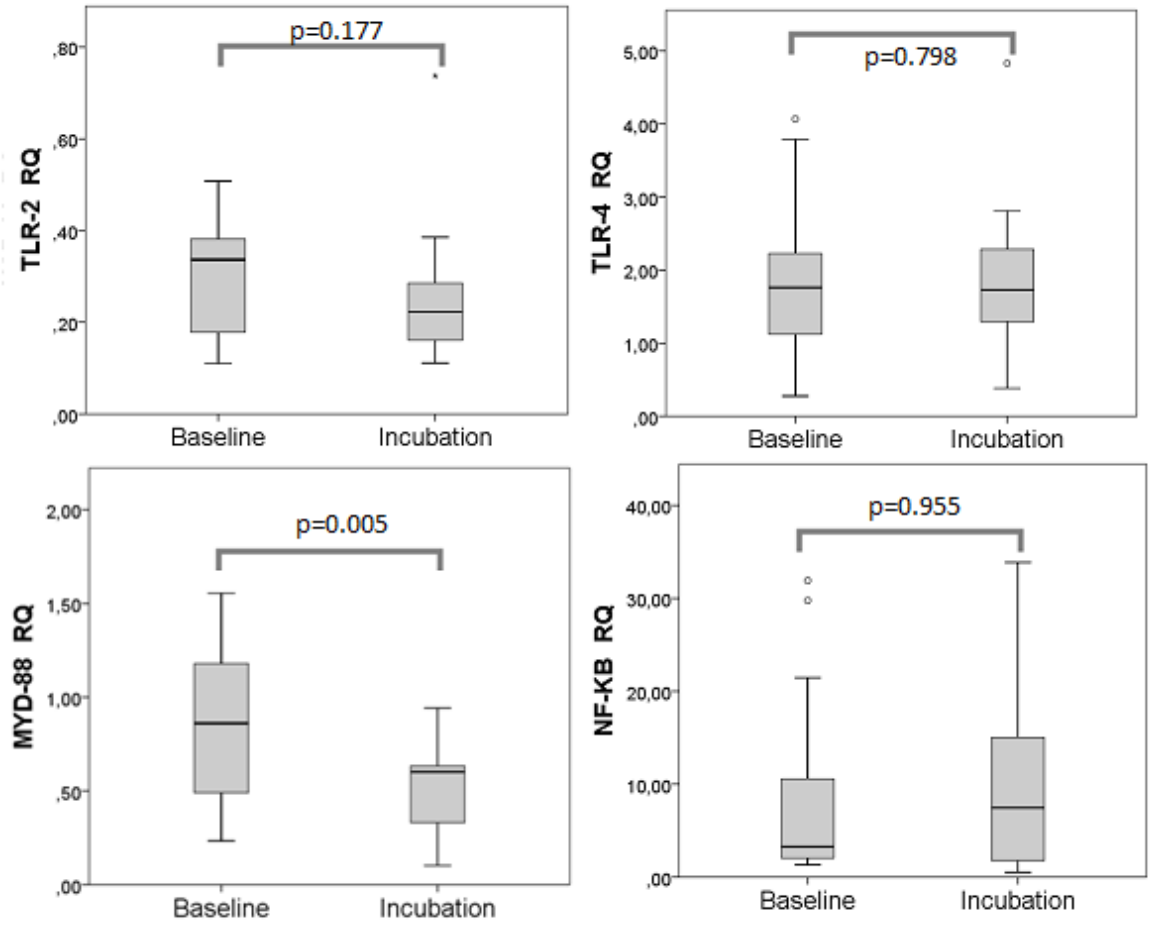

Figure 12.9. mRNA expression in the myocardium before and after incubation. Abbreviations: MYD-88: Myeloid Differentiation Primary Response 88; NF-κB: nuclear factor – κB; RQ: relative quantification; TLR: toll like receptor.

RIPC analysis

Forty-eight patients were analysed according to intervention allocation. Descriptive information on gene expression according to control or RIPC allocation is presented in *Table 12.10*.

Gene	Time point	Intervention	N	Mean – SD	Median – IQ range	P value
TLR-2	Baseline	RIPC	14		0.26 (0.18-0.38)	0.357
		Control	13		0.37 (0.21-0.49)	
	Incubation	RIPC	8		0.214(0.155-0.294)	1.000
		Control	6		0.224(0.164-0.285)	
TLR-4	Baseline	RIPC	14	1.877 ± 1.258		0.503
		Control	15	1.556 ± 1.279		
	Incubation	RIPC	9	2.020 ± 1.228		0.473
		Control	6	1.597 ± 0.808		
MYD-88	Baseline	RIPC	13	0.983 ± 0.508		0.549
		Control	15	0.872 ± 0.464		
	Incubation	RIPC	8	0.580 ± 0.257		0.341
		Control	6	0.442 ± 0.258		
NF-κB	Baseline	RIPC	14		3.54(1.78-7.92)	0.407
		Control	15		2.24(1.38-5.58)	
	Incubation	RIPC	9		9.592(2.109-14.381)	0.529
		Control	6		4.757(1.392-15.687)	
		Control	6		2.338(1.933-3.224)	

Table 12.10. Descriptive statistics for mRNA expression in the myocardium before and after incubation according to intervention allocation. Data is expressed as mean – standard deviations, or median – interquartile range as appropriate. Abbreviations: MYD-88: Myeloid Differentiation Primary Response 88; NF-κB: nuclear factor – κB; RIPC: remote ischaemic preconditioning; TLR: toll like receptor.

Expression of TLR-2 (*Figure 12.10*), TLR-4 (*Figure 12.11*), MYD-88 (*Figure 12.12*) and NF-κB (*Figure 12.13*) did not vary according to RIPC or control allocation.

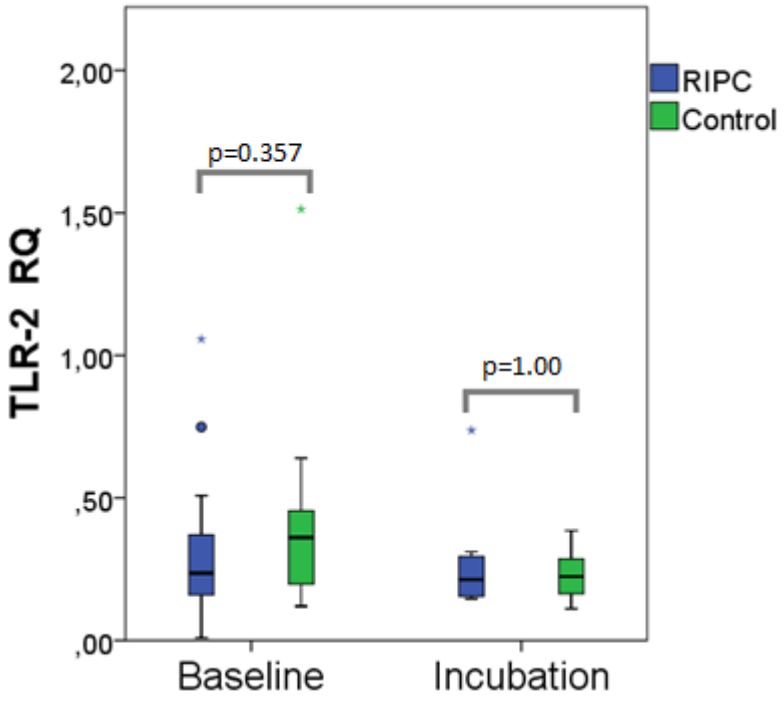

Figure 12.10. TLR-2 mRNA expression in the myocardium before and after incubation according to intervention allocation. Abbreviations: RIPC: remote ischaemic preconditioning; RQ: relative quantification; TLR-2: toll like receptor 2.

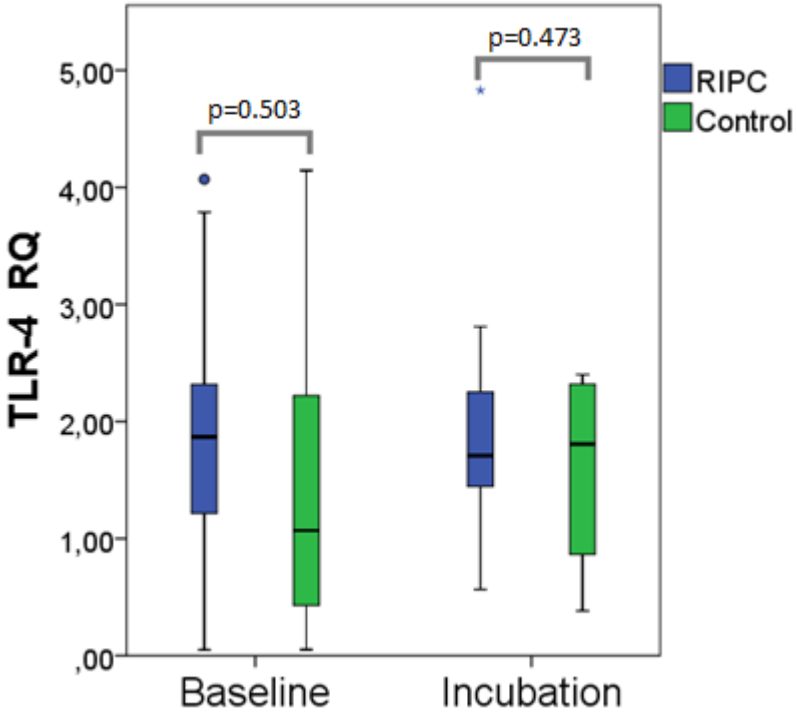

Figure 12.11. TLR-4 mRNA expression in the myocardium before and after incubation according to intervention allocation. Abbreviations: RIPC: remote ischaemic preconditioning; RQ: relative quantification; TLR-4: toll like receptor 4.

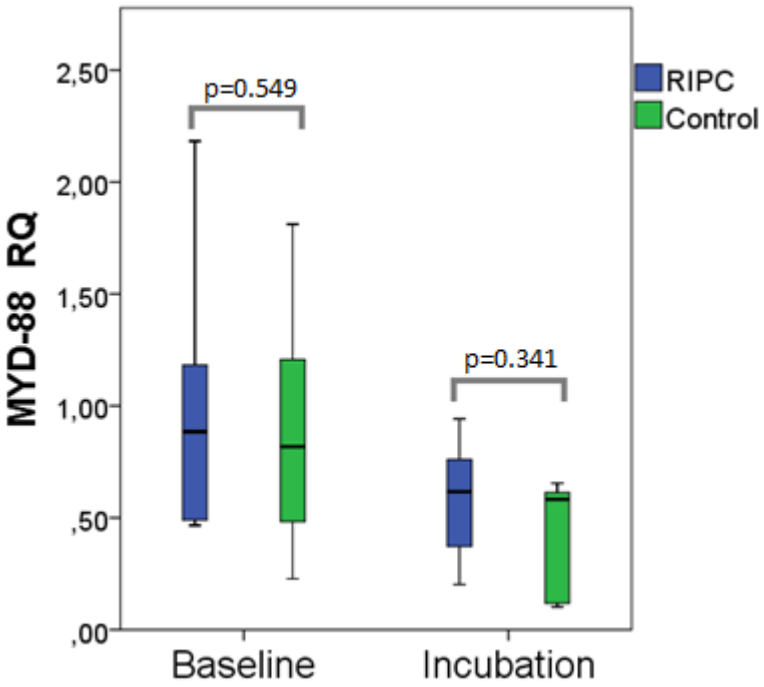

Figure 12.12. MYD-88 mRNA expression in the myocardium before and after incubation according to intervention allocation. Abbreviations: MYD-88: Myeloid Differentiation Primary Response 88; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

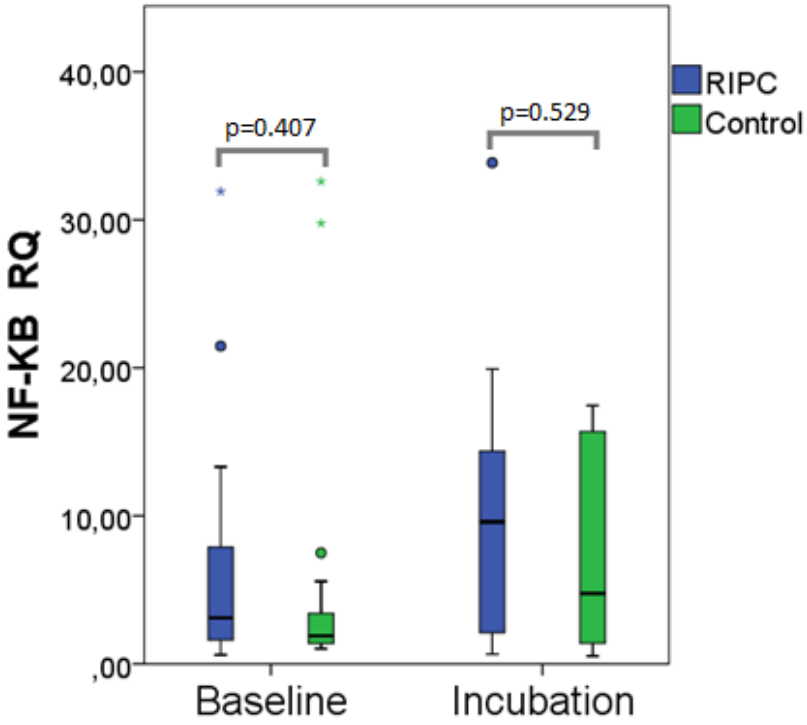

Figure 12.13. NF-κB mRNA expression in the myocardium before and after incubation according to intervention allocation. Abbreviations: NF-κB: nuclear factor – κB; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

12.4 Summary of key findings

- Preliminary experiments showed no difference in NF-κB expression according to cyanosis status or after incubation at normoxic, hyperoxic and hypoxic concentrations
- NF-κB baseline expression was higher in females.
- NF-κB baseline expression correlated to cTnI postoperative levels.
- TLR-4 and NF-κB presented higher expression in ventricular tissue.
- MYD-88 presented a significantly lower expression after hyperoxic incubation.
- RIPC did not have a significant effect on the expression of any of the analysed genes.

12.5 Limitations

Ct values representing the number of cycles required for the signal to cross the threshold during the Q-PCR reaction for TLR-2 and MYD-88 ranged among high 20s and low 30s values, meaning that in some samples the target nucleic acid varied from moderate to low amounts.

12.6 Discussion

The results showed that TLR-2, TLR-4, MYD-88 and NF-κB are expressed in the myocardium of CHD patients without exposure to any experimental conditions. Ct values suggesting relatively low levels of target cDNA for TLR-2 and MYD-88 were consistent with the low RQ values obtained for the expression of these genes. Microarray, RNA sequencing and serial analysis of gene expression (SAGE) have determined low levels of cardiac expression for TLR-2 and MYD-88 in humans (GeneCards).

NF-κB presented the highest baseline expression in this group of genes, interestingly NF-κB is located downstream to TLRs and MYD-88 and hence negative feedback could be taking place while NF-κB stimulates downstream inflammatory and stress reactions.

Previous work by our research group performed in the group of patients presented for the cyanotic vs acyanotic preliminary experiment in this chapter found significantly higher TLR-2

and TLR-4 expression in cyanotic patients (Walker, 2013), however our results in the RIPC group did not confirm differences in the expression of these genes between cyanotic and acyanotic groups.

Results from patients recruited for RIPC analysis confirmed the observation from the preliminary experiment in this chapter in which NF-κB did not present a significant variation according to cyanotic status.

NF-κB expression at baseline presented a correlation to cTnI levels after surgery, as the analysed tissue was resected during the ischaemic phase of CPB, NF-κB expression could not be a response to the damaging conditions of the total time of ischaemia and CPB that postoperative cTnI levels reflect. However NF-κB levels could have contributed to cardiac injury.

NF-κB has mainly been characterised as a cardioprotective component when activated early as it regulates several genes involved in cardioprotection (Tranter *et al*, 2010); nevertheless dual activities have been reported as it is an important component during inflammatory and immune reactions and is involved in several pathological processes. Its upregulation of inflammatory mediators has been shown to promote IRI (Ling *et al*, 2013; Zhang *et al*, 2013); furthermore it can increase injury through the inhibition of the salvage RISK pathway by interacting with TNF-α (Xing *et al*, 2016).

NF-κB expression was higher in females; the significantly lower weight in female patients observed in the study could have influenced the outcome, however a gender based difference in NF-κB expression cannot be ruled out. A study looking at gender differences in adult rats at basal and hypoxic conditions in cardiac fibroblasts found that NF-κB expression was higher in males at basal conditions and did not vary according to gender after 4 hours of hypoxia, to then show a lower expression in females after hypoxia for 16 hours (Zhao and Eghbali-Webb, 2002).

Expression in ventricular tissue tended to be higher in all genes reaching statistical significance for TLR-4 and NF-κB. Previous microarray analysis profiling had identified both genes in the left ventricle and not in right ventricular samples (Barth *et al*, 2005). To our knowledge spatial expression of these genes within the myocardium has not been explored before and the differences encountered could lead to determining more detailed patterns after further research.

Effects of hypoxia and reoxygenation have been reported to depend on the signalling of TLR-4, MYD-88 and NF-κB in the hepatocytes of a mice model (Ding *et al*, 2013).In our study, hyperoxic incubation resulted in a significantly lower MYD-88 expression compared to baseline measures, with no effects on the other genes, previous research in our laboratory group had not found a significant variation after similar incubation conditions.

The preliminary experiment comparing time points before and after different O_2 concentrations did not find significant changes in NF-κB expression, this was confirmed after hyperoxic exposure in the RIPC study.

NF-κB, TLR-2 and TLR-4 have been found to play both a detrimental and protective role during IRI according to the circumstances; NF-κB was described as an effector of direct preconditioning in an animal study that obtained infarct size reduction, as the effect was lost in animals presenting NF-κB deletions. One RIPC paediatric study analysed the activation of NF-κB in leukocytes for three randomly selected patients finding that its total quantification was inversely related to its inhibitor Iκ-Bα concentration; no analysis was done on the effects of RIPC (Pavione *et al*, 2012).

TLR-2 plays a role in the inflammation process taking place during IRI, animal models have linked it to ventricular dysfunction, while its inhibition has been found to reduce infarct size (Sakata *et al*, 2007; Arslan *et al*, 2012). On the other hand, it seems to play a role in preconditioning, as TLR-2 deficient mice lost the cardioprotective effects observed in normal animals (Dong *et al*, 2010).

Reports have consistently described TLR-4 as an inflammatory factor related to injury (Zhai *et al*, 2015), TLR-4 downregulation has been found to be beneficial and was reported as an effect of preconditioning (Yu *et al*, 2010; Yang *et al*, 2011). However one study using mice models found TLR-4 to be an important RIPC mediator through MYD-88 signalling, achieving cardiac protection (Wang *et al*, 2011).

The described effects of the analysed genes were not confirmed in this study as RIPC had no effect on their expression.

Conclusion

There is a potential differential pattern of expression according to ventricular or atrial origin of the tissue, hyperoxygenation reduced MYD-88 activity, while RIPC did not result in significant up or downregulation of any of the analysed genes.

Chapter 13. Hydrogen sulphide

13.1 Introduction

Hydrogen sulphide (H_2S), similarly to NO and CO is a gaseous signalling molecule or gasotransmitter. With a short half live H_2S is produced endogenously, diffuses rapidly intra and extracellularly and is present in all cellular types. Originally known for its toxicity it is now acknowledged that H_2S mediates various physiologic and pathological processes including the cell cycle, oxidative stress, apoptosis, inflammation, mitochondrial activity and energy metabolism, modulating cardiac, neural and gastrointestinal activity (reviewed by Olas, 2015).

H_2S is produced through different pathways, among them cysteine persulphidation has been the most extensively studied consisting of the enzymatic activity of cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE or CTH), cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase which mediate H_2S production from cysteine and homocysteine; CBS and CTH perform the main enzymatic reactions, they are present in the cytosol and are distributed according to tissue types with CBS being highly expressed in the nervous system while CTH is majorly present in the cardiovascular system (reviewed by Guo *et al*, 2013; Kabil *et al*, 2014).

H_2S has been established as a ion channel regulator including Ca^{2+} and mitoK_{ATP} channels, with the latter being an important modulatory mechanism; H_2S promotes the opening of mitoK_{ATP} channels resulting in vasodilation, preventing excessive smooth muscle contraction in the vessels and reducing blood pressure (Zhao *et al*, 2001); vasodilation is further achieved through H_2S regulation of Ca^{2+} causing membrane hyperpolarization (Jackson-Weaber *et al*, 2013). Interestingly studies have found that high H_2S concentration results in vasodilation while low concentrations are able to cause vasoconstriction, in a dose dependent effect due to unknown mechanisms (Webb *et al*, 2008).

Unlike NO the mechanisms for H_2S vasodilation using mitoK_{ATP} channels seem to be independent from cGMP mediation as its blockade does not affect results (Tang *et al*, 2005), however levels of cGMP have increased after H_2S exogenous administration without a clear conclusion on its involvement (reviewed by Bibli *et al*, 2015).

Potent antioxidant activity has been linked to H_2S as it intervenes in the production and scavenging of ROS and supports endogenous antioxidants systems (reviewed by Kabil *et al*, 2014; Bos *et al*, 2015). Additionally, angiogenesis has been shown to be favoured by H_2S mediated migration and growth of endothelial cells, producing neovascularisation in a process dependent on the Akt pathway in animal models (Cai *et al*, 2007).

Similarly to NO, H_2S seems to present a dual role, with inflammation, stimulating cytokine release ICAM-1 mediation and neutrophil migration through mitoK_{ATP} channels dependent mechanisms (Dal-Secco *et al*, 2008), while the same pathways have been reported to cause downregulation of inflammatory responses leading to inhibition of foam cell formation, leading to a limitation in the development of atherosclerosis (Zhao *et al*, 2011).

Pro and anti-apoptotic effects have also been described, with a link between CTH increased expression and H_2S synthesis leading to apoptosis induction in human endothelium (Yang *et al*, 2006); on the other hand apoptosis was prevented by H_2S induced activity of NF-κB, an effect abolished in CTH deficient animals (Sen *et al*, 2012).

A combination of the described activity of H_2S could contribute to IRI prevention, H_2S has been recognised as a potential cardioprotective agent that could prevent the development of several cardiac pathologies (reviewed by Yu *et al*, 2014).

mitoK_{ATP} channel opening has a direct effect on IRI as it intervenes in mitochondrial response, avoiding membrane potential depletion thus preventing cellular death after reperfusion. This was tested in a rat model of IRI which reported that H_2S promoted cardiac protection obtaining better cardiac function and arrhythmia scores (Zhang *et al*, 2007). Mitochondrial structure and functional conservation has been established as an important mechanism of H_2S cardioprotection (Elrod *et al*, 2007).

Various interventions have been used in order to analyse the effects of H_2S in IRI with its administration being used as a preconditioning modality. Apoptosis during IRI was reduced after the administration of H_2S in pigs showing variation in mediators including upregulation of MAPK and a decline in caspase 3 activity, reduced apoptosis correlated with lower infarct size and better left ventricular function (Osipov *et al*, 2009).This was further proved in a study of rat

IRI which presented reduced apoptosis, infarct size and better ventricular function after H_2S preconditioning through stress reduction in the endo/sarcoplasmic reticulum (Li *et al*, 2015). Administration of sulphur dioxide resulted in cardioprotection mediated by CTH and H_2S after altering redox activity (Jin *et al*, 2013).

Ischaemic preconditioning caused an upregulation of endogenous H_2S resulting in reduced duration and degree of arrhythmia, and increased cell viability in isolated rat hearts, these effects were lost when PKC or mitoK_{ATP} channels were blocked implying their important role in the process (Bian *et al*, 2006). The involvement of mitoK_{ATP} channels in H_2S mediated cardioprotection was confirmed in a metabolic inhibition conditioning study in rat hearts (Pan *et al*, 2008).

As CBS and CTH are necessary for H_2S production, they could be used as markers of its activity, CTH mRNA expression in the heart was confirmed in wild type mice and rats, H_2S production in myocardial tissue was significantly reduced in CTH knockout animals (Fu *et al*, 2012). CTH expression has been shown to correlate directly to H_2S production and cardiac protection in mice (Shen *et al*, 2015), in addition CTH overexpression has resulted in the prevention of atherosclerosis (Cheung *et al*, 2014).

13.2 Preliminary experiments

13.2.1 Aims

To compare CBS and CTH expression between cyanotic and acyanotic patients and to analyse their expression pattern before and after incubation at hypoxic, normoxic and hyperoxic conditions.

13.2.2 Materials and methods

Patients requiring cardiac surgery for CHD correction were recruited in order to perform two different analysis: a comparison of gene expression between cyanotic and acyanotic patients and a differential gene expression analysis before and after incubation at different O_2 concentrations.

Tissue was resected during aortic cross clamp as part of the surgical procedure, a cyanosis status experiment was performed using myocardium without previous exposure to any experimental conditions.

The second experiment divided the samples obtained during surgery into an immediate baseline sample after collection in theatre, a second sample processed after transportation to the laboratory in a flask previously exposed to 2% O_2, and subsequent samples incubated at hypoxic (2% O_2), normoxic (20% O_2) and hyperoxic (60% O_2) conditions during 4 hours.

mRNA was then extracted from the tissue samples. The stages of work described so far were performed by Susan Walker as part of her PhD work within our laboratory group for work in other genes.

As part of this thesis work cDNA was obtained from the mRNA samples through a RT-PCR reaction and Q-PCR was performed in order to obtain RQ values for CBS and CTH as described in *Chapter 2*.

13.2.3 Statistical analysis

Cyanotic vs acyanotic comparison was performed using an independent sample t-test.

For the second experiment analysis was divided into two sections: a comparison of time points at baseline (theatre collection), laboratory arrival and post-incubation for each O_2 concentration used, and a second comparison between the three O_2 concentrations used for incubation. Friedman repeated measures test was used with post-hoc analysis for significant results ($p<0.05$) using Wilcoxon signed-rank tests and Bonferroni correction at a significance level of $p=0.0167$.

13.2.4 Results

Gene expression analysis according to cyanosis status

Twelve patients were recruited for cyanosis status analysis, Demographic characteristics of the patients are shown in *Table 12.3*.

Table 13.1 presents descriptive data for CBS, CTH and NF-κB expression according to cyanosis status.

Gene	Cyanosis	N	Mean – SD	P value
CBS	Cyanotic	7	0.94±0.45	0.491
	Non-cyanotic	5	1.13±0.51	
CTH	Cyanotic	7	6.02±2.11	0.002
	Non-cyanotic	5	2.22±0.72	

Table 13.1. Descriptive statistics for the differential gene expression of CBS and CTH. Abbreviations: CBS: cystathionine-β synthase; CTH: cystathionine-γ lyase.

CTH was differentially expressed, presenting higher levels in cyanotic patients; CBS expression did not vary significantly (*Figure 13.1*).

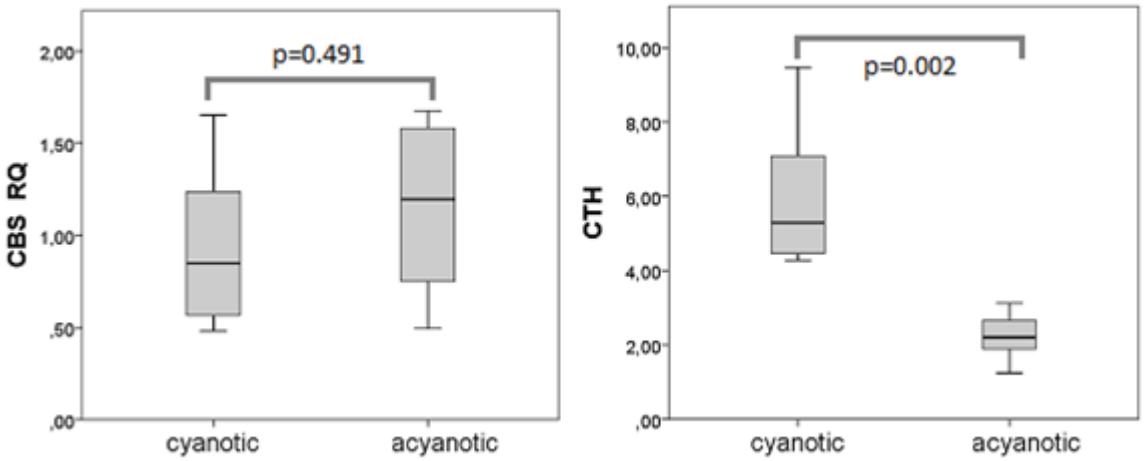

Figure 13.1. Expression of CBS and CTH according to cyanosis status. Abbreviations: CBS: cystathionine-β synthase; CTH: cystathionine-γ lyase.

Incubation at differential O_2 concentrations

Ten patients were recruited for the experiment, demographic data can be seen on *Table 12.4*.

Eight patients presented complete measurements for all time points, descriptive information is summarised in *Table 13.2*.

Gene	Time point	Median – IQ range
CBS	Baseline	0.86(0.58-1.40)
	Laboratory arrival	0.53(0.33-0.70)
	2% O_2 incubation	0.77(0.25-1.21)
	20% O_2 incubation	0.66(0.31-1.52)
	60% O_2 incubation	1.07(0.47-2.24)
CTH	Baseline	3.13(1.92-4.16)
	Laboratory arrival	1.67(1.45-5.21)
	2% O_2 incubation	2.40(1.63-3.40)
	20% O_2 incubation	3.74(1.86-5.57)
	60% O_2 incubation	6.34(4.19-7.93)

Table 13.2. Descriptive statistics for CBS and CTH gene expression analysis before and after differential O_2 incubation. Abbreviations: CBS: cystathionine-β synthase; CTH: cystathionine-γ lyase; IQ: interquartile; O_2: oxygen.

Friedman test was used in order to determine differences between time points for baseline, laboratory arrival and incubation. When the differences were significant, post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were used resulting in a significance level at $p<0.0167$. *Table 13.3* shows the results for the analysis.

Gene	Time point	X^2, P value	Significance	Post-hoc analysis	P value
CBS	BL-Lab-2% O_2	7.000, p=0.030	p<0.0167	Bl-Lab Bl-2% O_2 Lab-2% O_2	0.093 0.017 0.575
	BL-Lab-20% O_2	7.000, p=0.030	p<0.0167	Bl-Lab Bl-2% O_2 Lab-2% O_2	0.093 0.017 0.327
	BL-Lab-60% O_2	4.000, p=0.135			
CTH	BL-Lab-2% O_2	1.750, p=0.417			
	BL-Lab-20% O_2	1.000, p=0.607			
	BL-Lab-60% O_2	5.250, p=0.072			

Table 13.3. Results for CBS and CTH for differences between time points using related samples Friedman tests and post-hoc analysis with Bonferroni correction. Abbreviations: BL: baseline; CBS: cystathionine-β synthase; CTH: cystathionine-γ lyase; Lab: laboratory arrival; O_2: oxygen; X^2: chi square.

Variation in CBS expression between baseline, laboratory arrival and 2% O_2 incubation was significant between time points, however after post-hoc analysis differences were not significant between any pairs. Similar results were observed between baseline, laboratory arrival and 20% O_2 incubation. Variation between baseline, laboratory arrival and 60% O_2 incubation was not significant (*Figure 13.2*).

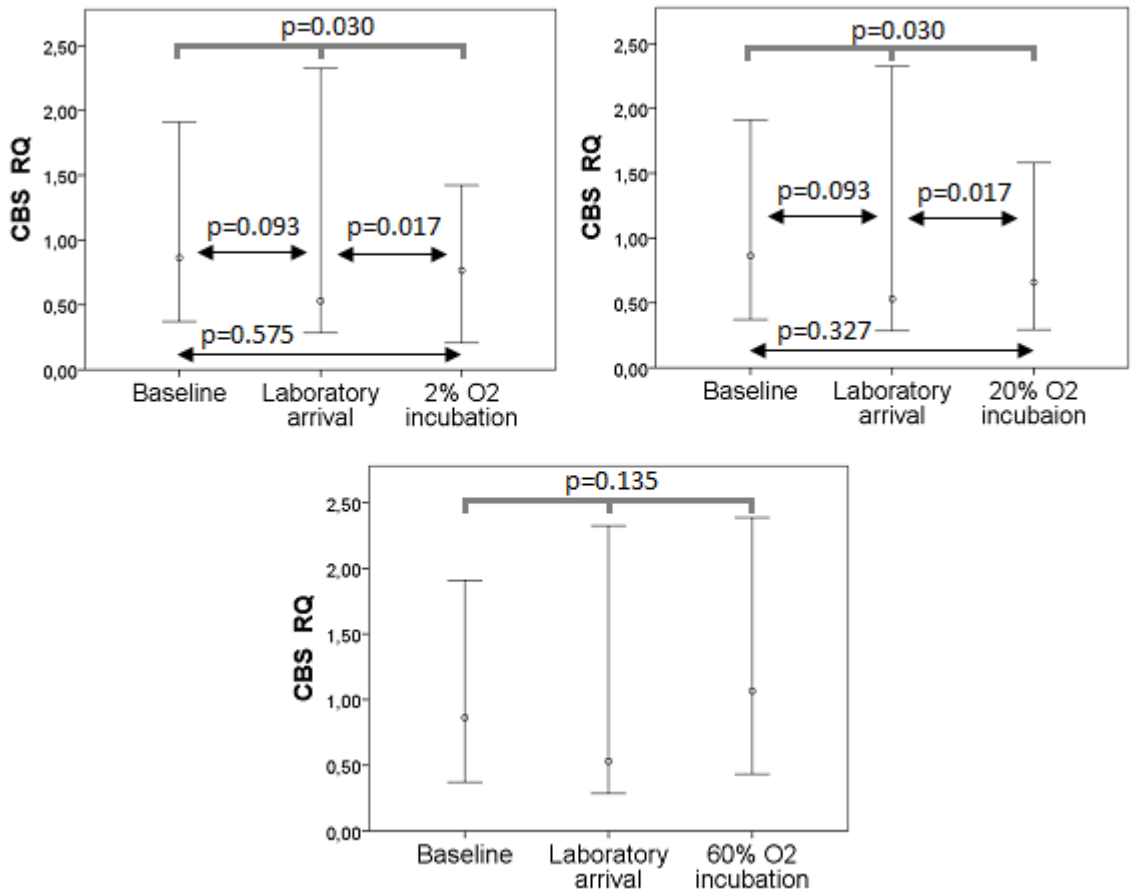

Figure 13.2. CBS expression according to time points before and after O_2 incubation. Post hoc analysis of differences between pairs is significant at p<0.0167. Abbreviations: CBS: cystathionine-β synthase; O_2: oxygen; RQ: relative quantification.

Analysis for CTH (*Figure 13.3*) did not result in any significant differences across time points.

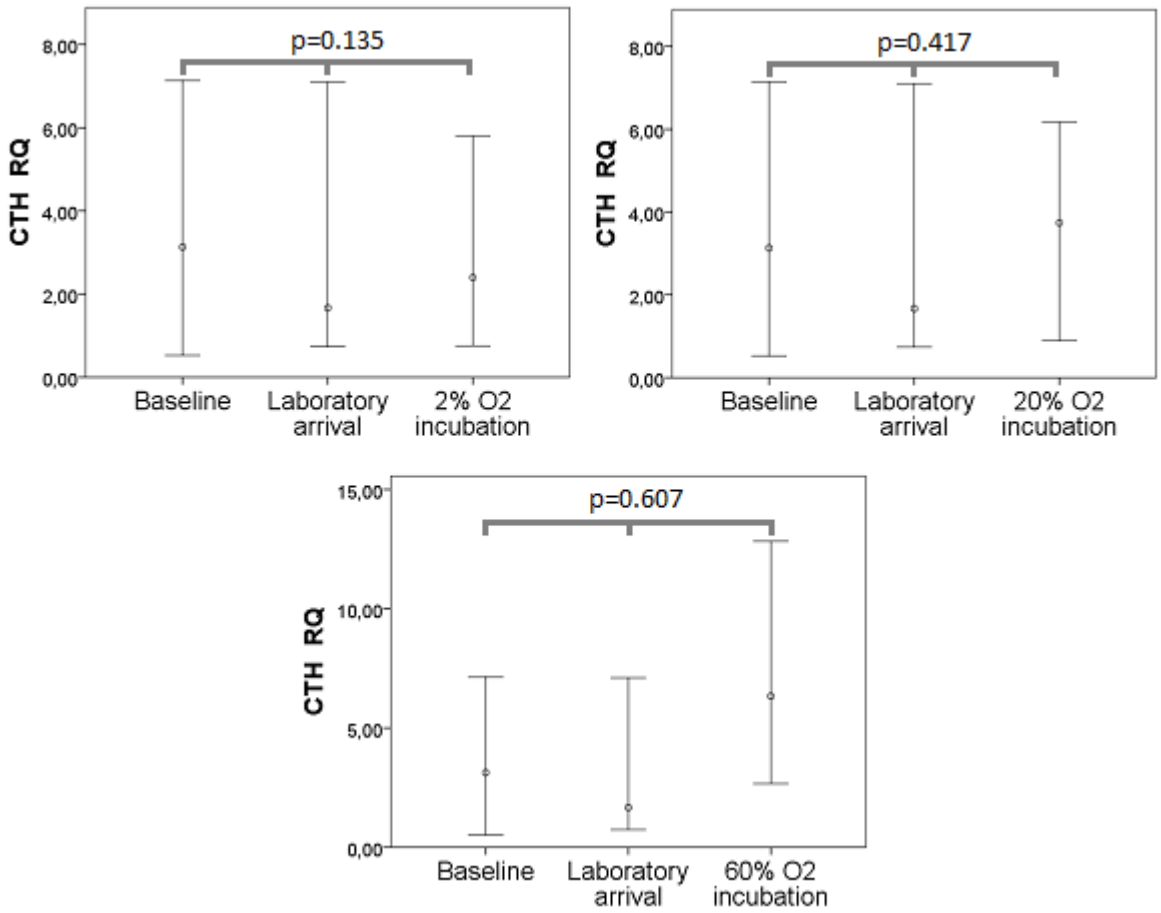

Figure 13.3. CTH expression according to time points before and after O_2 incubation. Abbreviations: CTH: cystathionine-γ lyase; O_2: oxygen; RQ: relative quantification.

All patients (n=10) presented complete data for incubation at 2%, 20% and 60% O_2 concentration, descriptive information is summarised in *Table 13.2*.

Repeated measures Friedman test was used in order to determine differences between incubation at different O_2 concentrations. When the differences were significant, post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction were used resulting in a significance level at $p<0.0167$. *Table 13.4* shows the results for the analysis.

Gene	Time point	X^2, P value	Significance	Post-hoc analysis	P value
CBS	2% O_2-20% O_2-60% O_2	2.400, p=0.301			
CTH	2% O_2-20% O_2-60% O_2	16.800, p<0.0001	p<0.0167	2% -20% 2%-60% 20%-60%	0.005 0.005 0.093

Table 13.4. Results for CBS and CTH for variation between incubation at different oxygen concentration using related samples Friedman tests and post-hoc analysis with Bonferroni correction. Abbreviations: CBS: cystathionine-β synthase; CTH: cystathionine-γ lyase; O_2: oxygen; X^2: chi square.

Differences in CTH expression were significant, post hoc analysis revealed that incubation at 2% and 20% O_2 resulted in significantly different expression, similar results were obtained when comparing 2% and 60% O_2 incubation. There was no significant variation in CTH expression between 20% and 60% O_2 concentration. Expression of CBS did not vary significantly according to incubation using different O_2 concentrations (*Figure 13.4*).

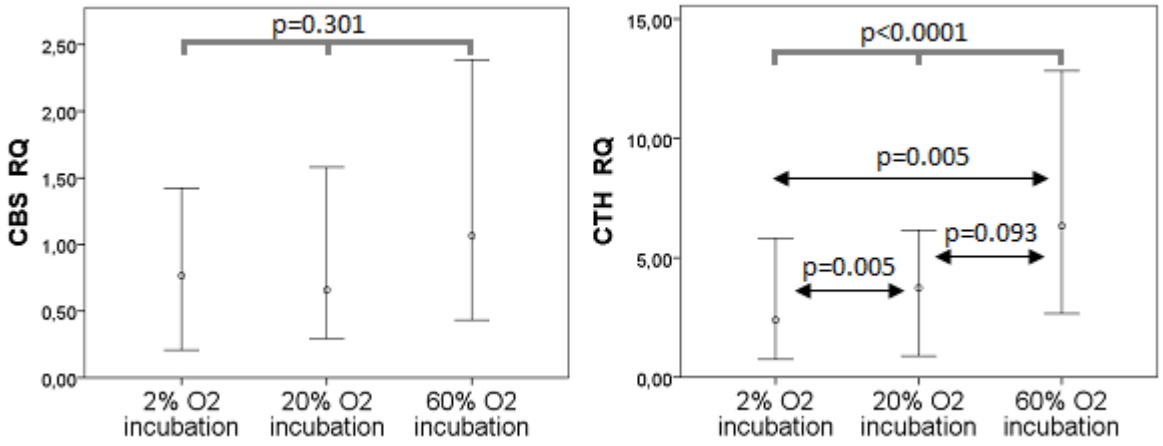

Figure 13.4. CBS and CTH gene expression analysis according to differential O_2 incubation. Abbreviations: CBS: cystathionine-β synthase; CTH: cystathionine-γ lyase; O_2: oxygen; RQ: relative quantification.

13.3 RIPC study

13.3.1 Aims

This study aimed to analyse the pattern of activity of CBS and CTH in CHD patients requiring corrective surgery using CPB and to assess the effects of RIPC on their expression.

13.3.2 Materials and Methods

Tissue was obtained from patients during surgical intervention, ventricular tissue was divided and part of it was immediately processed as a baseline reference, the remaining was incubated at 60% O_2 during 4 hours; atrial tissue was only used for baseline analysis due to insufficient available tissue.

mRNA was extracted from the myocardial tissue and used in order to obtain cDNA through a RT-PCR reaction followed by Q-PCR analysis in order to obtain relative quantification values for each gene. Genes used during the assay are shown in *Table 13.5*.

	Gene name	Gene aliases	Gene symbol	Assay Design	Amplicon length	Assay ID
CBS	cystathionine-beta-synthase	HIP4	CBS	Probe spans exons	59	Hs00163925_m1
CTH	cystathionase (cystathionine gamma-lyase)	CSE	CTH	Probe spans exons	110	Hs00542284_m1

Table 13.5. Genes analysed and assays used. Abbreviations: CBS: cystathionine-β synthase; CTH: cystathionine-γ lyase.

Details on materials and methods are described on *Chapter 2*.

13.3.3 Statistical analysis

The data did not follow a normal distribution, therefore analysis was performed using non-parametric tests.

Spearman analysis was used in order to determine correlation between demographic and baseline characteristics including the gender, age, weight and cyanosis status of patients, correlations to post-operative cTnI levels were also explored.

Differences in expression according to tissue type were analysed using Mann Whitney tests. Wilcoxon signed-rank test for paired measures was used to explore differences before and after tissue incubation.

Mann Whitney tests were used to determine differences according to RIPC or control allocation for each time point at baseline and after incubation.

Analysis was performed using SPSS version 23 software, a p value <0.05 (two tailed) was considered significant.

13.3.4 Results

Fifty patients were recruited for the study, demographic data and its analysis can be seen in *Tables 3.1* and *3.2*.

General analysis

The genes coding for both H_2S enzymes, CBS and CTH were expressed in the myocardium of CHD patients at baseline (*Figure 13.5*).

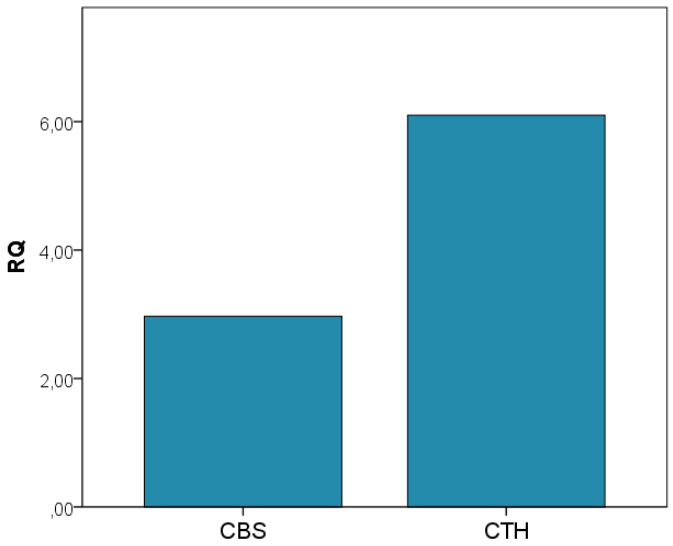

Figure 13.5. mRNA expression of hydrogen sulphide enzymes in myocardium of CHD patients. Figure shows the median for RQ values of each gene. Abbreviations: CBS: cystathionine β-synthase; CTH: cystathionine γ-lyase; RQ: relative quantification.

CBS expression presented a negative correlation to gender (r_s= -0.371, p=0.044); variation according to gender was significant with f: 4.08 (2.87-5.35) vs m: 1.93(1.67-3.02), p=0.047 (*Figure 13.6*).

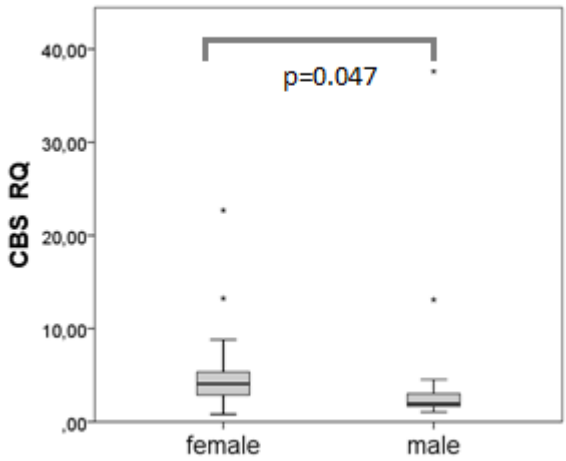

Figure 13.6. CBS expression according to gender. Abbreviations: CBS: cystathionine β-synthase; RQ: relative quantification.

Expression according to tissue type

Table 13.6 shows descriptive statistics for the analysed genes.

Gene	N	Tissue	Median – IQ range	P value
CBS	22	Ventricle	3.62(1.67-4.80)	0.393
	8	Atrium	2.07(1.85-2.91)	
CTH	22	Ventricle	8.70(4.89-17.05)	0.003
	7	Atrium	3.61(1.72-5.35)	

Table 13.6. Descriptive statistics for mRNA expression of hydrogen sulphide enzymes according to ventricular of atrial tissue. Data is expressed in median – IQ range. Abbreviations: CBS: cystathionine β-synthase; CTH: cystathionine γ-lyase; IQ: interquartile.

CTH expression was significantly higher in ventricular tissue (*Figure 13.7*).

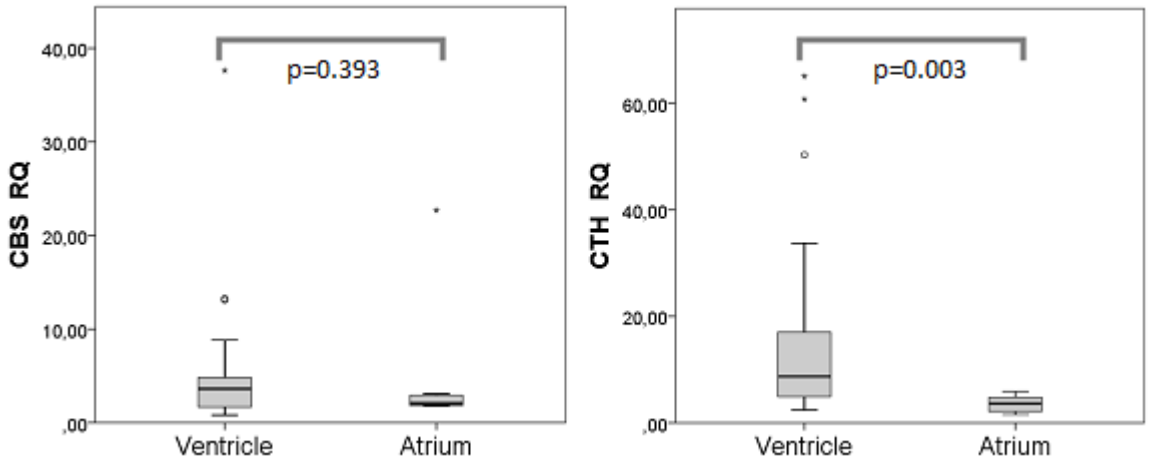

Figure 13.7. mRNA expression of hydrogen sulphide enzymes according to ventricular or atrial tissue. Abbreviations: CBS: cystathionine β-synthase; CTH: cystathionine γ-lyase; RQ: relative quantification.

Expression after tissue incubation

Descriptive information for CBS and CTH before and after incubation is shown in *Table 13.7*.

Gene	N	Time point	Median - IQ range	P value
CBS	15	Baseline	2.91(1.51-4.59)	0.002
	15	Incubated	1.84(1.27-2.32)	
CTH	15	Baseline	6.83(4.89-13.32)	0.307
	15	Incubated	8.59(5.40-14.74)	

Table 13.7. Descriptive statistics for mRNA expression of hydrogen sulphide enzymes in myocardium before and after incubation. Data is expressed in median – IQ range. Abbreviations: CBS: cystathionine β-synthase; CTH: cystathionine γ-lyase; IQ: interquartile.

After hyperoxic incubation CBS expression was significantly lower compared to baseline (*Figure 13.8*).

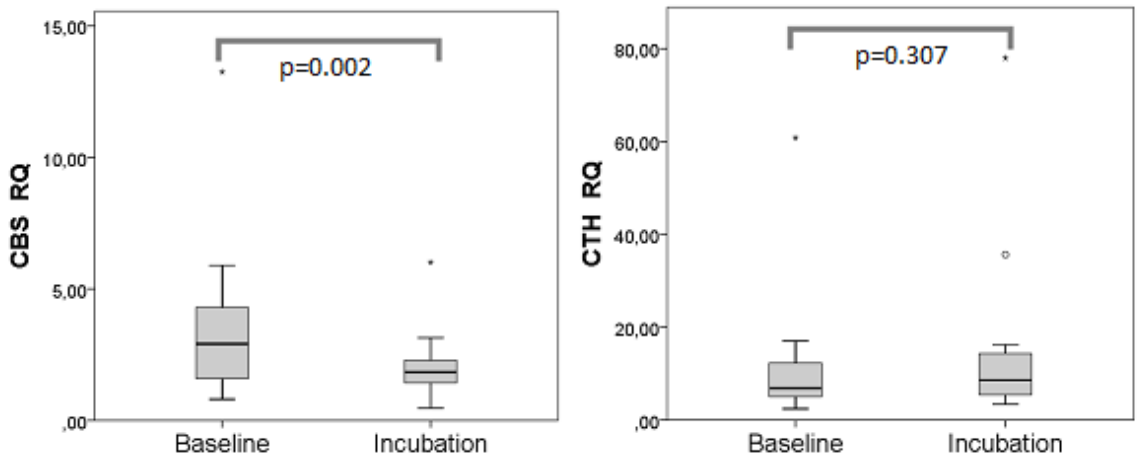

Figure 13.8. mRNA expression of hydrogen sulphide enzymes in myocardium before and after incubation. Abbreviations: CBS: cystathionine β -synthase; CTH: cystathionine γ-lyase; RQ: relative quantification.

RIPC analysis

Forty-eight patients were analysed according to intervention allocation. Descriptive information on gene expression according to control or RIPC allocation is presented in *Table 13.8*.

Gene	Time point	Intervention	N	Median – IQ range	P value
CBS	Baseline	RIPC	14	2.97(1.67-4.01)	0.222
		Control	15	4.53(2.04-8.81)	
	Incubation	RIPC	9	1.840(1.268-2.255)	1.000
		Control	6	1.947(1.589-2.315)	
CTH	Baseline	RIPC	14	5.26(4.17-10.16)	0.358
		Control	14	6.66(4.02-33.39)	
	Incubation	RIPC	9	6.554(4.168-16.231)	0.607
		Control	6	9.533(8.209-13.905)	

Table 13.8. Descriptive statistics for mRNA expression of hydrogen sulphide enzymes in myocardium before and after incubation according to intervention group allocation. Data is expressed in mean and standard deviations. Abbreviations: CBS: cystathionine β-synthase; CTH: cystathionine γ-lyase; RIPC: remote ischaemic preconditioning.

Expression of CBS (*Figure 13.9*) and CTH (*Figure 13.10*) did not vary according to group allocation at baseline or after incubation.

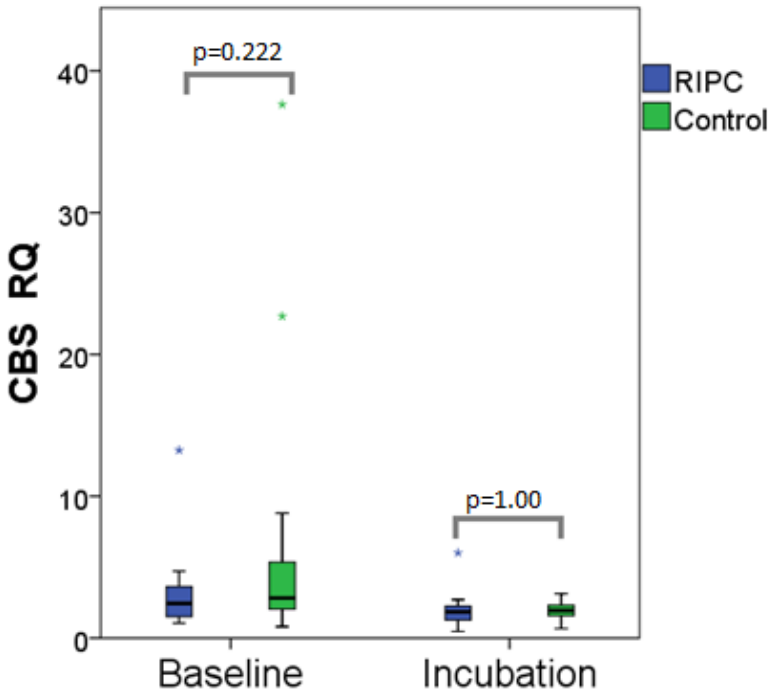

Figure 13.9. CBS mRNA expression in myocardium before and after incubation. Abbreviations: CBS: cystathionine β-synthase; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

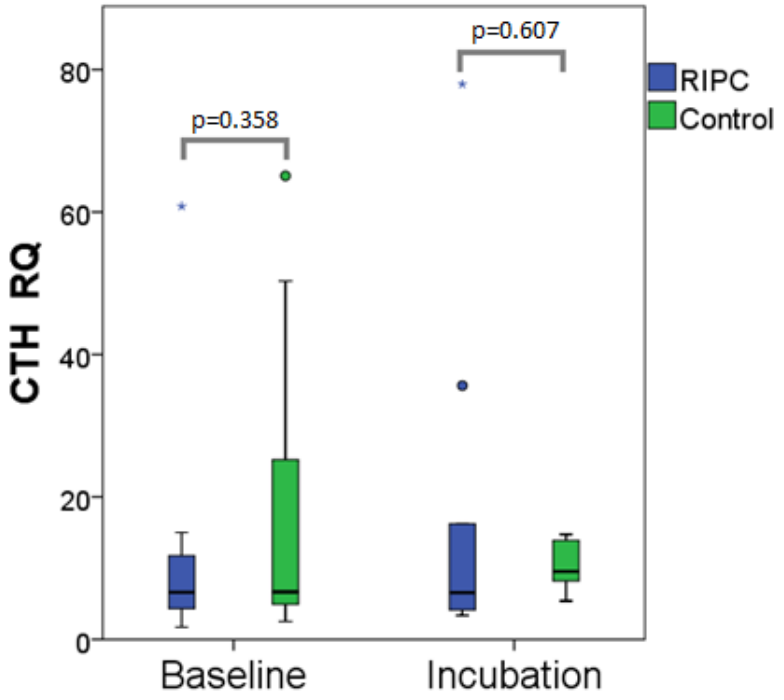

Figure 13.10. CTH mRNA expression in myocardium before and after incubation. Abbreviations: CTH: cystathionine γ-lyase; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

13.4 Summary of key findings

- Preliminary experiments resulted in a higher CTH expression in cyanotic patients; incubation at different O_2 concentrations resulted in different CTH expression patterns.
- CBS presented a lower expression in females.
- CTH was significantly higher in ventricular tissue.
- CBS expression was significantly reduced after hyperoxic incubation.
- RIPC did not affect the expression of CBS or CTH.

13.5 Limitations

Ct values representing the number of cycles required for the signal to cross the threshold during the Q-PCR reaction for CBS and CTH were above 30 in all samples; this reflects a low level of the target cDNA present in the tissue.

13.6 Discussion

In spite of requiring more than 30 cycles to reach the threshold, which means that low target nucleic acid was present during the reaction, CBS and CTH RQ values were higher than those of other genes presenting better reaction conditions. On account of this CBS and CTH values should not be compared to other genes, but only to different aspects of its own expression. CBS and CTH expression is present in the human myocardium at low levels as determined by microarray, RNA sequencing and SAGE (GeneCards).

CBS expression was higher in male patients, a significant lower weight was observed in female patients recruited in the study, and this could have influenced gene expression patterns; however, there was no correlation between CBS expression and weight. Other demographic characteristics did not correlate to expression values.

CBS expression tended to be higher in the ventricle, but it did not reach significance, this difference was very marked for CTH which showed a significantly higher expression in ventricular tissue. The limited evidence available on atrial and ventricular differences including genome profiling studies have not reported CTH findings (Barth *et al*, 2005).

Preliminary experiments presented an initial variation in the expression of CBS before and after hypoxic and normoxic incubation, this effect was lost after post-hoc analysis; hyperoxic incubation had no effect on CBS expression, however analysis in a bigger sample during the RIPC study showed an effect in CBS expression which was significantly reduced after incubation at 60% O_2 concentration.

Rat models of IRI have found a consistent reduction of CBS expression after ischaemia and reoxygenation which resulted in reduced H_2S activity and contributed to liver and renal injury (Xu *et al*, 2009; Teng *et al*, 2013; Wang *et al*, 2014).

Preliminary and RIPC analysis consistently resulted in no significant variation on CTH expression before and after incubation. It is to be noted that the preliminary experiment found that exposure to normoxic, hypoxic and hyperoxic incubation resulted in differential expression patterns for CTH when compared to each other.

The cardioprotective role of H_2S has been established in animal trials achieving infarct size reduction through a limitation of endo and sarcoplasmic reticulum stress (Li *et al*, 2015); however, this protective effect might not contribute to RIPC protection as reported in a rat model that showed limited contribution of endogenous H_2S (Bliksøen *et al*, 2008).

CBS has been linked to protection against cardiac IRI in an rat model while interacting with HO-1 (Zhu *et al*, 2008), while cardioprotective effects of CTH expression have been reported in mice (Shen *et al*, 2015).

To our knowledge this study is the first to explore CBS and CTH expression in human cardiac tissue.

Conclusion

CBS and CTH expression tended to be higher in the control group compared to RIPC, however these differences did not reach statistical significance before or after incubation.

Chapter 14. Apelin

14.1 Introduction

The human APJ gene coding for a G protein coupled receptor was discovered and cloned in 1993, presenting similarities to angiotensin II type 1 receptor, it did not bind to angiotensin (O´Dowd *et al*, 1993). Apelin, the only ligand for APJ remained unknown until it was identified in 1998 as a gene coding for a 77 aminoacid preprotein producing several forms of apelin (Tatemoto *et al*, 1998).

Apelin and APJ are expressed in various tissues, however they have mainly been characterised in the cardiovascular system presenting important functions as a cardiovascular signalling pathway with influence in physiological and pathological processes (reviewed by Yu *et al*, 2014).

Apelin has also been identified as an adipokine, it is secreted in adipose tissue and has a role in energy metabolism and modulates insulin secretion; it is upregulated in obesity and metabolic diseases such as diabetes (Boucher *et al*, 2005).

APJ and apelin expression is controlled by several mechanisms including endocannabinoid receptors and lipopolysaccharides in a process involving IL-1 and TNF-α; angiotensin II type 1 receptor upregulates apelin and APJ while type 2 receptors inhibit their production (Than *et al*, 2012).

Hypoxia and inflammatory mediators such as TNF-α have been shown to induce the expression of apelin in hepatic cells (Melgar-Lesmes *et al*, 2011), with an upregulating effect of hypoxia demonstrated in adipocytes through the action of HIF-1α (Geiger *et al*, 2011). On the other hand reoxygenation resulted in lower expression of both genes in human endothelium (Yamagata *et al*, 2012).

Within the cardiovascular system apelin regulation produces vasodilation, presenting opposing activity to that of angiotensin, it has an inotropic effect improving contraction and cardiac function, influences fluid homeostasis through the kidney and central activity related to vasopressin and it is involved in pathologic events such as heart failure, hypertension,

atherosclerosis, coronary disease, atrial fibrillation, myocardial infarction and IRI (reviewed by Barnes *et al*, 2010).

Contribution of apelin to cardiac protection has been established through several IRI animal studies. Administration of the apelin subtypes apelin-36 and apelin-13 during reperfusion resulted in infarct size reduction in *in vivo* and *in vitro* mice and rat models through the RISK pathway and MPTP opening delay (Simpkin *et al*, 2007). Protective effects involving inhibition of MPTP opening have been confirmed for apelin-13, which is more potent than other subtypes, in a process dependent on the RISK components PI3K/Akt and ERK (Yang *et al*, 2015).

A rat model of cardiac ischaemia and reperfusion showed that apelin levels were highly upregulated during ischaemia, however went back to baseline after 30 minutes of reperfusion, while APJ expression remained unchanged. Apelin-13 was effective in reducing infarct size when administered during reperfusion but not during ischaemia. Interestingly PI3K inhibition did not affect the protective effect suggesting an alternative mechanism for apelin function (Kleinz and Baxter, 2008).

The timing of apelin administration has been additionally assessed finding efficacy in cardiac protection when administered after ischaemia only, this suggests apelin could be used as a factor for post-conditioning and not preconditioning (Rastaldo *et al*, 2011).

Further investigation determined that APJ expression was increased after ischaemia and reperfusion at mRNA and protein levels with previous apelin administration improving cardiac function; cultured IRI cardiomyocytes presented induced apoptosis as well as APJ and apelin upregulation along with lactate dehydrogenase leakage; apelin administration reduced apoptosis, ROS, malonaldehyde content and lactate dehydrogenase leakage; in addition SOD activity was induced (Zeng *et al*, 2009). Apelin-13 administration was also linked to a reduction in endoplasmic reticulum stress induced apoptosis (Tao *et al*, 2011).

Previous investigation in our research group carried out in TOF patients going through surgical repair using CPB found that APJ was expressed in all ventricular myocardium samples analysed, with expression rising in correlation to ischaemia and cardioplegia duration while there was no

difference according to oxygen concentration used for reperfusion *in vitro* or between cyanotic and acyanotic patients (Walker *et al*, 2014).

14.2 Aims

To analyse the expression pattern of apelin and APJ in the myocardium of CHD patients requiring corrective surgery using CPB before and after hyperoxic incubation, and to assess the effects of RIPC on their expression.

14.3 Materials and Methods

Tissue was obtained from patients during the aortic clamp phase of the surgical intervention, atrial tissue was immediately processed, while ventricular tissue was divided into two sections: the first one was processed as a baseline reference while the second one was incubated at 60% O_2 for 4 hours.

mRNA was extracted from the tissue and after a RT-PCR reaction cDNA was obtained. Q-PCR analysis was then performed in order to obtain relative quantification values for each gene. Genes used during the assay are shown in *Table 14.1*.

Gene name	Gene aliases	Gene symbol	Assay Design	Amplicon length	Assay ID
Apelin	APEL, UNQ471, XNPEP2	APLN	Probe spans exons	79	Hs00175572 _m1
Apelin receptor	AGTRL1, APJ, APJR, HG11	APLNR	Both primers and probe map within a single exon	98	Hs00270873 _s1

Table 14.1. Genes analysed and assays used.

Details on materials and methods are described on *Chapter 2*.

14.4 Statistical analysis

The data did not follow a normal distribution, therefore analysis was performed using non-parametric tests.

Correlations between demographic characteristics, cTnI levels and gene expression were determined using Spearman analysis.

Differences in expression according to tissue type were analysed using Mann Whitney tests. Variation before and after tissue incubation was analysed using the Wilcoxon signed-rank test for paired measures.

Mann Whitney tests were used in order to determine differences according to RIPC or control allocation at baseline and after incubation.

Analysis was performed using SPSS version 23 software, a p value <0.05 (two tailed) was considered significant.

14.5 Results

Fifty patients were recruited for the study, demographic data and its analysis can be seen in *Tables 3.1* and *3.2*.

14.5.1 General analysis

Apelin and APJ were expressed in the myocardium of CHD patients (*Figure 14.1*).

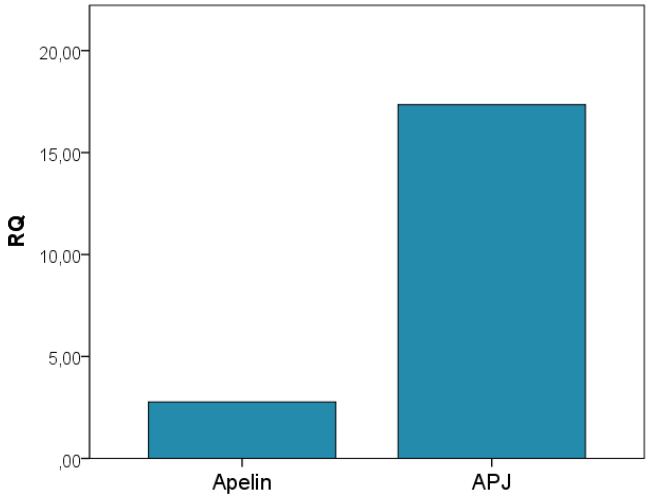

Figure 14.1. Apelin – APJ mRNA expression in myocardium of CHD patients. Figure shows the median for RQ values of each gene. Abbreviations: APJ: apelin receptor; RQ: relative quantification.

APJ correlated negatively to gender (r_s= -0.464, p=0.009), the variation according to gender was significant with f: 26.99(15.65-58.08) vs m: 12.21(2.30-18.74), p=0.010 (*Figure 14.2*).

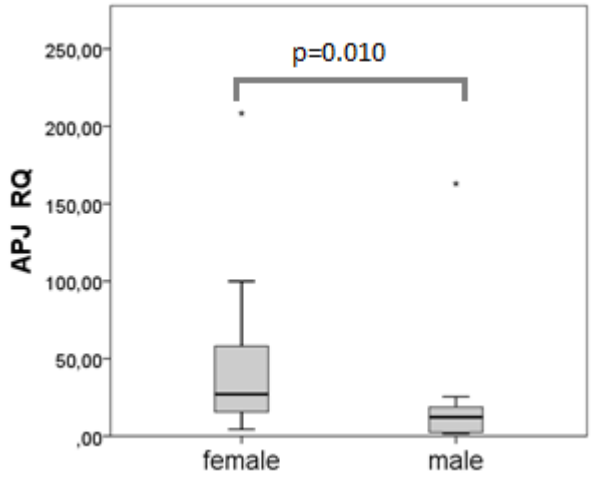

Figure 14.2. APJ expression according to gender. Data is expressed in median and interquartile range. Abbreviations: APJ: apelin receptor; IQ: interquartile.

Correlations explored resulted in a positive correlation of APJ to PO1 cTnI levels (r_s=0.422, p=0.018) (*Figure 14.3*).

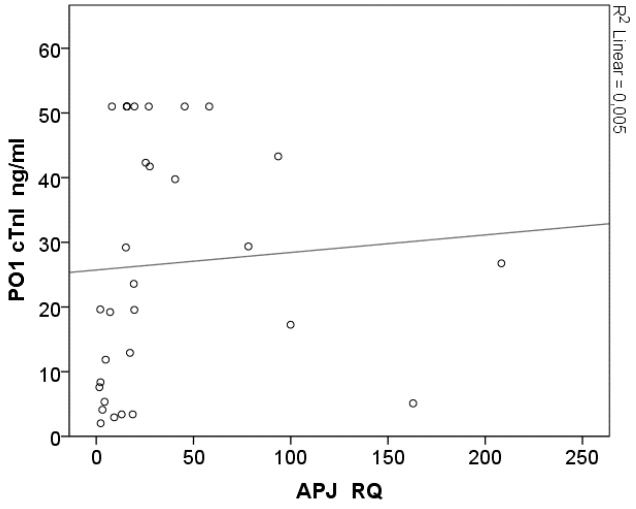

Figure 14.3. APJ correlation to cTnI. Abbreviations: APJ: apelin receptor; cTnI: cardiac troponin I.

Expression according to tissue type

Table 14.2 shows descriptive statistics for the analysed genes.

Gene	N	Intervention	Median – IQ range	P value
Apelin	22	Ventricle	5.52 (3.11-12.76)	0.006
	9	Atrium	1.94 (0.63-2.56)	
APJ	22	Ventricle	22.52 (15.68-58.08)	<0.0001
	9	Atrium	3.18 (2.19-9.20)	

Table 14.2. Descriptive statistics for mRNA expression of Apelin and APJ according to ventricular of atrial tissue. Data is expressed in median and interquartile range. Abbreviations: APJ: apelin receptor; IQ: interquartile.

Both genes presented a significantly higher expression in ventricular tissue (*Figure 14.4*).

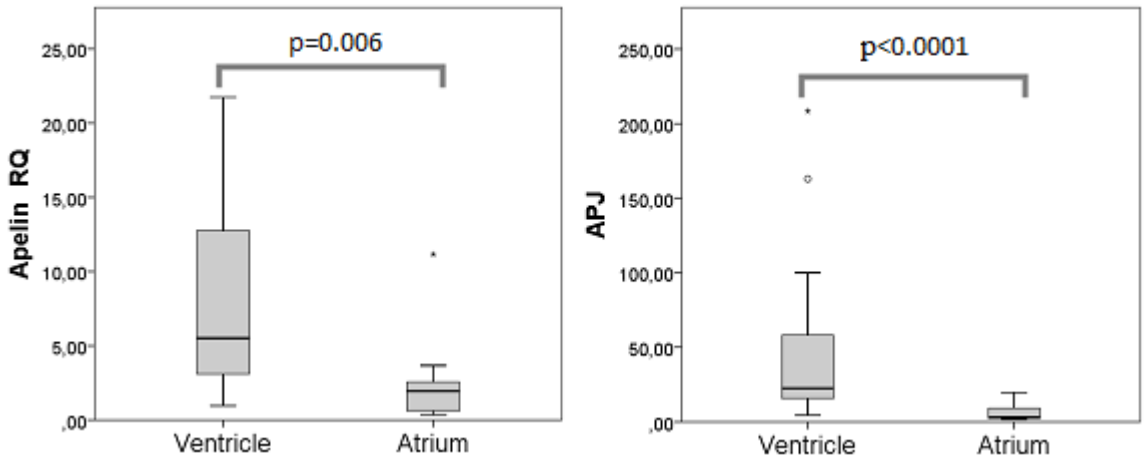

Figure 14.4. Apelin – APJ mRNA expression according to ventricular or atrial tissue. Abbreviations: APJ: apelin receptor; RQ: relative quantification.

Expression after tissue incubation

Descriptive information for Apelin and APJ before and after incubation is shown in *Table 14.3*.

Gene	N	Time point	Median – IQ range	P value
Apelin	15	Baseline	4.74(3.11-12.13)	0.001
	15	Incubated	2.77(1.55-4.64)	
APJ	15	Baseline	25.42(15.88-45.48)	0.001
	15	Incubated	10.99(9.47-20.20)	

Table 14.3. Descriptive statistics for mRNA expression of Apelin and APJ before and after incubation. Data is expressed in mean and standard deviations. Abbreviations: APJ: apelin receptor.

Incubation at 60% O_2 significantly reduced the expression of apelin and APJ compared to baseline (*Figure 14.5*).

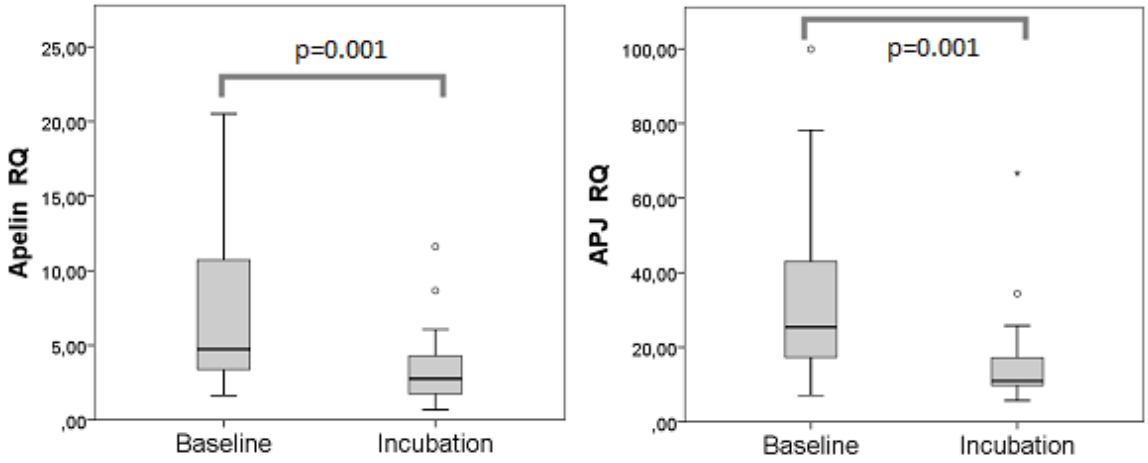

Figure 14.5. Apelin – APJ mRNA expression in the myocardium before and after incubation. Abbreviations: APJ: apelin receptor; RQ: relative quantification.

14.5.2 RIPC analysis

Forty-eight patients were analysed according to intervention allocation. Descriptive information on gene expression according to control or RIPC allocation is presented in *Table 14.4*.

Gene	Time point	Intervention	N	Median – IQ range	P value
Apelin	Baseline	RIPC	14	4.70(1.94-9.31)	0.965
		Control	15	3.67(1.94-16.65)	
	Incubation	RIPC	9	3.182(2.090-4.635)	0.224
		Control	6	1.972(0.842-2.765)	
APJ	Baseline	RIPC	14	19.03(13.07-27.48)	0.930
		Control	15	15.65(7.13-78.22)	
	Incubation	RIPC	9	12.803(10.964-14.154)	0.388
		Control	6	9.804(5.745-25.810)	

Table 14.4. Descriptive statistics for mRNA expression of Apelin and APJ before and after incubation according to intervention group allocation. Data is expressed in mean and standard deviations. Abbreviations: APJ: apelin receptor; RIPC: remote ischaemic preconditioning.

Expression of apelin and APJ did not vary significantly according to allocation to RIPC or control (*Figures 14.6 and 14.7*).

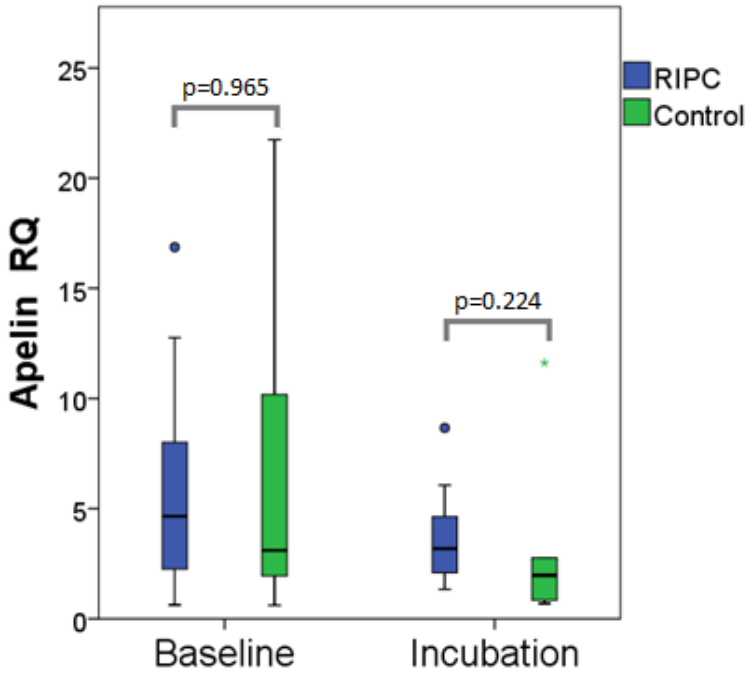

Figure 14.6. Apelin mRNA expression in the myocardium before and after incubation according to intervention group allocation. Abbreviations: RIPC: remote ischaemic preconditioning; RQ: relative quantification.

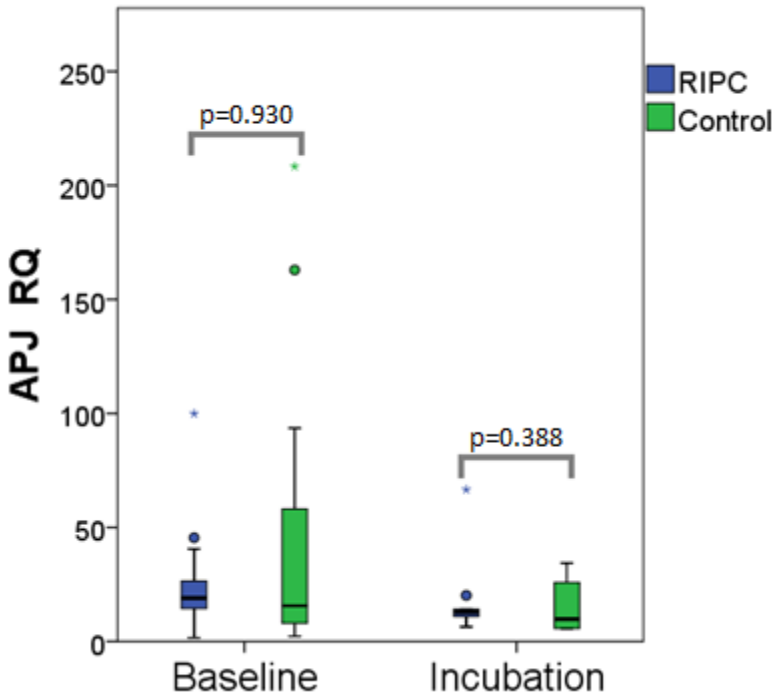

Figure 14.7. APJ mRNA expression in the myocardium before and after incubation according to intervention group allocation. Abbreviations: APJ: apelin receptor; RIPC: remote ischaemic preconditioning; RQ: relative quantification.

14.6 Summary of key findings

- APJ presented a higher expression in females, presenting no correlation to weight.
- APJ correlated to cTnI postoperative levels.
- Apelin and APJ presented a higher expression in ventricular tissue compared to atrial tissue.
- Hyperoxic incubation reduced the expression of apelin and APJ.
- RIPC did not cause a significant variation in the expression of either genes.

14.7 Limitations

Ct values representing the number of cycles required for the signal to cross the threshold during the Q-PCR reaction for apelin were above 30 in all samples while cycles for APJ were borderline; this reflects a low level of the target cDNA present in the tissue.

14.8 Discussion

Apelin required more than 30 cycles to reach the threshold, this reflects low target nucleic acid was present during the reaction. APJ Ct values ranged from just under 30 to just over 30, supporting the result of a higher expression of APJ compared to Apelin. Nevertheless comparison of these results to other genes should be made with caution.

Apelin and APJ are not highly expressed in tissues as a general pattern, their expression in the human heart has been reported at low levels according to microarray, RNA sequencing and SAGE analysis with APJ showing a slightly higher expression (GeneCards).

Interestingly APJ presented a higher expression in female patients, other demographic characteristics did not influence the expression of the gene and this remains to be explored.

APJ expression at baseline presented a correlation to post-operative cTnI levels, due to sampling timing the tissue was obtained before the full ischaemic phase was complete and prior to reperfusion, hence APJ expression could not reflect the extent of damage as cTnI after surgery would. This correlation is likely to reflect a participation of APJ in the production of injury or be linked to other underlying processes. Previous research by our laboratory group in TOF patients going through cardiac surgery also correlated APJ expression to ischaemia (Walker *et al*, 2014).

Both apelin and APJ showed a higher expression in ventricular tissue; they have been reported to be expressed in human atrial appendages and left ventricular tissue, however information comparing levels of expression is not available (GeneCards).

Incubation of tissue at hyperoxic levels resulted in a reduction in the expression of both genes. Research using human endothelium found that reoxygenation had an inhibitory effect on apelin expression (Yamagata *et al*, 2012); similar results were seen in a rat IRI model in which apelin was upregulated after ischaemia and downregulated after reperfusion in the heart, the same study reported APJ levels to remain constant (Kleinz and Baxter, 2008).

In discordance to our findings, an enhanced expression of APJ after ischaemia and reperfusion has been reported (Zeng *et al*, 2009).

In spite of the benefits for cardiac protection reported from apelin and APJ activity (Zeng *et al*, 2009; Yang *et al*, 2015), their involvement in RIPC has not been previously investigated. RIPC did not have an effect in the expression of these genes in our study.

Conclusion

The expression of apelin and APJ was affected by mimicking the *in vitro* process of IRI, however RIPC did not have a significant influence their expression.

Chapter 15. Discussion

This study aimed to assess the efficacy of RIPC in cardiac surgery using CPB for children requiring CHD correction. The approach of the study is unique as it has integrated clinical variables of patient outcome and cardiac function, quantitation of circulating markers and analysis of gene expression in the myocardial tissue.

The information gathered regarding all these variables also provided an opportunity for describing patterns of activity and functionality and exploring factors influencing outcome in our patients.

The population in our study included a wide age range within the paediatric age group, this was decided in order to reflect the real activity within a cardiac referral centre such as the Royal Hospital for Sick Children, in which the intervention would be applied if proved effective. In addition, this was necessary in order to recruit sufficient participants into the study.

The sample size of this trial was calculated based on a significant decrease in tricuspid velocity previously described within our research group (Vassalos *et al*, 2009). We aimed to assess if RIPC could reduce this difference significantly. We recruited the required amount of patients, however due to clinical circumstances it was not possible to measure tricuspid velocity in all patients. For this reason the analysis would not have enough power to detect the specified difference in our calculation and the results must be considered exploratory.

Demographic characteristics influenced several markers; weight and age correlated positively or negatively to numerous measured variables, with the presence of cyanosis having an impact in most aspects analysed in this study, revealing a basic differential response caused by chronic hypoxia and a need to take into account the differences between age categories for paediatric evaluation of these variables.

Gender differences were also observed across several measures, in this context it is not possible to determine the nature of these variations as a significantly lower weight was found in female participants; in addition to this cross clamp time tended to be higher in females, although this difference was not statistically significant. As weight and aortic clamp duration are two important independent variables, they could have influenced the observed differences in female

patients during the study, nevertheless it is not possible to completely reject the possibility of a gender based difference.

RIPC evidence has produced mixed results, with initial reports being very positive, however when larger studies were undertaken benefits were not found (see *Tables 1.7* and *1.8*).

Cardiac injury and function were not affected by RIPC in this study, a marked trend existed for RIPC allocated patients to present lower cTnI values, however this was not significant for any time points or by AUC analysis. This was not the pattern for BNP which presented very similar levels for both groups at all time points.

Inotrope score tended to be lower in the RIPC group, but again differences did not reach statistical significance, while echocardiographic parameters showed no measurable benefit from RIPC. Overall, RIPC did not result in any cardiac benefits.

Effects on the function of other organs including lungs, kidneys and liver did not favour RIPC clearly either; there was a higher arterial PCO_2 concentration in the control group without differences in any other blood gas analysis parameter and no other clinical respiratory measures were different between groups.

Potential markers for cardiac and lung function were analysed. Arginine metabolites, the promising cardiovascular markers, demonstrated interesting patterns of activity without showing an effect from RIPC. Similarly, NO metabolites and cGMP, inflammatory indicators which play a role in pulmonary function, did not show an influence of RIPC on its activity.

Renal function was evaluated through values of creatinine and Cystatin C, both markers showed a strong correlation to each other without being altered by RIPC; this was also the case for urea and electrolyte values. Evidence regarding the effects of RIPC on renal function have not favoured the intervention with large studies and meta-analysis demonstrating no clear benefits, especially in the context of CPB interventions (Li *et al*, 2013; McCrindle *et al*, 2014).

Cytokines tended to present values close to zero at baseline, peaking during the early post-operative period to then start a lowering trend; this shows that there was a definite inflammatory reaction after surgery, with humoral factors responding quickly to then yield. However this was

not the case for cellular adhesion molecules; ICAM-1 and VCAM-1 presented measurable values at baseline, reduced values during the immediate post-operative period and peaked the day after surgery regressing below baseline values by the second day after surgery; ADMA followed a similar pattern to adhesion molecules.

Most inflammatory markers in the study tended to present higher levels in the control group; even though only TNF-α reached statistical significance an overall trend is recognisable, this was also the case for white blood cell count, supporting the notion of a process triggered by RIPC influencing inflammatory reactions which may not have been strong enough.

The molecular component of this study is novel as no other RIPC clinical trial has explored myocardial gene expression to this extent. Hyperoxic incubation allowed us to mimic the high O_2 concentration myocardial tissue is exposed to after aortic clamp, completing the cycle of ischaemia and reperfusion. We were able to explore links between gene expression patterns and demographic characteristics as well as markers of injury, integrating molecular and clinical aspects of IRI and relating them to preconditioning.

TLR-2 and MYD-88 presented the lowest myocardial expression among all the genes analysed, however CBS, CTH, APJ and Apelin produced Ct values over 30; this does not correlate to high expression values, and these expression levels should be interpreted with caution.

HO-1 presented a lower baseline expression than its regulating transcription factor HIF-1, however this was not the case for HSP-70, HSP-90 and its regulatory gene NF-κB, with both HSPs presenting higher expression than NF-κB. This could be due to different patterns of interaction within a wide stress and inflammatory response triggered by surgery. An earlier response of genes such as HSP-70 and HSP-90 could have occurred having an inhibitory feedback effect on NF-κB, while a slower response could explain higher HIF-1 levels as it simulated the expression of downstream genes.

Our findings suggest expression of certain genes are different according to ventricular or atrial origin of the tissue. There is limited evidence regarding differential gene expression between atrial and ventricular tissue, a previous study used right atrial and left ventricular tissue in a

microarray profiling analysis which included several but not all the genes in our study, suggesting there are gene expression differences according to areas within the myocardium (Barth *et al*, 2005). Further analysis with more atrial specimens would be appropriate in order to confirm the observed differences.

Our research found a higher expression of NF-κB and APJ in females as well as a higher CBS expression in males. Evidence is limited on differential gene expression according to gender in the myocardium; a study looking at gender differences in adult rats at basal and hypoxic conditions in cardiac fibroblasts had contradictory results with a higher expression of NF-κB in males, while there was no difference in HIF-1 expression (Zhao and Eghbali-Webb, 2002).

A genome profiling study in heart failure patients determined gender variation in the expression of genes (Fermin *et al*, 2008); however among hundreds of genes, the only one analysed in the present study that was included in the report was HSP-70 which presented a higher expression in female patients. The marked differences in age and physiopathology between the population of this gene profiling study and our research should be emphasised.

The weight difference according to gender in the present study could have contributed to gene expression variation, however weight did not present a direct correlation to the expression of these genes, therefore a gender based difference cannot be ruled out.

Although cyanosis affected many inflammatory mediators and clinical variables, the only gene influenced by cyanosis was HSP-90, which presented a higher expression in acyanotic patients at baseline, it was not possible to analyse for this difference after hyperoxic incubation due to insufficient acyanotic specimens available for incubation.

The expression of certain genes correlated to post-operative levels of cTnI; as tissue samples were obtained before the complete ischaemic period had elapsed and prior to reperfusion, gene expression from our specimens cannot reflect the final degree of injury the patient presented. Therefore, correlations between the expression of NF- κB, HSPB7 and APJ to post-operative cTnI levels are more likely to reflect a causative role of these genes rather than a consequence of injury.

The in vitro conditions applied during this study influenced the expression of several genes, however RIPC failed to have a clear impact in these expression patterns.

Expression of all the analysed genes was not significantly affected by RIPC. RIPC clinical trials have rarely looked at gene expression patterns, an adult study analysed HIF-1 expression (Albrecht *et al*, 2013) and a paediatric study investigated HSP-70 expression (Zhou *et al*, 2010); both finding significantly higher expression after RIPC along with lower cardiac injury markers. This was not the case in our study for any of the markers.

Animal studies have identified numerous key mediators within the RIPC process, reporting them as essential after blocking its activity resulted in loss of the protective effect; however there are so many factors which have been validated through this process that it is not possible to select one specific gene or mediator responsible for the effect. RIPC protection is rather a multifactorial process involving the organised activity of numerous components.

The activity of many genes analysed in this study have the potential for both protective and detrimental effects according to their location and concentration; their function is linked to inflammatory processes while they take part in survival effects. The role of each gene during IRI and any possible cardioprotective effects are still to be determined and each gene is most likely a component of a bigger process.

Although several studies have found protection was abolished after inhibition of particular genes, the mechanisms of RIPC are likely to depend on a finely tuned collaboration between the components analysed in this thesis and many more.

Definite conclusions and recommendations regarding RIPC have not been reached. All the large RCTs evaluating RIPC which found no benefit from the intervention looked at the immediate phase of conditioning only, using protocols right after or during anaesthesia induction, just before the surgical procedure started (McCrindle *et al*, 2014; Hausenloy *et al*, 2015; Meybohm *et al*, 2015).

Our study is only the second human trial evaluating a dual RIPC protocol implemented the day before surgery and immediately before the surgery started with the purpose of allowing for both early and late phase of protection to act during CPB. Our study did not reproduce the results of

the previous trial following this approach which found significantly lower levels of cardiac injury markers and a lower inflammatory response in children after CPB (Zhou *et al*, 2010).

Additional influencing variables have been analysed in previous studies, the use of certain anaesthetics during surgery including volatile agents such as sevoflurane and isoflurane, or intravenous propofol have been reported to limit RIPC (Kottenberg *et al*, 2012; Zhou *et al*, 2013). Our study did not control the use of anaesthetics, surgical procedures followed usual protocols without alteration and this could have inhibited the effects of RIPC.

There was no definite overall benefit from applying RIPC for cardiac parameters or end organ function in this study; however a very important clinical outcome, length of PICU stay, was significantly reduced by the intervention.

Limitations:

- The sample size for assessing tricuspid velocity in echocardiography was not achieved.
- Limited atrial specimens were available, this could have reduced the power of the analysis for the identification of differences between tissue types.
- Application of the RIPC protocol on the day before surgery did not allow for the intervention to be blinded to patients as they were conscious during the process and parents were present; the intervention was performed by the CI and cardiac surgeon on occasions due to lack of staff appointed for such roles, hence the cardiac surgeon was not blinded to the intervention. The PI was blinded during all stages of the trial including laboratory analysis and data collection until unblinding was necessary for data analysis.
- The study did not control for the use of anaesthetic agents, the choice was done according to relevant protocols.
- Cyanosis status in this study was defined according to the clinical diagnosis of the patient and degree of repair, oxygen saturation was not considered pre-operatively in order to determine cyanosis in each patient.

See each section for specific details.

Conclusion

RIPC did not reduce markers of cardiac injury or improved echocardiographic parameters and it did not have an effect on end organ function; some effects were seen with lower TNF-α at one post-operative time point. Nevertheless it resulted in a shorter PICU length of stay, an important outcome indicator which could justify further investigation. Larger studies with more statistical power could determine if the tendency of lower injury and inflammatory markers linked to RIPC is real. The present results mostly support findings of larger multicentre trials which have reported little difference and no clear cardiac benefit produced by RIPC in paediatric cardiac surgery.

List of References

Achan V, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, Vallance P (2003) Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolised by dimethylarginine dimethylaminohydrolase. *Arteriosclerosis, Thrombosis and Vascular Biology* **23(8)**:1455-1459.

Acunzo J, Katsogiannou M, Rocchi P (2012) Small heat shock proteins HSP27 (HspB1), αB-crystallin (HspB5) and HSP22 (HspB8) as regulators of cell death. *The International Journal of Biochemistry and Cell Biology* **44(10)**:1622-1631.

Adams DH, Shaw S (1994) Leucocyte-endothelial interactions and regulation of leucocyte migration. *Lancet* **343(8901)**:831-836.

Adelman GA (2011) Cardiology Essentials in Clinical Practice, Chapter 10, Springer, First Edition. , pages: 281-283

Adin CA, Croker BP, Agarwal A (2005) Protective effects of exogenous bilirubin on ischemia-reperfusion injury in the isolated, perfused rat kidney. *American Journal of Physiology. Renal Physiology* **288(4)**:F778-784.

Afzal AR, Mandal K, Nyamweya S, Foteinos G, Poloniecki J, Camm AJ, Jahangiri M, Xu Q (2008) Association of Met439Thr substitution in heat shock protein 70 gene with postoperative atrial fibrillation and serum HSP70 protein levels. *Cardiology* **110(1)**:45-52.

Agarwal A, Bolisetty S (2013) Adaptive responses to tissue injury: role of heme oxygenase-1. *Transactions of the American Clinical and Climatological Association* **124**:111-122.nf-

Aimo A, Borrelli C, Giannoni A, Pastormerlo LE, Barison A, Mirizzi G, Emdin M, Passino C (2015) Cardioprotection by remote ischemic conditioning: Mechanisms and clinical evidences. *World Journal of Cardiology* **7(10)**:621-632.

Alam M, Hedman A, Nordlander R, Samad B (2003) Right ventricular function before and after an uncomplicated coronary artery bypass graft as assessed by pulsed wave Doppler tissue imaging of the tricuspid annulus. *American Heart Journal* **146(3)**:520-526.

Albelda SM, Smith CW, Ward PA (1994) Adhesion molecules and inflammatory injury. *FASEB Journal* **8(8)**:504-512.

Albrecht M, Zitta K, Bein B, Wennemuth G, Broch O, Renner J, Schuett T, Lauer F, Maahs D, Hummitzsch L, Cremer J, Zacharowski K, Meybohm P (2013) Remote ischemic preconditioning regulates HIF-1α levels, apoptosis and inflammation in heart tissue of cardiosurgical patients: a pilot experimental study. *Basic Research in Cardiology* **108(1)**:314.

Ali MM, Roe SM, Vaughan CK, Meyer P, Panaretou B, Piper PW, Prodromou C, Pearl LH (2006) Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex. *Nature* **440(7087)**:1013-1017.

Ali N, Rizwi F, Iqbal A, Rashid A (2010) Induced remote ischemic pre-conditioning on ischemia-reperfusion injury in patients undergoing coronary artery bypass. *Journal of the College of Physicians and Surgeons – Pakistan* **20(7)**:427-431.

Alpoim PN, Sousa LP, Mota AP, Rios DR, Dusse LM (2015) Asymmetric Dimethylarginine (ADMA) in cardiovascular and renal disease. *Clinica Chimica Acta; International Journal of Clinical Chemistry* **440**:36-39.

American Academy of Pediatrics. Committee on Hospital Care and Section of Critical Care. Society of Critical Care Medicine. Pediatric Section Admission Criteria Task Force (1999) Guidelines for developing admission and discharge policies for the pediatric intensive care unit. *Pediatrics* **103(4 PT 1):**840-842.

Ammirante M, Rosati A, Gentilella A, Festa M, Petrella A, Marzullo L, Pascale M, Belisario MA, Leone A, Turco MC (2008) The activity of hsp90 alpha promoter is regulated by NF-kappa B transcription factors. *Oncogene* **27(8)**:1175-1178.

Andresen TK, Svennevig JL, Videm V (2002) Soluble VCAM-1 is a very early marker of endothelial cell activation in cardiopulmonarybypass. *Perfusion* **17(1)**:15-21.

APEX AMI Investigators, Armstrong PW, Granger CB, Adams PX, Hamm C, Holmes D Jr, O'Neill WW, Todaro TG, Vahanian A, Van de Werf F (2007) Pexelizumab for acute ST-elevation myocardial infarction in patients undergoing primary percutaneous coronary intervention: a randomised controlled trial. *JAMA* **297(1)**:43-51.

Apostolakis E, Filos KS, Koletsis E, Dougenis D (2010) Lung dysfunction following cardiopulmonary bypass. *Journal of Cardiac Surgery* **25(1)**:47-55.

Arnold D, Faath S, Rammensee H, Schild H (1995) Cross-priming of minor histocompatibility antigen-specific cytotoxic T cells upon immunization with the heat shock protein gp96. *The Journal of Experimental Medicine* **182(3)**:885-889.

Arslan F, Houtgraaf JH, Keogh B, Kazemi K, de Jong R, McCormack WJ, O'Neill LA, McGuirk P, Timmers L, Smeets MB, Akeroyd L, Reilly M, Pasterkamp G, de Kleijn DP (2012) Treatment with OPN-305, a humanised anti-Toll-Like receptor-2 antibody, reduces myocardial ischemia/reperfusion injury in pigs. *Circulation. Cardiovascular Interventions* **5(2)**:279-287.

Arslan F, Keogh B, McGuirk P, Parker AE (2010) TLR2 and TLR4 in ischemia reperfusion injury. *Mediators of Inflammation* **2010**:704202.

Arslan F, Smeets MB, O'Neill LA, Keogh B, McGuirk P, Timmers L, Tersteeg C, Hoefer IE, Doevendans PA, Pasterkamp G, de Kleijn DP (2010) Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. *Circulation* **121(1)**:80-90.

Ashley EA, Niebauer J (2004) Understanding the echocardiogram, In Cardiology explained, Remedica, London, available from http://www.ncbi.nlm.nih.gov/books/NBK2215/

Attaran S, Sherwood R, Desai J, Langworthy R, Mhandu P, John L, El-Gamel A (2009) Brain natriuretic peptide a predictive marker in cardiac surgery. *Interactive Cardiovascular and Thoracic Surgery*. **9(4)**:662-666.

Aydin SI, Seiden HS, Blaufox AD, Parnell VA, Choudhury T, Punnoose A, Schneider J (2012) Acute kidney injury after surgery for congenital heart disease. *The Annals of Thoracic Surgery* **94(5)**:1589-1595.

Baines CP, Song CX, Zheng YT, Wang GW, Zhang J, Wang OL, Guo Y, Bolli R, Cardwell EM, Ping P (2003) Protein kinase Cepsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria. *Circulation Research* **92(8)**:873-880.

Bakthisaran R, Tangirala R, Rao ChM (2015) Small heat shock proteins: Role in cellular functions functions and pathology. *Biochimica et Biophysica Acta* **1854(4)**:291-319.

Bär FW, Tzivoni D, Dirksen MT, Fernández-Ortiz A, Heyndrickx GR, Brachmann J, Reiber JH, Avasthy N, Tatsuno J, Davies M, Hibberd MG, Krucoff MW; CASTEMI Study Group (2006) Results of the first clinical study of adjunctive CAldaret (MCC-135) in patients undergoing primary percutaneous coronary intervention for ST-Elevation

Myocardial Infarction: the randomised multicentre CASTEMI study. *European Heart Journal* **27(21)**:2516-2523.

Barañano DE, Rao M, Ferris CD, Snyder SH (2002) Biliverdin reductase: a major physiologic cytoprotectant. *Proceedings of the National Academy of Sciences of the United States of America* **99(25)**:16093-16098.

Barnes G, Japp AG, Newby DE (2010) Translational promise of the apelin--APJ system. *Heart* **96(13)**:1011-1016.

Barth AS, Merk S, Arnoldi E, Zwermann L, Kloos P, Gebauer M, Steinmeyer K, Bleich M, Kääb S, Pfeufer A, Uberfuhr P, Dugas M, Steinbeck G, Nabauer M (2005) Functional profiling of human atrial and ventricular gene expression. *European Journal of Physiology* **450(4):**201-208.

Basta S, Stoessel R, Basler M, van den Broek M, Groettrup M (2005) Cross-presentation of the long-lived lymphocytic choriomeningitis virus nucleoprotein does not require neosynthesis and is enhanced via heat shock proteins. *Journal of Immunology* **175(2)**:796-805.

Beauchamp P, Richard V, Tamion F, Lallemand F, Lebreton JP, Vaudry H, Daveau M, Thuillez C (1999) Protective effects of preconditioning in cultured rat endothelial cells: effects on neutrophil adhesion and expression of ICAM-1 after anoxia and reoxygenation. *Circulation* **100(5)**:541-546.

Bellis A, Castaldo D, Trimarco V, Monti MG, Chivasso P, Sadoshima J, Trimarco B, Morisco C (2009) Cross-talk between PKA and Akt protects endothelial cells from apoptosis in the late ischemic preconditioning. *Arteriosclerosis, Thrombosis and Vascular Biology* **29(8)**:1207-1212.

Berger HJ, Taratuska A, Smith TW, Halperin JA (1993) Activated complement directly modifies the performance of isolated heart muscle cells from guinea pig and rat. *The American Journal of Physiology* **265(1 Pt 2)**:H267-272.

Berkowitz DH, Gaynor JW (2013) Management of Pediatric Cardiopulmonary Bypass, In: Pediatric cardiac surgery (ed Mavroudis C, Backer C, Idriss RF), Blackwell Publishing, Chichester, pp:169-205.

Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K (2002) Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. *The New England Journal of Medicine* **346(8)**:557-563.

Bernardi P, Rasola A, Forte M, Lippe G (2015) The Mitochondrial Permeability Transition Pore: Channel Formation F-ATP Synthase, Integration in Signal Transduction, and Role in Pathophysiology. *Physiological Reviews* **95(4)**:1111-1155.

Bian JS, Yong QC, Pan TT, Feng ZN, Ali MY, Zhou S, Moore PK (2006) Role of hydrogen sulphide in the cardioprotection caused by ischemic preconditioning in the rat heart and cardiac myocytes. *The Journal of Pharmacology and Experimental Therapeutics* **316(2)**:670-678.

Bibli SI, Yang G, Zhou Z, Wang R, Topouzis S, Papapetropoulos A (2015) Role of cGMP in hydrogen sulphide signaling. *Nitric Oxide: Biology and Chemistry* **46**:7-13.

Binder RJ (2014) Functions of heat shock proteins in pathways of the innate and adaptive immune system. *Journal of Immunology* **193(12)**:5765-71.

Binder RJ, Kelly JB 3rd, Vatner RE, Srivastava PK (2007) Specific immunogenicity of heat shock protein gp96 derives from chaperoned antigenic peptides and not from contaminating proteins. *Journal of Immunology* **179(11)**:7254-7261.

Bizzarro M, Gross I, Barbosa FT (2014) Inhaled nitric oxide for the postoperative management of pulmonary hypertension in infants and children with congenital heart disease. *Cochrane Database of Systematic Reviews* **7**:CD005055.

Blackwell S (2010) The biochemistry, measurement and current clinical significance of asymmetric dimethylarginine. *Annals of Clinical Biochemistry* **47(Pt 1)**:17-28.

Blackwell S, O'Reilly DS, Talwar DK (2009) HPLC analysis of asymmetric dimethylarginine (ADMA) and related arginine metabolites in human plasma using a novel non-endogenous internal standard. *Clinica Chimica Acta* **401(1-2)**:14-19.

Bliksøen M, Kaljusto ML, Vaage J, Stensløkken KO (2008) Effects of hydrogen sulphide on ischaemia-reperfusion injury and ischaemic preconditioning in the isolated, perfused rat heart. *European Journal of Cardiothoracic Surgery* **34(2)**:344-349.

Bohonowych JE, Hance MW, Nolan KD, Defee M, Parsons CH, Isaacs JS (2014) Extracellular Hsp90 mediates an NF-κB dependent inflammatory stromal program: implications for the prostate tumor microenvironment. *The Prostate* **74(4)**:395-407.

Boldt J, Kumle B, Papsdorf M, Hempelmann G (1998) Are circulating adhesion molecules specifically changed in cardiac surgical patients? *The Annals of Thoracic Surgery* **65(3)**:608-614.

Boldt J, Osmer C, Linke LC, Dapper F, Hempelmann G (1995) Circulating adhesion molecules in pediatric cardiac surgery. *Anesthesia & Analgesia* **81(6)**:1129-1135.

Bolli R, Li QH, Tang XL, Guo Y, Xuan YT, Rokosh G, Dawn B (2007) The late phase of preconditioning and its natural clinical application--gene therapy. *Heart Failure Reviews* **12(3-4)**:189-199.

Bond JM, Herman B, Lemasters JJ (1991) Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. *Biochemical and Biophysical Research Communications* **179(2)**:798-803.

Borges TJ, Wieten L, van Herwijnen MJ, Broere F, van der Zee R, Bonorino C, van Eden W (2012) The anti-inflammatory mechanisms of Hsp70. *Frontiers in Immunology* **3**:95.

Bos EM, van Goor H, Joles JA, Whiteman M, Leuvenink HG (2015) Hydrogen sulfide: physiological properties and therapeutic potential in ischaemia. *British Journal of Pharmacology* **172(6)**:1479-1493.

Boshoff A (2015) Chaperonin-co-chaperonin interactions. *Sub-cellular Biochemistry* **78**:153-178.

Bøtker HE, Kharbanda R, Schmidt MR, Bøttcher M, Kaltoft AK, Terkelsen CJ, Munk K, Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH, Krusell LR, Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sørensen HT, Redington AN,Nielsen TT (2010) Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. *Lancet* **375(9716):**727-734.

Boucher J, Masri B, Daviaud D, Gesta S, Guigné C, Mazzucotelli A, Castan-Laurell I, Tack I, Knibiehler B, Carpéné C, Audigier Y, Saulnier-Blache JS, Valet P (2005) Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology* **146(4)**:1764-1771.

Boyle EM Jr, Kovacich JC, Hèbert CA, Canty TG Jr, Chi E, Morgan EN, Pohlman TH, Verrier ED (1998) Inhibition of interleukin-8 blocks myocardial ischemia-reperfusion injury. The Journal of Thoracic and Cardiovascular Surgery **116(1)**:114-121.

Brandenburger T, Grievink H, Heinen N, Barthel F, Huhn R, Stachuletz F, Kohns M, Pannen B, Bauer I (2014) Effects of remote ischemic preconditioning and myocardial ischemia on microRNA-1 expression in the rat heart *in vivo*. *Shock* **42(3)**:234-238.

Bresciani G, da Cruz IB, González-Gallego J (2015) Manganese superoxide dismutase and oxidative stress modulation. *Advances in Clinical Chemistry* **68**:87-130.

Bubb KJ, Trinder SL, Baliga RS, Patel J, Clapp LH, MacAllister RJ, Hobbs AJ (2014) Inhibition of phosphodiesterase 2 augments cGMP and cAMP signaling to ameliorate pulmonary hypertension. *Circulation* **130(6)**:496-507.

Buchhorn R, Wessel A, Hulpke-Wette M, Bürsch J, Werdan K, Loppnow H (2001) Endogenous nitric oxide and soluble tumor necrosis factor receptor levels are enhanced in infants with congenital heart disease. *Critical Care Medicine* **29(11)**:2208-2210.

Budas GR, Churchill EN, Disatnik MH, Sun L, Mochly-Rosen D (2010) Mitochondrial import of PKC epsilon is mediated by HSP90: a role in cardioprotection from ischaemia and reperfusion injury. *Cardiovascular Research* **88(1)**:83-92.

Buja LM (2005) Myocardial ischemia and reperfusion injury. *Cardiovascular Pathology* **14(4)**:170-175.

Bukach OV, Seit-Nebi AS, Marston SB, Gusev NB (2004) Some properties of human small heat shock protein Hsp20 (HspB6). *European Journal of Biochemistry* **271(2)**:291-302.

Bulwer BE, Solomon SD, Janardhanan R (2007) Echocardiographic assessment of ventricular systolic function, In: Essential Echocardiography (ed Solomon SD and Bulwer B), Springer, New York, pp 89-117.

Burma O, Onat E, Uysal A, Ilhan N, Erol D, Ozcan M, Sahna E (2014) Effects of rosuvastatin on ADMA, rhokinase, NADPH oxidase, caveolin-1, hsp 90 and NFkB levels in a rat model of myocardial ischaemia-reperfusion. *Cardiovascular Journal of Africa* **25(5)**:212-216.

Busche MN, Stahl GL (2010) Role of the complement components C5 and C3a in a mouse model of myocardial ischemia and reperfusioninjury. German Medical Science 8. pii Doc20.

Cai WJ, Wang MJ, Moore PK, Jin HM, Yao T, Zhu YC (2007) The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation. *Cardiovascular Research* **76(1)**:29-40.

Cai Z, Luo W, Zhan H, Semenza GL (2013) Hypoxia-inducible factor 1 is required for remote ischemic preconditioning of the heart. *Proceedings of the National Academy of Sciences of the United States of America* **110(43)**:17462-17467.

Cantinotti M, Storti S, Lorenzoni V, Murzi B, Marotta M, Crocetti M, Molinaro S, Assanta N, Lopez L, Clerico A (2013) Response of cardiac endocrine function to surgery stress is age dependent in neonates and children with congenital heart defects: consequences in diagnostic and prognostic accuracy of brain natriuretic peptide measurement. *Pediatric Critical Care Medicine* **14(5)**:508-517.

Cantinotti M, Walters HL, Crocetti M, Marotta M, Murzi B, Clerico A (2015) BNP in children with congenital cardiac disease: is there now sufficient evidence for its routine use? *Cardiology in the Young* **25(3)**:424-437.

Cao X, Yue L, Song J, Wu Q, Li N, Luo L, Lan L, Yin Z (2012) Inducible HSP70 antagonizes IL-1β cytocidal effects through inhibiting NF-kB activation via destabilizing TAK1 in HeLa cells. *PLoS One* **7(11)**:e50059.

Cappola TP, Matkovich SJ, Wang W, van Booven D, Li M, Wang X, Qu L, Sweitzer NK, Fang JC, Reilly MP, Hakonarson H, Nerbonne JM, Dorn GW 2nd (2011) Loss-of-

function DNA sequence variant in the CLCNKA chloride channel implicates the cardio-renal axis in interindividual heart failure risk variation. *Proceedings of the National Academy of Sciences of the United States of America* **108(6)**:2456-2461.

Carter YM, Thomas R, Bargatze R, Jutila M, Murry C, Allen MD (2000) Intracoronary E- and L-selectin blockade attenuates myocardial neutrophil infiltration in cardiac ischemia/reperfusion injury. *Current Surgery* **57(6)**:639-640.

Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N (2009) Importing mitochondrial proteins: machineries and mechanisms. Cell **138(4)**:628-644.

Chai PJ (2013) Intraoperative myocardial protection, In: Pediatric cardiac surgery (ed Mavroudis C, Backer C, Idriss RF), Blackwell Publishing, Chichester, pp:216-219.

Chandra D, Choy G, Tang DG (2007) Cytosolic accumulation of HSP60 during apoptosis with or without apparent mitochondrial release: evidence that its pro-apoptotic or pro-survival functions involve differential interactions with caspase-3. *The Journal of Biological Chemistry* **282(43)**:31289-301.

Chang ZL (2010) Important aspects of Toll-like receptors, ligands and their signaling pathways. *Inflammation Research* **59(10)**:791-808.

Checchia PA, Bronicki RA, Goldstein B (2012) Review of inhaled nitric oxide in the pediatric cardiac surgery setting. *Pediatric Cardiology* **33(4)**:493-505.

Chen CN, Watson G, Zhao L (2013) Cyclic guanosine monophosphate signalling pathway in pulmonary arterial hypertension. *Vascular Pharmacology* **58(3)**:211-218.

Chen HS, Jia J, Su HF, Lin HD, Chen JW, Lin SJ, Yang JY, Lai HC, Mestril R, Wang PH (2006) Downregulation of the constitutively expressed Hsc70 in diabetic myocardium is mediated by insulin deficiency. *The Journal of Endocrinology* **190(2)**:433-440.

Chen JX, Meyrick B (2004) Hypoxia increases Hsp90 binding to eNOS via PI3K-Akt in porcine coronary artery endothelium. *Laboratory Investigations* **84(2)**:182-190.

Chen X, Tao Q, Yu H, Zhang L, Cao X (2002) Tumor cell membrane-bound heat shock protein 70 elicits antitumor immunity. *Immunology Letters* **84(2)**:81-87.

Chen Z, Siu B, Ho YS, Vincent R, Chua CC, Hamdy RC, Chua BH (1998) Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice. *Journal of Molecular and Cellular Cardiology* **30(11)**:2281-2289.

Cheung MM, Kharbanda RK, Konstantinov IE, Shimizu M, Frndova H, Li J, Holtby HM, Cox PN, Smallhorn JF, Van Arsdell GS and Redington AN (2006) Randomised controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery. *Journal of the American College of Cardiology* **47(11)**:2277-2282.

Cheung SH, Kwok WK, To KF, Lau JY (2014) Anti-atherogenic effect of hydrogen sulfide by over-expression of cystathionine gamma-lyase (CSE) gene. *PLoS One* **9(11)**:e113038.

Chiu TF, Li CH, Chen CC, Chen CH, Cheng CJ, Yan YT, Yang RB (2012) Association of plasma concentration of small heat shock protein B7 with acute coronary syndrome. *Circulation Journal* **76(9)**:2226-2233.

Choi YS, Shim JK, Kim JC, Kang KS, Seo YH, Ahn KR, Kwak YL (2011) Effect of remote ischemic preconditioning on renal dysfunction after complex valvular heart surgery: a randomised controlled trial. *The Journal of Thoracic and Cardiovascular Surgery* **142(1)**:148-154.

Chong AJ, Shimamoto A, Hampton CR, Takayama H, Spring DJ, Rothnie CL, Yada M, Pohlman TH, Verrier ED (2004) Toll-like receptor 4 mediates ischemia/reperfusion injury of the heart. *The Journal of Thoracic and Cardiovascular Surgery* **128(2)**:170-179.

Chukwuemeka AO, Brown KA, Venn GE, Chambers DJ (2005) Changes in P-selectin expression on cardiac microvessels in blood-perfused rat hearts subjected to ischemia-reperfusion. *The Annals of Thoracic Surgery* **79(1)**:204-211.

Ciocca DR, Clark GM, Tandon AK, Fuqua SA, Welch WJ, McGuire WL (1993) Heat shock protein hsp70 in patients with axillary lymph node-negative breast cancer: prognostic implications. *Journal of the National Cancer Institute* **85(7)**:570-574.

Clerico EM, Tilitsky JM, Meng W, Gierasch LM (2015) How hsp70 molecular machines interact with their substrates to mediate diverse physiological functions. *Journal of Molecular Biology* **427(7)**:1575-1588.

Closs EI, Basha FZ, Habermeier A, Förstermann U (1997) Interference of L-arginine analogues with L-arginine transport mediated by the y+ carrier hCAT-2B. *Nitric Oxide: Biology and Chemistry* **1(1)**:65-73.

Codispoti M, Sundaramoorthi T, Saad RA, Reid A, Sinclair C and Mankad P (2006) Optimal myocardial protection strategy for coronary artery bypass grafting without cardioplegia: prospective randomised trial. *Interactive Cardiovascular and Thoracic Surgery* **5(3)**:217-21.

Collins PL, Hightower LE (1982) Newcastle disease virus stimulates the cellular accumulation of stress (heat shock) mRNAs and proteins. *Journal of Virology* **44(2)**:703-707.

Colvin TA, Gabai VL, Gong J, Calderwood SK, Li H, Gummuluru S, Matchuk ON, Smirnova SG, Orlova NV, Zamulaeva IA, Garcia-Marcos M, Li X,Young ZT, Rauch JN, Gestwicki JE, Takayama S, Sherman MY (2014) Hsp70-Bag3 interactions regulate cancer-related signaling networks. *Cancer Research* **74(17)**:4731-4740.

Coneski PN, Schoenfisch MH (2012) Nitric oxide release: part III. Measurement and reporting. *Chemical Society Reviews* **41(10)**:3753-3758.

Cook-Mills JM, Marchese ME, Abdala-Valencia H (2011) Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. *Antioxidants & Redox Signaling* **15(6)**:1607-1638.

Coskun C, Avci B, Yalcin M, Yermezler A, Yilmaz MS, Savci V (2014) Protective effect of CDP-choline on ischemia-reperfusion-induced myocardial tissue injury in rats. *Irish Journal of Medical Science* **183(4)**:539-548.

Costa JF, Fontes-Carvalho R, Leite-Moreira AF (2013) Myocardial remote ischemic preconditioning: from pathophysiology to clinical application. *Portuguese Journal of Cardiology* **32(11)**:893-904.

Crowley LE, McIntyre CW (2013) Remote ischaemic conditioning-therapeutic opportunities in renal medicine. *Nature Reviews. Nephrology* **9(12)**:739-746.

Crowther JR (2009) The ELISA Guidebook. 2nd Ed, Humana Press - Springer, Vienna. pp 61-63.

Czibik G, Derumeaux G, Sawaki D, Valen G, Motterlini R (2014) Heme oxygenase-1: an emerging therapeutic target to curb cardiac pathology. *Basic Research in Cardiology* **109(6)**:450.

Czibik G, Wu Z, Berne GP, Tarkka M, Vaage J, Laurikka J, Järvinen O, Valen G (2008) Human adaptation to ischemia by preconditioning or unstable angina: involvement of nuclear factor kappa B, but not hypoxia-inducible factor 1 alpha in the heart. *European Journal of Cardiothoracic Surgery* **34(5)**:976-984.

Dai C, Sampson SB (2016) HSF1: Guardian of Proteostasis in Cancer. *Trends in Cell Biology* **26(1)**:17-28.

Dale HH, Shuster EHJ (1928) A double perfusion pump. *The Journal of Physiology* **64(4)**:356-364.

Dal-Secco D, Cunha TM, Freitas A, Alves-Filho JC, Souto FO, Fukada SY, Grespan R, Alencar NM, Neto AF, Rossi MA, Ferreira SH, Hothersall JS, Cunha FQ (2008) Hydrogen sulfide augments neutrophil migration through enhancement of adhesion molecule expression and prevention of CXCR2 internalization: role of ATP-sensitive potassium channels. *Journal of Immunology* **181(6)**:4287-4298.

D'Ascenzo F, Cavallero E, Moretti C, Omedè P, Sciuto F, Rahman IA, Bonser RS, Yunseok J, Wagner R, Freiberger T, Kunst G, Marber MS, Thielmann M, Ji B,Amr YM, Modena MG, Zoccai GB, Sheiban I, Gaita F (2012) Remote ischaemic preconditioning in coronary artery bypass surgery: a meta-analysis. *Heart* **98(17)**:1267-1271.

Davidson SM, Selvaraj P, He D, Boi-Doku C, Yellon RL, Vicencio JM, Yellon DM (2013) Remote ischaemic preconditioning involves signalling through the SDF-1α/CXCR4 signalling axis. *Basic Research in Cardiology* **108(5)**:377.

de Jong PR, Schadenberg AW, Jansen NJ, Prakken BJ (2009) Hsp70 and cardiac surgery: molecular chaperone and inflammatory regulator with compartmentalised effects. *Cell Stress & Chaperones* **14(2)**:117-131.

de Kleer IM, Kamphuis SM, Rijkers GT, Scholtens L, Gordon G, De Jager W, Häfner R, van de Zee R, van Eden W, Kuis W, Prakken BJ (2003) The spontaneous remission of juvenile idiopathic arthritis is characterised by CD30+ T cells directed to humanheat-shock protein 60 capable of producing the regulatory cytokine interleukin-10. *Arthritis and Rheumatism* **48(7)**:2001-2010.

DeBakey ME (1934) Simple continuous-flow blood transfusion instrument. *The New Orleans Medical and Surgical Journal* **87**:386-386.

Deguine J, Barton GM (2014) MYD-88: a central player in innate immune signaling. *F1000prime Reports* **6**:97.

Demidov ON, Tyrenko VV, Svistov AS, Komarova YY, Karpishenko AI, Margulis BA, Shevchenko YL (1999) Heat shock proteins in cardiosurgery patients. *European Journal of Cardiothoracic Surgery* **16(4)**:444-449.

den Engelsman J, Boros S, Dankers PY, Kamps B, Vree Egberts WT, Böde CS, Lane LA, Aquilina JA, Benesch JL, Robinson CV, de Jong WW, Boelens WC (2009) The small heat-shock proteins HSPB2 and HSPB3 form well-defined heterooligomers in a unique 3 to 1 subunit ratio. *Journal of Molecular Biology* **393(5)**:1022-1032.

Depre C, Kim SJ, John AS, Huang Y, Rimoldi OE, Pepper JR, Dreyfus GD, Gaussin V, Pennell DJ, Vatner DE, Camici PG, Vatner SF (2004) Program of cell survival underlying human and experimental hibernating myocardium. *Circulation Research* **95(4)**:433-440.

Depre C, Wang L, Sui X, Qiu H, Hong C, Hedhli N, Ginion A, Shah A, Pelat M, Bertrand L, Wagner T, Gaussin V, Vatner SF (2006) H11 kinase prevents myocardial infarction by preemptive preconditioning of the heart. *Circulation Research* **98(2)**:280-288.

Deshane J, Chen S, Caballero S, Grochot-Przeczek A, Was H, Li Calzi S, Lach R, Hock TD, Chen B, Hill-Kapturczak N, Siegal GP, Dulak J, Jozkowicz A, Grant MB, Agarwal A (2007) Stromal cell-derived factor 1 promotes angiogenesis via a heme oxygenase 1-dependent mechanism. *The Journal of Experimental Medicine* **204(3)**:605-618.

Dhingra R, Shaw JA, Aviv Y, Kirshenbaum LA (2010) Dichotomous actions of NF-kappaB signaling pathways in heart. *Journal of Cardiovascular Translational Research* **3(4)**:344-54.

Dickson EW, Reinhardt CP, Renzi FP, Becker RC, Porcaro WA, Heard SO (1999) Ischemic preconditioning may be transferable via whole blood transfusion: preliminary evidence. *Journal of Thrombosis and Thrombolisis* **8(2)**:123-129.

Diller GP, Wasan BS, Kyriacou A, Patel N, Casula RP, Athanasiou T, Francis DP, Mayet J (2008) Effect of coronary artery bypass surgery on myocardial function as assessed by tissue Doppler echocardiography. *European Journal of Cardio-thoracic Surgery* **34(5)**:995-999.

Ding HS, Yang J, Chen P, Yang J, Bo SQ, Ding JW, Yu QQ (2013) The HMGB1-TLR4 axis contributes to myocardial ischemia/reperfusion injury via regulation of cardiomyocyte apoptosis. *Gene* **527(1)**:389-893.

Ding YF, Zhang MM, He RR (2001) Role of renal nerve in cardioprotection provided by renal ischemic preconditioning in anesthetised rabbits. *Sheng Li Xue Bao: Acta Physiologica Sinica* **53(1)**:7-12.

Dolk H, Loane M, Garne E; European Surveillance of Congenital Anomalies (EUROCAT) Working Group (2011) Congenital heart defects in Europe: prevalence and perinatal mortality, 2000 to 2005. *Circulation* **123(8)**:841-849.

Dong JW, Vallejo JG, Tzeng HP, Thomas JA, Mann DL (2010) Innate immunity mediates myocardial preconditioning through Toll-like receptor 2 and TIRAP-dependent signaling pathways. *American Journal of Physiology. Heart and Circulatory Physiology* **298(3)**:H1079.

Doyle SM, Genest O, Wickner S (2013) Protein rescue from aggregates by powerful molecular chaperone machines. Nature Reviews. *Molecular Cell Biology* **14(10)**:617-629.

Drapalova J, Kopecky P, Bartlova M, Lacinova Z, Novak D, Maruna P, Lips M, Mraz M, Lindner J, Haluzik M (2014) The influence of deep hypothermia on inflammatory status, tissue hypoxia and endocrine function of adipose tissue during cardiac surgery. *Cryobiology* **68(2)**:269-275.

Dreiza CM, Komalavilas P, Furnish EJ, Flynn CR, Sheller MR, Smoke CC, Lopes LB, Brophy CM (2010) The small heat shock protein, HSPB6, in muscle function and disease. *Cell Stress & Chaperones* **15(1)**:1-11.

Duckers HJ, Boehm M, True AL, Yet SF, San H, Park JL, Clinton Webb R, Lee ME, Nabel GJ, Nabel EG (2001) Heme oxygenase-1 protects against vascular constriction and proliferation. *Nature Medicine* **7(6)**:693-698.

Dunn LL, Midwinter RG, Ni J, Hamid HA, Parish CR, Stocker R (2014) New insights into intracellular locations and functions of heme oxygenase-1. *Antioxidants & Redox Signaling* **20(11)**:1723-1742.

Dupont A, Chwastyniak M, Beseme O, Guihot AL, Drobecq H, Amouyel P, Pinet F (2008) Application of saturation dye 2D-DIGE proteomics to characterize proteins modulated by oxidised low density lipoprotein treatment of human macrophages. *Journal of Proteome Research* **7(8)**:3572-3582.

Edwards HV, Cameron RT, Baillie GS (2011) The emerging role of HSP20 as a multifunctional protective agent. *Cellular Signalling* **23(9)**:1447-1454.

Edwards HV, Scott JD, Baillie GS (2012) The A-kinase-anchoring protein AKAP-Lbc facilitates cardioprotective PKA phosphorylation of Hsp20 on Ser(16). *The Biochemical Journal* **446(3)**:437-443.

Egom EE (2015) BNP and Heart Failure: Preclinical and Clinical Trial Data. *Journal of Cardiovascular Translational Research* **8(3)**:149-157.

Eikemo H, Sellevold OF, Videm V (2004) Markers for endothelial activation during open heart surgery. *The Annals of Thoracic Surgery* **77(1)**:214-219.

Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, Tao L, Jiao X, Scalia R, Kiss L, Szabo C, Kimura H, Chow CW, Lefer DJ (2007) Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proceedings of the National Academy of Sciences of the United States of America* **104(39)**:15560-15565.

Endre ZH (2011) Renal ischemic preconditioning: finally some good news for prevention of acute kidney injury. *Kidney International* **80(8)**:796-798.

Entman ML, Youker K, Shoji T, Kukielka G, Shappell SB, Taylor AA, Smith CW (1992) Neutrophil induced oxidative injury of cardiac myocytes. A compartmented system requiring CD11b/CD18-ICAM-1 adherence. *The Journal of Clinical Investigation* **90(4)**:1335-1345.

Eskedal L, Hagemo P, Eskild A, Aamodt G, Seiler KS, Thaulow E (2004) A population-based study of extra-cardiac anomalies in children with congenital cardiac malformations. *Cardiology in the Young* **14(6)**:600-607.

Fan GC, Chu G, Mitton B, Song Q, Yuan Q, Kranias EG (2004) Small heat-shock protein Hsp20 phosphorylation inhibits beta-agonist-induced cardiac apoptosis. *Circulation Research* **94(11)**:1474-1482.

Fan GC, Ren X, Qian J, Yuan Q, Nicolaou P, Wang Y, Jones WK, Chu G, Kranias EG (2005) Novel cardioprotective role of a small heat-shock protein, Hsp20, against ischemia/reperfusion injury. Circulation **111(14)**:1792-1799.

Fan GC, Yuan Q, Song G, Wang Y, Chen G, Qian J, Zhou X, Lee YJ, Ashraf M, Kranias EG (2006) Small heat-shock protein Hsp20 attenuates beta-agonist-mediated cardiac remodeling through apoptosis signal-regulating kinase 1. *Circulation Research* **99(11)**:1233-1242.

Faraci FM, Brian JE Jr, Heistad DD (1995) Response of cerebral blood vessels to an endogenous inhibitor of nitric oxide synthase. *American Journal of Physiology* **269(5 Pt 2)**:H1522-1527.

Fathil MF, Arshad MK, Gopinath SC, Hashim U, Adzhri R, Ayub RM, Ruslinda AR, Nuzaihan M N M, Azman AH, Zaki M, Tang TH (2015) Diagnostics on acute myocardial infarction: Cardiac troponin biomarkers. *Biosensors & Bioelectronics* **70**:209-220.

Favre J, Musette P, Douin-Echinard V, Laude K, Henry JP, Arnal JF, Thuillez C, Richard V (2007) Toll-like receptros 2-deficient mice are protected against postischemic coronary endothelial dysfunction. *Arterioclerosis, Thrombosis and Vascular Biology* **27(5)**:1064-1071.

Feil R, Lohmann SM, de Jonge H, Walter U, Hofmann F (2003) Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice. *Circulation Research* **93(10)**:907-916.

Fermin DR, Barac A, Lee S, Polster SP, Hannenhalli S, Bergemann TL, Grindle S, Dyke DB, Pagani F, Miller LW, Tan S, Dos Remedios C, Cappola TP,Margulies KB, Hall JL

(2008) Sex and age dimorphism of myocardial gene expression in nonischemic human heart failure. *Circulation. Cardiovascular Genetics* **1(2)**:117-125.

Ferrari R, Alfieri O, Curello S, Ceconi C, Cargnoni A, Marzollo P, Pardini A, Caradonna E, Visioli O (1990) Occurrence of oxidative stress during reperfusion of the human heart. *Circulation* **81(1)**:201-211.

Ferreiro CR, Chagas AC, Carvalho MH, Dantas AP, Jatene MB, Bento De Souza LC, Lemos Da Luz P (2001) Influence of hypoxia on nitric oxide synthase activity and gene expression in children with congenital heart disease: a novel pathophysiological adaptive mechanism. *Circulation* **103(18)**:2272-2276.

Finegan BA, Lopaschuk GD, Gandhi M, Clanachan AS (1995) Ischemic preconditioning inhibits glycolysis and proton production in isolated working rat hearts. *The American Journal of Physiology* **269(5 Pt 2)**:H1767-1775.

Flaherty JT, Pitt B, Gruber JW, Heuser RR, Rothbaum DA, Burwell LR, George BS, Kereiakes DJ, Deitchman D, Gustafson N, et al (1994) Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. *Circulation* **89(5)**:1982-1991.

Fliss H, Gattinger D (1996) Apoptosis in ischemic and reperfused rat myocardium. *Circulation Research* **79(5)**:949-956.

Fontaine JM, Sun X, Benndorf R, Welsh MJ (2005) Interactions of HSP22 (HSPB8) with HSP20, alphaB-crystallin, and HSPB3. *Biochemical and Biophysical Research Communications* **337(3)**:1006-1011.

Franchello A, Gilbo N, David E, Ricchiuti A, Romagnoli R, Cerutti E, Salizzoni M (2009) Ischemic preconditioning (IP) of the liver as a safe and protective technique against ischemia/reperfusion injury (IRI). *American Journal of Transplantation* **9(7)**:1629-1639.

Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB, Mendoza LH, Spengler RN, Smith CW, Entman ML (1998) Resident cardiac mast cells degranulate and release preformed TNF-alpha, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. *Circulation* **98(7)**:699-710.

Frangogiannis NG, Smith CW, Entman ML (2002) The inflammatory response in myocardial infarction. *Cardiovascular Research* **53(1)**:31-47.

Fredenburgh LE, Merz AA, Cheng S (2015) Haeme oxygenase signalling pathway: implications for cardiovascular disease. *European Heart Journal* **36(24)**:1512-1518.

Freude B, Masters TN, Robicsek F, Fokin A, Kostin S, Zimmermann R, Ullmann C, Lorenz-Meyer S, Schaper J (2000) Apoptosis is initiated by myocardial ischemia and executed during reperfusion. *Journal of Molecular and Cellular Cardiology* **32(2)**:197-208.

Fu M, Zhang W, Yang G, Wang R (2012) Is cystathionine gamma-lyase protein expressed in the heart? *Biochemical and Biophysical Research Communications* **428(4)**:469-474.

Fujita T, Toda K, Karimova A, Yan SF, Naka Y, Yet SF, Pinsky DJ (2001) Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. *Nature Medicine* **7(5)**:598-604.

Gammazza AM, Bucchieri F, Grimaldi LM, Benigno A, de Macario EC, Macario AJ, Zummo G, Cappello F (2012) The molecular anatomy of human Hsp60 and its similarity with that of bacterial orthologs and acetylcholine receptor reveal a potential pathogenetic role of anti-chaperonin immunity in myasthenia gravis. *Cellular and Molecular Neurobiology* **32(6)**:943-947.

Geiger K, Muendlein A, Stark N, Saely CH, Wabitsch M, Fraunberger P, Drexel H (2011) Hypoxia induces apelin expression in human adipocytes. *Hormone and Metabolic Research* **43(6)**:380-385.

Gelb BD, Chung WK (2014) Complex genetics and the etiology of human congenital heart disease. *Cold Spring Harbor Perspectives in Medicine* **4(7)**:a013953.

Gelman S (1995) The pathophysiology of aortic cross-clamping and unclamping. *Anesthesiology* **82(4)**:1026-1060.

Gene Expression Omnibus (GEO) profiles, National Center for Biotechnology Information – (NCBI) http://www.ncbi.nlm.nih.gov/geoprofiles/63872695
http://www.ncbi.nlm.nih.gov/geoprofiles/22665498
last accessed on 12/04/2016.

GeneCards, Human Gene Database
http://www.genecards.org/cgi-bin/carddisp.pl?gene=APLN
http://www.genecards.org/cgi-bin/carddisp.pl?gene=APLNR
http://www.genecards.org/cgi-bin/carddisp.pl?gene=CBS
http://www.genecards.org/cgi-bin/carddisp.pl?gene=CTH
http://www.genecards.org/cgi-bin/carddisp.pl?gene=MYD88
http://www.genecards.org/cgi-bin/carddisp.pl?gene=TLR2
last accessed on 14/04/2016

Ghasemi A, Zahedi Asl S, Mehrabi Y, Saadat N, Azizi F (2008) Serum nitric oxide metabolite levels in a general healthy population: relation to sex and age. *Life Sciences* **83(9-10)**:326-331.

Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD (1996) Myocardial protection by brief ischemia in noncardiac tissue. *Circulation* **94(9)**:2193-2200.

Giannessi D, Caselli C, Vitale RL, Crucean A, Murzi B, Del Ry S, Vanini V, Biagini A (2003) A possible cardioprotective effect of heat shock proteins during cardiac surgery in pediatric patients. *Pharmacological Research* **48(5)**:519-529.

Gibbon JH Jr (1954) Application of a mechanical heart and lung apparatus to cardiac surgery. *Minnesota Medicine* **37(3)**:171-185.

Giffard RG, Han RQ, Emery JF, Duan M, Pittet JF (2008) Regulation of apoptotic and inflammatory cell signaling in cerebral ischemia: the complex roles of heat shock protein 70. *Anesthesiology* **109(2)**:339-348

Gill R, Kuriakose R, Gertz ZM, Salloum FN, Xi L, Kukreja RC (2015) Remote ischemic preconditioning for myocardial protection: update on mechanisms and clinical relevance. *Molecular and Cellular Biochemistry* **402(1-2)**:41-49.

Giomarelli P, Scolletta S, Borrelli E, Biagioli B (2003) Myocardial and lung injury after cardiopulmonary bypass: role of interleukin (IL)-10. *The Annals of Thoracic Surgery* **76(1)**:117-123.

Girgis RE, Champion HC, Diette GB, Johns RA, Permutt S, Sylvester JT (2005) Decreased exhaled nitric oxide in pulmonary arterial hypertension: response to bosentan therapy. *American Journal of Respiratory and Critical Care Medicine* **172(3)**:352-357.

Giricz Z, Varga ZV, Baranyai T, Sipos P, Pálóczi K, Kittel Á, Buzás EI, Ferdinandy P (2014) Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles. *Journal of Molecular and Cellular Cardiology* **68**:75-78.

Glover JR, Lindquist S (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell* **94(1)**:73-82.

Golenhofen N, Perng MD, Quinlan RA, Drenckhahn D (2004) Comparison of the small heat shock proteins alphaB-crystallin, MKBP, HSP25, HSP20, and cvHSP in heart and skeletal muscle. *Histochemistry and Cell Biology* **122(5)**:415-425.

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *The Journal of Clinical Investigation* **94(4)**:1621-8.

Gozzelino R, Jeney V, Soares MP (2010) Mechanisms of cell protection by heme oxygenase-1. *Annual Review of Pharmacology and Toxicology* **50**:323-254.

Granger CB, Mahaffey KW, Weaver WD, Theroux P, Hochman JS, Filloon TG, Rollins S, Todaro TG, Nicolau JC, Ruzyllo W, Armstrong PW; COMMA Investigators (2003) Pexelizumab, an anti-C5 complement antibody, as adjunctive therapy to primary percutaneous coronary intervention in acute myocardial infarction: the COMplement inhibition in Myocardial infarction treated with Angioplasty (COMMA) trial. *Circulation* **108(10)**:1184-1190.

Grieve SM, Lønborg J, Mazhar J, Tan TC, Ho E, Liu CC, Lay W, Gill AJ, Kuchel P, Bhindi R, Figtree GA (2013) Cardiac magnetic resonance imaging of rapid VCAM-1 up-regulation in myocardial ischemia-reperfusion injury. *European Biophysics Journal* **42(1)**:61-70.

Griffiths EJ, Ocampo CJ, Savage JS, Stern MD, Silverman HS (2000) Protective effects of low and high doses of cyclosporin A against reoxygenation injury in isolated rat cardiomyocytes are associated with differential effects on mitochondrial calcium levels. *Cell calcium* **27(2)**:87-95.

Groban L, Sanders DM, Houle TT, Antonio BL, Ntuen EC, Zvara DA, Kon ND, Kincaid EH (2010) Prognostic value of tissue Doppler-Derived E/e' on early morbid events after cardiac surgery. *Echocardiography* **27(2)**:131-138.

Groesbeck D, Köttgen A, Parekh R, Selvin E, Schwartz GJ, Coresh J, Furth S (2008) Age, gender, and race effects on cystatin C levels in US adolescents. *Clinical Journal of the American Society of Nephrology* **(6)**:1777-1785.

Gu Q, Yang XP, Bonde P, DiPaula A, Fox-Talbot K, Becker LC (2006) Inhibition of TNF-alpha reduces myocardial injury and proinflammatory pathways following ischemia-reperfusionin the dog. *Journal of Cardiovascular Pharmacology* **48(6)**:320-328.

Gumina RJ, Buerger E, Eickmeier C, Moore J, Daemmgen J, Gross GJ (1999) Inhibition of the Na(+)/H(+) exchanger confers greater cardioprotection against 90 minutes of myocardial ischemia than ischemic preconditioning in dogs. *Circulation* **(25)**:2519-2526; discussion 2469-72.

Gumina RJ, el Schultz J, Yao Z, Kenny D, Warltier DC, Newman PJ, Gross GJ (1996) Antibody to platelet/endothelial cell adhesion molecule-1 reduces myocardial infarct size in a rat model of ischemia-reperfusion injury. *Circulation* **94(12)**:3327-3333.

Guo W, Cheng ZY, Zhu YZ (2013) Hydrogen sulfide and translational medicine. *Acta Pharmacologica Sinica* **34(10)**:1284-1291.

Guo Y, Tukaye DN, Wu WJ, Zhu X, Book M, Tan W, Jones SP, Rokosh G, Narumiya S, Li Q, Bolli R (2012) The COX-2/PGI2 receptor axis plays an obligatory role in mediating the cardioprotection conferred by the late phase of ischemic preconditioning. *PLoS One* **7(7)**:e41178.

Gupta S, Knowlton AA (2005) HSP60, Bax, apoptosis and the heart. *Journal of Cellular and Molecular Medicine* **9(1)**:51-58.

Gupta-Malhotra M, Kern JH, Flynn PA, Schiller MS, Quaegebeur JM, Friedman DM (2013) Cardiac troponin I after cardiopulmonary bypass in infants in comparison with older children. *Cardiology in the Young* **23(3)**:431-435.

Ha T, Hu Y, Liu L, Lu C, McMullen JR, Kelley J, Kao RL, Williams DL, Gao X, Li C (2010) TLR2 ligands induce cardioprotection against ischaemia/reperfusion injury through a PI3K/Akt-dependent mechanism. Cardiovascular Research **87(4)**:694-703.

Hafez MO, Morsy SM, Mahfoz RA, Ali AR (2015) Myocardial Injury in Children with Unoperated Congenital Heart Diseases. *Cardiology Research and Practice* **2015**:104818.

Hahne M, Jäger U, Isenmann S, Hallmann R, Vestweber D (1993) Five tumor necrosis factor-inducible cell adhesion mechanisms on the surface of mouse endothelioma cells mediate the binding of leukocytes. *The Journal of Cell Biology* **121(3)**:655-664.

Haji Mohd Yasin NA, Herbison P, Saxena P, Praporski S, Konstantinov IE (2014) The role of remote ischemic preconditioning in organ protection after cardiac surgery: a meta-analysis. *The Journal of Surgical Research* **186(1)**:207-216.

Halestrap AP, Pasdois P (2009) The role of the mitochondrial permeability transition pore in heart disease. *Biochimica et Biophysica Acta* **1787(11)**:1402-1415.

Hamacher-Brady A, Brady NR, Gottlieb RA (2006) Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. *The Journal of Biological Chemistry* **281(40)**:29776-29787.

Han TH, Perloff JK, Liao JC (2007) Nitric oxide metabolism in adults with cyanotic congenital heart disease. *The American Journal of Cardiology* **99(5)**:691-695.

Hartl FU, Bracher A, Hayer-Hartl M (2011) Molecular chaperonees in protein folding and proteostasis. *Nature* **475(7356):**324-332.

Hase M, Depre C, Vatner SF, Sadoshima J (2005) H11 has dose-dependent and dual hypertrophic and proapoptotic functions in cardiac myocytes. The Biochemical Journal **388(Pt 2)**:475-483.

Haslbeck M, Vierling E (2015) A first line of stress defense: small heat shock proteins and their function in protein homeostasis. *Journal of Molecular Biology* **427(7)**:1537-1548.

Hassinger AB, Backer CL, Lane JC, Haymond S, Wang D, Wald EL (2012) Predictive power of serum cystatin C to detect acute kidney injury and pediatric-modified RIFLE class in children undergoing cardiac surgery. *Pediatric Critical Care Medicine* **13(4)**:435-440.

Hausenloy DJ, Candilio L, Evans R, Ariti C, Jenkins DP, Kolvekar S, Knight R, Kunst G, Laing C, Nicholas J, Pepper J, Robertson S, Xenou M, Clayton T, Yellon DM; ERICCA Trial Investigators (2015) Remote Ischemic Preconditioning and Outcomes of Cardiac Surgery. *The New England Journal of Medicine* **373(15)**:1408-1417.

Hausenloy DJ, Mwamure PK, Venugopal V, Harris J, Barnard M, Grundy E, Ashley E, Vichare S, Di Salvo C, Kolvekar S, Hayward M, Keogh B, MacAllister RJ,Yellon DM (2007) Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. *Lancet* **370(9587)**:575-579.

Hausenloy DJ, Yellon DM (2008) Remote ischaemic preconditioning: underlying mechanisms and clinical application. *Cardiovascular Research* **79(3)**:377-386.

Hausenloy DJ, Yellon DM (2013) Myocardial ischemia-reperfusion injury: a neglected therapeutic target. The Journal of Clinical Investigation **123(1)**:92-100.

Healy DA, Khan WA, Wong CS, Moloney MC, Grace PA, Coffey JC, Dunne C, Walsh SR, Sadat U, Gaunt ME, Chen S, Tehrani S, Hausenloy DJ, Yellon DM, Kramer RS, Zimmerman RF, Lomivorotov VV, Shmyrev VA, Ponomarev DN, Rahman IA,Mascaro JG, Bonser RS, Jeon Y, Hong DM, Wagner R, Thielmann M, Heusch G, Zacharowski K, Meybohm P, Bein B, Tang TY, Remote Preconditioning Trialists' Group (2014) Remote preconditioning and major clinical complications following adult cardiovascular surgery: systematic review and meta-analysis. *International Journal of Cardiology* **176(1)**:20-31.

Hearse DJ, Humphrey SM, Chain EB (1973) Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release. *Journal of Molecular and Cellular Cardiology* **5(4)**:395-407.

Hearse DJ, Stewart DA, Braimbridge MV, (1976) Cellular protection during myocardial ischemia: the development and characterization of a procedure for the induction of reversible ischemic arrest. *Circulation* **54(2)**:193-202.

Heidbreder M, Naumann A, Tempel K, Dominiak P, Dendorfer A (2008) Remote vs ischaemic preconditioning: the differential role of mitogen-activated protein kinase pathways. *Cardiovascular Research* **78(1)**:108-115.

Heiserman JP, Chen L, Kim BS, Kim SC, Tran AL, Siebenborn N, Knowlton A3A (2015) TLR4 mutation and HSP60-induced cell death in adult mouse cardiac myocytes. *Cell Stress & Chaperones* **20(3)**:527-535.

Henein M, Gibson D (1999) Normal long axis function. *Heart* **81(2)**:111–113.

Hepponstall M, Ignjatovic V, Binos S, Monagle P, Jones B, Cheung MH, d'Udekem Y, Konstantinov IE (2015) Remote ischemic preconditioning (RIPC) modifies plasma proteome in humans. *PLoS One* **7(11)**:e48284.

Hessel EA II, Edmunds LH Jr. (2003) Extracorporeal Circulation: Perfusion Systems - Cardiac Surgery in the Adult. New York: McGraw-Hill, second edition: 317-338. http://cardiacsurgery.ctsnetbooks.org/cgi/content-nw/full/2/2003/317/F2).

Heusch G, Boengler K, Schulz R (2010) Inhibition of mitochondrial permeability transition pore opening: the Holy Grail of cardioprotection. *Basic Research in Cardiology* **105(2)**:151-154

Heusch G, Musiolik J, Kottenberg E, Peters J, Jakob H, Thielmann M (2012) STAT5 activation and cardioprotection by remote ischemic preconditioning in humans: short communication. *Circulation Research* **110(1)**:111-115.

Hibert P, Prunier-Mirebeau D, Beseme O, Chwastyniak M, Tamareille S, Pinet F, Prunier F (2014) Modifications in rat plasma proteome after remote ischemic preconditioning (RIPC) stimulus: identification by a SELDI-TOF-MS approach. *PLos One* **9(1)**:e85669.

Hirani N, Antonicelli F, Strieter RM, Wiesener MS, Ratcliffe PJ, Haslett C, Donnelly SC (2001) The regulation of interleukin-8 by hypoxia in human macrophages--a potential role in the pathogenesis of the acute respiratory distress syndrome (ARDS). *Molecular Medicine* **7(10)**:685-697.

Hoffman JI1, Kaplan S (2002) The incidence of congenital heart disease. *Journal of the American College of Cardiology* **39(12)**:1890-1900.

Hoffmann A, Baltimore D (2006) Circuitry of nuclear factor kappaB signaling. *Immunological Reviews* **210**:171-186.

Holmgren D, Westerlind A, Lundberg PA, Wåhlander H (2005) Increased plasma levels of natriuretic peptide type B and A in children with congenital heart defects with left

compared with right ventricular volume overload or pressure overload. *Clinical Physiology and Functional Imaging* **25(5)**:263-269.

Hosoda K, Nakao K, Mukoyama M, Saito Y, Jougasaki M, Shirakami G, Suga S, Ogawa Y, Yasue H, Imura H (1991) Expression of brain natriuretic peptide gene in human heart. Production in the ventricle. *Hypertension* **17(6 Pt 2)**:1152-1155.

Hu CP, Peng J, Xiao L, Ye F, Deng HW, Li YJ (2002) Effect of age on alpha-calcitonin gene-related peptide-mediated delayed cardioprotection induced by intestinal preconditioning in rats. *Regulatory Peptides* **107(1-3)**:137-143.

Huang JB, Liu YL, Sun PW, Lv XD, Du M and Fan XM (2010) Molecular mechanisms of congenital heart disease. *Cardiovascular Pathology* **19(5)**:e183-193.

Huang QQ, Sobkoviak R, Jockheck-Clark AR, Shi B, Mandelin AM 2nd, Tak PP, Haines GK 3rd, Nicchitta CV, Pope RM (2009) Heat shock protein 96 is elevated in rheumatoid arthritis and activates macrophages primarily via TLR2 signaling. *Journal of Immunology* **182(8)**:4965-4973.

Hussein AM, Harraz AM, Awadalla A, Barakat N, Khater S, Shokeir AA (2016) Remote limb ischemic preconditioning (rIPC) activates antioxidant and antiapoptotic genes and inhibits proinflammatory cytokine genes in renal ischemia/reperfusion injury. *General Physiology and Biophysics* **35(1)**:77-86.

Hwang TS, Han HS, Choi HK, Lee YJ, Kim YJ, Han MY, Park YM (2003) Differential, stage-dependent expression of Hsp70, Hsp110 and Bcl-2 in colorectal cancer. *Journal of Gastroenterology and Hepatology* **18(6)**:690-700.

Hyun J, Kanagavelu S, Fukata M (2013) A unique host defense pathway: TRIF mediates both antiviral and antibacterial immune responses. *Microbes and Infection / Institut Pasteur* **15(1)**:1-10.

Ibanez B, Fuster V, Jiménez-Borreguero J, Badimon JJ (2011) Lethatl myocardial reperfusion injury: a necessary evil? *International Journal of Cardiology* **151(1)**:3-11.

Igarashi G, Iino K, Watanabe H, Ito H (2013) Remote ischemic pre-conditioning alleviates contrast-induced acute kidney injury in patients with moderate chronic kidney disease. *Circulation Journal* **77(12)**:3037-3044.

Igarashi K, Watanabe-Matsui M (2014) Wearing red for signaling: the heme-bach axis in heme metabolism, oxidative stress response and iron immunology. *The Tohoku Journal of Experimental Medicine* **232(4)**:229-253.

Imai H, Kurokawa S, Taneoka M, Baba H (2011) Tissue Doppler imaging is useful for predicting the need for inotropic support after cardiac surgery. *Journal of Anesthesia* **25(6)**:805-811.

Imatoh T, Sugie T, Miyazaki M, Tanihara S, Baba M, Momose Y, Uryu Y, Une H (2009) Is heat shock protein 60 associated with type 2 diabetes mellitus? *Diabetes Research and Clinical Practice* **85(2)**:208-212.

Imura H, Caputo M, Parry A, Pawade A, Angelini GD, Suleiman MS (2001) Age-dependent and hypoxia-related differences in myocardial protection during pediatric open heart surgery. *Circulation* **103(11)**:1551-1556.

Inserte J, Barba I, Poncelas-Nozal M, Hernando V, Agulló L, Ruiz-Meana M, Garcia-Dorado D (2011) cGMP/PKG pathway mediates myocardial postconditioning protection in rat hearts by delaying normalization of intracellular acidosis during reperfusion. *Journal of Molecular and Cellular Cardiology* **50(5)**:903-909.

Ishii H, Ichimiya S, Kanashiro M, Amano T, Imai K, Murohara T, Matsubara T (2005) Impact of a single intravenous administration of nicorandil before reperfusion in patients with ST-segment-elevation myocardial infarction. *Circulation* **112(9)**:1284-1288.

Ishii T, Udono H, Yamano T, Ohta H, Uenaka A, Ono T, Hizuta A, Tanaka N, Srivastava PK, Nakayama E (1999) Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. *Journal of Immunology* **162(3)**:1303-1309.

Issan Y, Kornowski R, Aravot D, Shainberg A, Laniado-Schwartzman M, Sodhi K, Abraham NG, Hochhauser E (2014) Heme oxygenase-1 induction improves cardiac function following myocardial ischemia by reducing oxidative stress. *PLoS One* **9(3)**:e92246.

Itoh H, Komatsuda A, Ohtani H, Wakui H, Imai H, Sawada K, Otaka M, Ogura M, Suzuki A, Hamada F (2002) Mammalian HSP60 is quickly sorted into the mitochondria under conditions of dehydration. *European Journal of Biochemistry/FEBS* **269(23)**:5931-5938.

Jackson SE (2013) Hsp90: structure and function. *Topics in Current Chemistry* **328**:155-240.

Jackson-Weaver O, Osmond JM, Riddle MA, Naik JS, Gonzalez Bosc LV, Walker BR, Kanagy NL (2013) Hydrogen sulfide dilates rat mesenteric arteries by activating endothelial large-conductance Ca^{2+}-activated K^+ channels and smooth muscle Ca^{2+} sparks. *American Journal of Physiology. Heart and Circulatory Physiology* **304(11)**:H1446-1454.

Jacobs JP (2013) Pediatric Cardiac Surgery, Chapter 2, Blackwell Publishing Ltd, Fourth Edition, pages: 33-34.

Jancsó G, Cserepes B, Gasz B, Benkó L, Borsiczky B, Ferenc A, Kürthy M, Rácz B, Lantos J, Gál J, Arató E, Sínayc L, Wéber G, Róth E (2007) Expression and protective role of heme oxygenase-1 in delayed myocardial preconditioning. *Annals of the New York Academy of Science* **1095:**251-261.

Jarosz DF, Lindquist S (2010) Hsp90 and environmental stress transform the adaptive value of natural genetic variation. *Science* **330(6012)**:1820-1824.

Jenkins KJ, Correa A, Feinstein JA, Botto L, Britt AE, Daniels SR, Elixson M, Warnes CA, Webb CL; American Heart Association Council on CardiovascularDisease in the Young (2007) Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American Heart Association Council on Cardiovascular Disease in the Young: endorsed by the AmericanAcademy of Pediatrics. *Circulation* **115(23)**:2995-3014.

Jennings rb, Sommers HM, Smyth GA, Flack HA, Linn H (1960) Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Archives of Pathology* **70**:68-78.

Jia H, Halilou AI, Hu L, Cai W, Liu J, Huang B (2011) Heat shock protein 10 (Hsp10) in immune-related diseases: one coin, two sides. *International Journal of Biochemistry and Molecular Biology* **2(1)**:47-57.

Jiao JD, Garg V, Yang B, Hu K (2008) Novel functional role of heat shock protein 90 in ATP-sensitive K+ channel-mediated hypoxic preconditioning. *Cardiovascular Research* **77(1)**:126-133.

Jin C, Cleveland JC, Ao L, Li J, Zeng Q, Fullerton DA, Meng X (2014) Human myocardium releases heat shock protein 27 (HSP27) after global ischemia: the proinflammatory effect of extracellular HSP27 through toll-like receptor (TLR)-2 and TLR4. *Molecular Medicine* **20**:280-289.

Jin Hf, Wang Y, Wang Xb, Sun Y, Tang Cs, Du Jb (2013) Sulfur dioxide preconditioning increases antioxidative capacity in rat with myocardial ischemia reperfusion (I/R) injury. *Nitric Oxide: Biology and Chemistry* **32**:56-61.

Jones BO, Pepe S, Sheeran FL, Donath S, Hardy P, Shekerdemian L, Penny DJ, McKenzie I, Horton S, Brizard CP, d'Udekem Y, Konstantinov IE, Cheung MM (2013) Remote ischemic preconditioning in cyanosed neonates undergoing cardiopulmonary bypass: a randomised controlled trial. *The Journal of Thoracic and Cardiovascular Surgery* **146(6)**:1334-1340.

Jones WK, Fan GC, Liao S, Zhang JM, Wang Y, Weintraub NL, Kranias EG, Schultz JE, Lorenz J, Ren X (2009) Peripheral nociception associated with surgical incision elicits remote nonischemic cardioprotection via neurogenic activation of protein kinase C signaling. Circulation **120**(11 Suppl):S1-9.

Kabil O, Motl N, Banerjee R (2014) H2S and its role in redox signaling. *Biochimica et Biophysica Acta* **1844(8)**:1355-1366.

Kaczorowski DJ, Nakao A, Mollen KP, Vallabhaneni R, Sugimoto R, Kohmoto J, Tobita K, Zuckerbraun BS, McCurry KR, Murase N, Billiar TR (2007) Toll-like receptor 4 mediates the early inflammatory response after cold ischemia/reperfusion. *Transplantation* **84(10)**:1279-1287.

Kadappu KK, Thomas L (2015) Tissue Doppler imaging in echocardiography: value and limitations. *Heart, Lung & Circulation* **24(3)**:224-233.

Kaeffer N, Richard V, François A, Lallemand F, Henry JP, Thuillez C (1996) Preconditioning prevents chronic reperfusion-induced coronary endothelial dysfunction in rats. *The American Journal of Physiology* **271(3 Pt 2)**:H842-849.

Kalakech H, Tamareille S, Pons S, Godin-Ribuot D, Carmeliet P, Furber A, Martin V, Berdeaux A, Ghaleh B, Prunier F (2013) Role of hypoxia inducible factor-1α in remote limb ischemic preconditioning. *Journal of Molecular and Cellular Cardiology* **65**:98-104.

Kampinga HH, Craig EA (2010) The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nature Reviews. Molecular Cell Biology* **11(8)**:579-592.

Kaneda H, Ohno M, Taguchi J, Togo M, Hashimoto H, Ogasawara K, Aizawa T, Ishizaka N, Nagai R (2002) Heme oxygenase-1 gene promoter polymorphism is associated with coronary artery disease in Japanese patients with coronary risk factors. *Arteriosclerosis, Thrombosis, and Vascular Biology* **22(10)**:1680-1685.

Kaplan P, Hendrikx M, Mattheussen M, Mubagwa K, Flameng W (1992) Effect of ischemia and reperfusion on sarcoplasmic reticulum calcium uptake. *Circulation Research* **71(5)**:1123-1130.

Karuppasamy P, Chaubey S, Dew T, Musto R, Sherwood R, Desai J, John L, Shah AM, Marber MS, Kunst G (2011) Remote intermittent ischemia before coronary artery bypass graft surgery: a strategy to reduce injury and inflammation? *Basic Research in Cardiology* **106(4)**:511-519.

Kato K, Goto S, Inaguma Y, Hasegawa K, Morishita R, Asano T (1994) Purification and characterization of a 20-kDa protein that is highly homologous to alpha B crystallin. *The Journal of Biological Chemistry* **269(21)**:15302-15309.

Kats S, Schönberger JP, Brands R, Seinen W, van Oeveren W (2011) Endotoxin release in cardiac surgery with cardiopulmonary bypass: pathophysiology and possible therapeutic strategies. An update. *European Journal of Cardio-thoracic Surgery* **39(4)**:451-458.

Kawai T, Akira S (2006) TLR signaling. *Cell Death and Differentiation* **13(5)**:816-825.

Kawakatsu M, Ishihara T, Kani K, Nakagawa A, Hiura M, Hazui H, Okabe T, Hoshiga M, Waku S, Iida N, Hanafusa T (2002) Plasma Nitrate/Nitrite Concentration in Healthy Population and Patients with Diabetes Mellitus - Relationships with Gender, Aging and Diabetic Complications. *Bulletin of the Osaka Medical College* **48（1, 2):**1-6.

Kawamura T, Wakusawa R, Okada K, Inada S (1993) Elevation of cytokines during open heart surgery with cardiopulmonary bypass: participation of interleukin 8 and 6 in reperfusion injury. *Canadian Journal of Anaesthesia* **40(11)**:1016-1021.

Kazakov AS, Markov DI, Gusev NB, Levitsky DI (2009) Thermally induced structural changes of intrinsically disordered small heat shock protein Hsp22. *Biophysical Chemistry* **145(2-3)**:79-85.

Kelm M (1999) Nitric oxide metabolism and breakdown. *Biochimica et Biophysica Acta* **1411(2-3)**:273-289.

Kharbanda RK (2010) Cardiac conditioning: a review of evolving strategies to reduce ischaemia-reperfusion injury. *Heart* **96(15)**:1179-1186.

Kharbanda RK, Mortensen UM, White PA, Kristiansen SB, Schmidt MR, Hoschtitzky JA, Vogel M, Sorensen K, Redington AN, MacAllister R (2002) Transient limb ischemia induces remote ischemic preconditioning *in vivo*. *Circulation* **106(23)**:2881-2883.

Khurana N, Bhattacharyya S (2015) Hsp90, the concertmaster: tuning transcription. *Frontiers in Oncology* **5**:100.

Kielstein JT, Salpeter SR, Bode-Boeger SM, Cooke JP, Fliser D (2006) Symmetric dimethylarginine (SDMA) as endogenous marker of renal function--a meta-analysis. *Nephrology, Dialysis, Transplantation* **21(9)**:2446-2451.

Kielstein JT, Simmel S, Bode-Böger SM, Roth HJ, Schmidt-Gayk H, Haller H, Fliser D (2004) Subpressor dose asymmetric dimethylarginine modulates renal function in humans through nitric oxidesynthase inhibition. *Kidney & Blood Pressure Research* **27(3)**:143-147.

Kiessling AH, Dietz J, Reyher C, Stock UA, Beiras-Fernandez A, Moritz A (2014) Early postoperative serum cystatin C predicts severe acute kidney injury following cardiac surgery: a post-hoc analysis of a randomised controlled trial. *Journal of Cardiothoracic Surgery* **9**:10.

Kim JS, Jin Y, Lemasters S (2006) Reactive oxygen species, but not Ca2+ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. *American Journal of physiology. Heart and Circulatory Physiology* **290(5)**:H2024-2034.

Kim SH, Carney DF, Hammer CH, Shin ML (1987) Nucleated cell killing by complement: effects of C5b-9 channel size and extracellular Ca2+ on the lytic process. *Journal of Immunology* **138(5)**:1530-1536.

Kim YM, Pae HO, Park JE, Lee YC, Woo JM, Kim NH, Choi YK, Lee BS, Kim SR, Chung HT (2011) Heme oxygenase in the regulation of vascular biology: from molecular mechanisms to therapeutic opportunities. *Antioxidants & Redox Signaling* **14(1)**:137-167.

Kim MV, Seit-Nebi AS, Marston SB, Gusev NB (2004) Some properties of human small heat shock protein Hsp22 (H11 or HspB8). *Biochemical and Biophysical Research Communications* **315(4)**:796-801.

Kimura A, Sakurada S, Ohkuni H, Todome Y, Kurata K (2002) Moderate hypothermia delays proinflammatory cytokine production of human peripheral blood mononuclear cells. *Critical Care Medicine* **30(7)**:1499-1502.

Kingma JG (1999) Cardiac adaptation to ischemia-reperfusion injury. *Annals of the New York Academy of Sciences* **874**:83-99.

Kityk R, Kopp J, Sinning I, Mayer MP (2012) Structure and dynamics of the ATP-bound open conformation of Hsp70 chaperones. *Molecular Cell* **48(6)**:863-874.

Klein HH, Pich S, Lindert S, Nebendahl K, Warneke G, Kreuzer H (1989) Treatment of reperfusion injury with intracoronary calcium channel antagonists and reduced coronary free calcium concentration in regionally ischemic, reperfused porcine hearts. *Journal of the American College of Cardiology* **13(6)**:1395-1401.

Kleinz MJ, Baxter GF (2008) Apelin reduces myocardial reperfusion injury independently of PI3K/Akt and P70S6 kinase. *Regulatory Peptides* **146(1-3)**:271-277.

Knowlton AA, Gupta S (2003) HSP60, Bax, and cardiac apoptosis. *Cardiovascular Toxicology* **3(3)**:263-268.

Koch A, Zink S, Singer H (2006) B-type natriuretic peptide in paediatric patients with congenital heart disease. *European Heart Journal* **27(7)**:861-866.

Kodani E, Xuan YT, Takano H, Shinmura K, Tang XL, Bolli R (2002) Role of cyclic guanosine monophosphate in late preconditioning in conscious rabbits. *Circulation* **105(25)**:3046-3052.

Konstantinov IE (2013) Remote ischemic preconditioning in children with cyanotic heart disease: lost in translation? *The Journal of Thoracic and Cardiovascular Surgery* **145(2)**:613-614.

Konstantinov IE, Arab S, Kharbanda RK, Li J, Cheung MM, Cherepanov V, Downey GP, Liu PP, Cukerman E, Coles JG, Redington AN (2004) The remote ischemic preconditioning stimulus modifies inflammatory gene expression in humans. *Physiological Genomics* **19(1)**:143-150.

Konstantinov IE, Arab S, Li J, Coles JG, Boscarino C, Mori A, Cukerman E, Dawood F, Cheung MM, Shimizu M, Liu PP, Redington AN (2005) The remote ischemic preconditioning stimulus modifies gene expression in mouse myocardium. *The Journal of Thoracic and Cardiovascular Surgery* **130(5)**:1326-1332.

Konstantinov IE, Li J, Cheung MM, Shimizu M, Stokoe J, Kharbanda RK, Redington AN (2005) Remote ischemic preconditioning of the recipient reduces myocardial ischemia-reperfusion injury of the denervated donor heart via a Katp channel-dependent mechanism. *Transplantation* **79(12)**:1691-1695.

Korennykh A, Walter P (2012) Structural basis of the unfolded protein response. *Annual Review of Cell and Developmental Biology* **28**:251-277.

Kottenberg E, Thielmann M, Bergmann L, Heine T, Jakob H, Heusch G, Peters J (2012) Protection by remote ischemic preconditioning during coronary artery bypass graft surgery with isoflurane but not propofol - a clinical trial. *Acta Anaesthesiologica Scandinavica* **56(1)**:30-38.

Köttgen A, Selvin E, Stevens LA, Levey AS, Van Lente F, Coresh J (2008) Serum cystatin C in the United States: the Third National Health and Nutrition Examination Survey (NHANES III). *American Journal of Kidney Diseases* **51(3)**:385-394.

Kouchoukos NT, Blackstone EH, Doty DB, Hanley FL, Karp RB (2003) Kirklin/Barratt-Boyes cardiac surgery : morphology, diagnostic criteria, natural history, techniques, results, and indications Philadelphia, Pa. : Churchill Livingstone, third edition.

Krause S, Hess ML (1984) Characterization of cardiac sarcoplasmic reticulum dysfunction during short-term, normothermic, global ischemia. *Circulation Research* **55(2)**:176-184.

Krief S, Faivre JF, Robert P, Le Douarin B, Brument-Larignon N, Lefrère I, Bouzyk MM, Anderson KM, Greller LD, Tobin FL, Souchet M, Bril A (1999) Identification and characterization of cvHsp. A novel human small stress protein selectively expressed in cardiovascular and insulin-sensitive tissues. *The Journal of Biological Chemistry* **274(51)**:36592-36600.

Kroemer G, Reed JC (2000) Mitochondrial control of cell death (2000) *Nature Medicine* **6(5)**:513-519.

Ku DD (1982) Coronary vascular reactivity after acute myocardial ischemia. *Science* **218(4572)**:576-8.

Kupatt C, Dessy C, Hinkel R, Raake P, Daneau G, Bouzin C, Boekstegers P, Feron O (2004) Heat shock protein 90 transfection reduces ischemia-reperfusion-induced myocardial dysfunction via reciprocal endothelial NO synthase serine 1177 phosphorylation and threonine 495 dephosphorylation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **24(8)**:1435-1441.

Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M (1993) Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circulation Research* **72(6)**:1293-1299.

Ladilov YV, Siegmund B, Piper HM (1995) Protection of reoxygenated cardiomyocytes against hypercontracture by inhibition of Na+/H+ exchange. *The American journal of Physiology* **268(4 Pt 2)**:H1531-1539.

Lakkisto P, Csonka C, Fodor G, Bencsik P, Voipio-Pulkki LM, Ferdinandy P, Pulkki K (2009) The heme oxygenase inducer inducer hemin protects against cardiac dysfunction and ventricular fibrillation in ischemic/reperfused rat hearts: role of connexin 43. *Scandinavian Journal of Clinical and Laboratory Investigation* **69(2)**:209-218.

Lam CK, Zhao W, Cai W, Vafiadaki E, Florea SM, Ren X, Liu Y, Robbins N, Zhang Z, Zhou X, Jiang M, Rubinstein J, Jones WK, Kranias EG (2013) Novel role of HAX-1 in ischemic injury protection involvement of heat shock protein 90. *Circulation Research* **112(1)**:79-89.

Lassnigg A, Schmid ER, Hiesmayr M, Falk C, Druml W, Bauer P, Schmidlin D (2008) Impact of minimal increases in serum creatinine on outcome in patients after cardiothoracic surgery: do we have to revise current definitions of acute renal failure? *Critical Care Medicine* **36(4)**:1129-1137.

Laterza OF, Price CP, Scott MG (2002) Cystatin C: an improved estimator of glomerular filtration rate? *Clinical Chemistry* **48(5)**:699-707.

Laude K, Thuillez C, Richard V (2001) Reperfusion-induced coronary endothelial injury: A new target for ischemic preconditioning. Experimental and Clinical Cardiology 6(3):149-52.

Laure L, Long R, Lizano P, Zini R, Berdeaux A, Depre C, Morin D (2012) Cardiac H11 kinase/Hsp22 stimulates oxidative phosphorylation and modulates mitochondrial reactive oxygen species production: Involvement of a nitric oxide-dependent mechanism. *Free Radical Biology & Medicine* **52(11-12)**:2168-2176.

Lawson S, Ellis C, McRobb C, Mejak B (2013) Mechanical Aspects of Pediatric Cardio Pulmonary Bypass, In: Pediatric and congenital cardiology, cardiac surgery and intensive care (ed Da Cruz E, Ivy D, Jaggers J), Springer, New York, pp: 772-781.

Leach MD, Budge S, Walker L, Munro C, Cowen LE, Brown AJ (2012) Hsp90 orchestrates transcriptional regulation by Hsf1 and cell wall remodelling by MAPK signalling during thermal adaptation in a pathogenic yeast. *PLoS Pathogens* **8(12)**:e1003069.

Lee, H (2014) Foundations of Applied Statistical Methods. Springer, Cham. 90pp.

Lee JH, Park YH, Byon HJ, Kim HS, Kim CS, Kim JT (2012) Effect of remote ischaemic preconditioning on ischaemic-reperfusion injury in pulmonary hypertensive infants receiving ventricular septal defect repair. *British Journal of Anaesthesia* **108(2)**:223-228.

Lee KH, Jeong J, Yoo CG (2013) Positive feedback regulation of heat shock protein 70 (Hsp70) is mediated through Toll-like receptor 4-PI3K/Akt-glycogen synthase kinase-3β pathway. *Experimental Cell Research* **319(1)**:88-95.

Lee SW, Won JY, Lee HY, Lee HJ, Youn SW, Lee JY, Cho CH, Cho HJ, Oh S, Chae IH, Kim HS (2011) Angiopoietin-1 Protects Heart against Ischemia/Reperfusion Injury through VE-Cadherin Dephosphorylation and Myocardiac Integrin-β1/ERK/Caspase-9 Phosphorylation Cascade. Molecular Medicine 17(9-10): 1095–1106.

Lehotský J, Burda J, Danielisová V, Gottlieb M, Kaplán P, Saniová B (2009) Ischemic tolerance: the mechanisms of neuroprotective strategy. *Anatomical Record* **292(12)**:2002-2012.

Lepper PM, Bals R (2012) On the edge: targeting Toll-like receptor 2 in ischemia/reperfusion injury. *Circulation. Cardiovascular Interventions* **5(2)**:146-149.

Li C, Hu M, Wang Y, Lu H, Deng J, Yan X (2015) Hydrogen sulphide preconditioning protects against myocardial ischemia/reperfusion injury through inhibition of endo/sarcoplasmic reticulum stress. *International Journal of Clinical and Experimental Pathology* **8(7)**:7740-7751.

Li C, Hu M, Wang Y, Lu H, Deng J, Yan X (2015) Hydrogen sulfide preconditioning protects against myocardial ischemia/reperfusion injury in rats through inhibition of endo/sarcoplasmic reticulum stress. *International Journal of Clinical and Experimental Pathology* **8(7)**:7740-7751.

Li G, Labruto F, Sirsjö A, Chen F, Vaage J, Valen G (2004) Myocardial protection by remote preconditioning: the role of nuclear factor kappa-B p105 and inducible nitricoxide synthase. *European Journal of Cardiothoracic Surgery* **26(5)**:968-973.

Li J, Buchner J (2013) Structure, function and regulation of the hsp90 machinery. *Biomedical Journal* **36(3)**:106-117.

Li J, Rohailla S, Gelber N, Rutka J, Sabah N, Gladstone RA, Wei C, Hu P, Kharbanda RK, Redington AN (2014) MicroRNA-144 is a circulating effector of remote ischemic preconditioning. *Basic Research in Cardiology* **109(5)**:423.

Li L, Li G, Yu C, Li Y (2013) The role of remote ischemic preconditioning on postoperative kidney injury in patients undergoing cardiac and vascular interventions: a meta-analysis. *Journal of Cardiothoracic Surgery* **8**:43.

Li L, Li G, Yu C, Li Y (2014) The role of remote ischemic preconditioning on postoperative kidney injury in patients undergoing cardiac and vascular interventions: a meta-analysis. *Journal of Cardiothoracic Surgery* **8**:43.

Li L, Luo W, Huang L, Zhang W, Gao Y, Jiang H, Zhang C, Long L, Chen S (2010) Remote preconditioning reduces myocardial injury in adult valve replacement: a randomised controlled trial. *The Journal of Surgical Research* **164(1)**:e21-26.

Li S, Krawczeski CD, Zappitelli M, Devarajan P, Thiessen-Philbrook H, Coca SG, Kim RW, Parikh CR; TRIBE-AKI Consortium (2011) Incidence, risk factors, and outcomes of acute kidney injury after pediatric cardiac surgery: a prospective multicenter study. *Critical Care Medicine* **39(6)**:1493-1499.

Li Y, Cai M, Xu Y, Swartz HM, He G (2011) Late phase ischemic preconditioning preserves mitochondrial oxygen metabolism and attenuates post-ischemicmyocardial tissue hyperoxygenation. *Life Sciences* **88(1-2)**:57-64.

Liem DA, Verdouw PD, Ploeg H, Kazim S, Duncker DJ (2002) Sites of action of adenosine in interorgan preconditioning of the heart. *American Journal of Physiology. Heart and Circulatory Physiology* **283(1)**:H29-37.

Lim SY, Yellon DM, Hausenloy DJ (2010) The neural and humoral pathways in remote limb ischemic preconditioning. *Basic Research in Cardiology* **105(5)**:651-655.

Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, Smith A, Bordner J, Polte T, Gaunitz F, Dennery PA (2007) Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. *The Journal of Biological Chemistry* **282(28)**:20621-20633.

Ling H, Gray CB, Zambon AC, Grimm M, Gu Y, Dalton N, Purcell NH, Peterson K, Brown JH (2013) Ca2+/Calmodulin-dependent protein kinase II δ mediates myocardial ischemia/reperfusion injury through nuclearfactor-κB. *Circulation Research* **112(6)**:935-944.

Liu RR, Li J, Gong JY, Kuang F, Liu JY, Zhang YS, Ma QL, Song CJ, Truax AD, Gao F, Yang K, Jin BQ, Chen LH (2015) MicroRNA-141 regulates the expression level of ICAM-1 on endothelium to decrease myocardial ischemia-reperfusion injury. *American Journal of Physiology. Heart and Circulatory Physiology* **309(8)**:H1303-1313.

Liu T, Daniels CK, Cao S (2012) Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. *Pharmacology & Therapeutics* **136(3)**:354-374.

Lomivorotov VV, Shmyrev VA, Nepomnyaschih VA, Ponomarev DN, Knyazkova LG, Lomivorotov VN, Karaskov AM (2012) Remote ischaemic preconditioning does not protect the heart in patients undergoing coronary artery bypass grafting. *Interactive Cardiovascular and Thoracic Surgery* **15(1)**:18-22.

Lorts A, Hehir D, Krawczeski C (2013) Biomarkers in Pediatric Cardiology and Cardiac Surgery, In: Pediatric and Congenital Cardiology, Cardiac Surgery and Intensive Care (ed Da Cruz EM, Ivy D, Jaggers J), Springer, London, pp: 857-873.

Loukogeorgakis SP, Panagiotidou AT, Broadhead MW, Donald A, Deanfield JE, MacAllister RJ (2005) Remote ischemic preconditioning provides early and late protection against endothelial ischemia-reperfusion injury in humans: role of the autonomic nervous system. *Journal of the American College of Cardiology* **46(3)**:450-456.

Lucchinetti E, Bestmann L, Feng J, Freidank H, Clanachan AS, Finegan BA, Zaugg M (2012) Remote ischemic preconditioning applied during isoflurane inhalation provides no benefit to the myocardium of patients undergoing on-pump coronary artery bypass graft surgery: lack of synergy or evidence of antagonism in cardioprotection? *Anesthesiology* **116(2)**:296-310.

Luo W, Zhu M, Huang R, Zhang Y (2011) A comparison of cardiac post-conditioning and remote pre-conditioning in paediatric cardiac surgery. *Cardiology in the Young* **21(3)**:266-270.

Lyck R, Enzmann G (2015) The physiological roles of ICAM-1 and ICAM-2 in neutrophil migration into tissues. *Current Opinion in Hematology* **22(1)**:53-59.

Lytrivi ID, Lai WW, Ko HH, Nielsen JC, Parness IA, Srivastava S (2005) Color Doppler tissue imaging for evaluation of right ventricular systolic function in patients with congenital heart disease. *Journal of the American Society of Echocardiography* **18(10)**:1099-1104.

Magierowski M, Jasnos K, Sliwowski Z, Surmiak M, Krzysiek-Maczka G, Ptak-Belowska A, Kwiecien S, Brzozowski T (2014) Exogenous asymmetric dimethylarginine (ADMA) in pathogenesis of ischemia-reperfusion-induced gastric lesions: interaction with protective nitric oxide (NO) and calcitonin gene-related peptide (CGRP). *International Journal of Molecular Sciences* **15(3)**:4946-4964.

Mahesh B, Choong CK, Goldsmith K, Gerrard C, Nashef SA, Vuylsteke A (2012) Prolonged stay in intensive care unit is a powerful predictor of adverse outcomes after cardiac operations. *The Annals of Thoracic Surgery* **94(1)**:109-116.

Malik ZA, Kott KS, Poe AJ, Kuo T, Chen L, Ferrara KW, Knowlton AA (2013) Cardiac myocyte exosomes: stability, HSP60, and proteomics. *American Journal of Physiology. Heart and Circulatory Phyiology* **304(7)**:H954-965.

Malinovschi A, Henrohn D, Eriksson A, Lundberg JO, Alving K, Wikström G (2011) Increased plasma and salivary nitrite and decreased bronchial contribution to exhaled NO in pulmonary arterial hypertension. *European Journal of Clinical Investigation* **41(8)**:889-897.

Malouf PJ, Madani M, Gurudevan S, Waltman TJ, Raisinghani AB, DeMaria AN, Blanchard DG (2006) Assessment of diastolic function with Doppler tissue imaging after cardiac surgery: effect of the "postoperative septum" in on-pump and off-pump procedures. *Journal of the American Society of Echocardiography* **19(4)**:464-467.

Manavalan B, Basith S, Choi S (2011) Similar Structures but Different Roles - An Updated Perspective on TLR Structures. *Frontiers in Physiology* **2**:41.

Mangoni AA (2009) The emerging role of symmetric dimethylarginine in vascular disease. *Advances in Clinical Chemistry* **48**:73-94.

Mao H, Katz N, Ariyanon W, Blanca-Martos L, Adýbelli Z, Giuliani A, Danesi TH, Kim JC, Nayak A, Neri M, Virzi GM, Brocca A, Scalzotto E,Salvador L, Ronco C (2013) Cardiac surgery-associated acute kidney injury. *Cardiorenal Medicine* **3(3)**:178-199.

Marczin N, El-Habashi N, Hoare GS, Bundy RE, Yacoub M (2003) Antioxidants in myocardial ischemia-reperfusion injury: therapeutic potential and basic mechanisms. Archives of Biochemistry and Biophysics 420(2):222-36.

Marin-Vinader L, Shin C, Onnekink C, Manley JL, Lubsen NH (2006) Hsp27 enhances recovery of splicing as well as rephosphorylation of SRp38 after heat shock. *Molecular Biology of the Cell* **17(2)**:886-894.

Martin TP, Currie S, Baillie GS (2014a) The cardioprotective role of small heat-shock protein 20. *Biochemical Society Transactions* **42(2)**:270-273.

Martin TP, Hortigon-Vinagre MP, Findlay JE, Elliott C, Currie S, Baillie GS (2014b) Targeted disruption of the heat shock protein 20-phosphodiesterase 4D (PDE4D) interaction

protects against pathological cardiac remodelling in a mouse model of hypertrophy. *FEBS Open Bio* **4**:923-927.

Martin-Puig S, Tello D, Aragonés J (2015) Novel perspectives on the PHD-HIF oxygen sensing pathway in cardioprotection mediated by IPC and RIPC. *Frontiers in Physiology* **6**:137.

Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B, Sadoshima J (2007) Distict roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinaseand Beclin 1 in mediating autophagy. Circulation research 100(6):914-22.

Mattoo RU, Goloubinoff P (2014) Molecular chaperones are nanomachines that catalytically unfold misfolded and alternatively folded proteins. *Cellular and Molecular Life Sciences* **71(17)**:3311-3325.

Mattoo RU, Sharma SK, Priya S, Finka A, Goloubinoff P (2013) Hsp110 is a bona fide chaperone using ATP to unfold stable misfolded polypeptides and reciprocally collaborate with Hsp70 to solubilize protein aggregates. *The Journal of Biological Chemistry* **288(29)**:21399-21411.

Mayer MP (2010) Gymnastics of molecular chaperones. Cell, Molecular Cell **39(3)**:321-331.

Mayer MP (2013) Hsp70 chaperone dynamics and molecular mechanism. *Trends in Biomedical Sciences* **38(10)**:507-514.

Mayer MP, Kityk R (2015) Insights into the molecular mechanism of allostery in Hsp70s. *Frontiers in Molecular Biosciences* **2**:58.

Mayer MP, Schröder H, Rüdiger S, Paal K, Laufen T, Bukau B (2000) Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nature Structural Biology* **7(7)**:586-593.

McCrindle BW, Clarizia NA, Khaikin S, Holtby HM, Manlhiot C, Schwartz SM, Caldarone CA, Coles JG, Van Arsdell GS, Scherer SW, Redington AN (2014) Remote ischemic preconditioning in children undergoing cardiac surgery with cardiopulmonary bypass: a single-center double-blinded randomised trial. *Journal of the American Heart Association* **3(4)**. pii: e000964.

McGinnis GR, Ballmann C, Peters B, Nanayakkara G, Roberts M, Amin R, Quindry JC (2015) Interleukin-6 mediates exercise preconditioning against myocardial ischemia reperfusion injury. *American Journal of Physiology. Heart and Circulatory Physiology* **308(11)**:H1423-1433.

McGuinness J, Bouchier-Hayes D and Redmond JM (2008) Understanding the inflammatory response to cardiac surgery. *Surgeon* **6(3)**:162-171.

McGuinness J, Bouchier-Hayes D, Redmond JM (2008) Understanding the inflammatory response to cardiac surgery. *Surgeon* **6(3)**:162-171.

Mehta JL, Lawson DL, Nichols WW (1989) Attenuated coronary relaxation after reperfusion: effects of superoxide dismutase and TxA2 inhibitor U 63557A. The American Journal of Physiology 257(4 Pt 2):H1240-6.

Mehta RH, Grab JD, O'Brien SM, Bridges CR, Gammie JS, Haan CK, Ferguson TB, Peterson ED; Society of Thoracic Surgeons National Cardiac Surgery Database Investigators (2006) Bedside tool for predicting the risk of postoperative dialysis in patients undergoing cardiac surgery. *Circulation* **114(21)**:2208-2216.

Melgar-Lesmes P, Pauta M, Reichenbach V, Casals G, Ros J, Bataller R, Morales-Ruiz M, Jiménez W (2011) Hypoxia and proinflammatory factors upregulate apelin receptor expression in human stellate cells and hepatocytes. *Gut* **60(10)**:1404-1411.

Meng L, Hunt C, Yaglom JA, Gabai VL, Sherman MY (2011) Heat shock protein Hsp72 plays an essential role in Her2-induced mammary tumorigenesis. *Oncogene* **30(25)**:2836-2845.

Mersmann J, Berkels R, Zacharowski P, Tran N, Koch A, Iekushi K, Dimmeler S, Granja TF, Boehm O, Claycomb WC, Zacharowski K (2010) Preconditioning by toll-like receptor 2 agonist Pam3CSK4 reduces CXCL1-dependent leukocyte recruitment in murine myocardial ischemia/reperfusion injury. *Critical Care Medicine* **38(3)**:903-909.

Mersmann J, Iskandar F, Latsch K, Habeck K, Sprunck V, Zimmermann R, Schumann RR, Zacharowski K, Koch A (2013) Attenuation of myocardial injury by HMGB1 blockade during ischemia/reperfusion is toll-like receptor 2-dependent. *Mediators of Inflammation* **2013**:174168.

Meybohm P, Bein B, Brosteanu O, Cremer J, Gruenewald M, Stoppe C, Coburn M, Schaelte G, Böning A, Niemann B, Roesner J, Kletzin F, Strouhal U, Reyher C, Laufenberg-Feldmann R, Ferner M, Brandes IF, Bauer M, Stehr SN, Kortgen A, Wittmann M, Baumgarten G, Meyer-Treschan T, Kienbaum P, Heringlake M, Sander M, Treskatsch S, Smul T, Wolwender E, Schilling T, Fuernau G, Hasenclever D, Zacharowski K; RIPHeart Study Collaborators (2015) A Multicenter Trial of Remote Ischemic Preconditioning for Heart Surgery. *The New England Journal of Medicine* **373(15)**:1397-1407.

Meybohm P, Renner J, Broch O, Caliebe D, Albrecht M, Cremer J, Haake N, Scholz J, Zacharowski K, Bein B (2013) Postoperative neurocognitive dysfunction in patients undergoing cardiac surgery after remote ischemic preconditioning: a double-blind randomised controlled pilot study. *PLoS One* **8(5)**:e64743.

Meyer G, André L, Kleindienst A, Singh F, Tanguy S, Richard S, Obert P, Boucher F, Jover B, Cazorla O, Reboul C (2015) Carbon monoxide increases inducible NOS expression that mediates CO-induced myocardial damage duringischemia-reperfusion. *American Journal of Physiology. Heart and Circulatory Physiology* **308(7)**:H759-767.

Meyer P, Prodromou C, Liao C, Hu B, Roe SM, Vaughan CK, Vlasic I, Panaretou B, Piper PW, Pearl LH (2004) Structural basis for recruitment of the ATPase activator Aha1 to the Hsp90 chaperone machinery. *The EMBO Journal* **23(6)**:1402-1410.

Mir TS, Flato M, Falkenberg J, Haddad M, Budden R, Weil J, Albers S, Laer S (2006) Plasma concentrations of N-terminal brain natriuretic peptide in healthy children, adolescents, and young adults: effect of age and gender. *Pediatric Cardiology* **27(1)**:73-77.

Mocanu MM, Baxter GF, Yellon DM (2000) Caspase inhibition and limitation of myocardial infarct size: protection against lethal reperfusion injury. British Journal of Pharmacology 130(2):197-200.

Modi P, Imura H, Angelini GD, Pawade A, Parry AJ, Suleiman MS, Caputo M (2003) Pathology-related troponin I release and clinical outcome after pediatric open heart surgery. *Journal of Cardiac Surgery* **18(4)**:295-300.

Modi P, Suleiman MS, Reeves B, Pawade A, Parry AJ, Angelini GD, Caputo M (2004) Myocardial metabolic changes during pediatric cardiac surgery: a randomised study of 3 cardioplegic techniques. *The Journal of Thoracic and Cardiovascular Surgery* **128(1)**:67-75.

Molnar T, Sütö B, Biri B, Nagy L, Keki S, Ruzsics I (2013) Methylarginine metabolism is different in acute and chronic hypoxia: 12AP1-3. *Intensive Care Medicine* **30**:181.

Moncada S, Palmer RM, Higgs EA (1989) Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. Biochemical Pharmacology 38(11):1709-15.

Mueller M, Herzog C, Larmann J, Schmitz M, Hilfiker-Kleiner D, Gessner JE, Theilmeier G (2013) The receptor for activated complement factor 5 (C5aR) conveys myocardial ischemic damage by mediating neutrophil transmigration. Immunobiology 218(9):1131-8.

Müllebner A, Moldzio R, Redl H, Kozlov AV, Duvigneau JC (2015) Heme Degradation by Heme Oxygenase Protects Mitochondria but Induces ER Stress via Formed Bilirubin. *Biomolecules* **5(2)**:679-701.

Muller P, Ruckova E, Halada P, Coates PJ, Hrstka R, Lane DP, Vojtesek B (2013) C-terminal phosphorylation of Hsp70 and Hsp90 regulates alternate binding to co-chaperones CHIP and HOP to determine cellular protein folding/degradation balances. *Oncogene* **32(25)**:3101-3110.

Murohara T, Delyani JA, Albelda SM, Lefer AM (1996) Blockade of platelet endothelial cell adhesion molecule-1 protects against myocardial ischemia and reperfusioninjury in cats. *Journal of Immunology* **156(9)**:3550-7.

Murohara Y, Yui Y, Hattori R, Kawai C (1991) Effects of superoxide dismutase on reperfusion arrhythmias and left ventricular function in patients undergoing thrombolysis for anterior wall acute myocardial infarction. *The American Journal of Cardiology* **67(8)**:765-7.

Murphy E, Steenbergen C (2008) Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiological Reviews* **88(2)**:581-609.

Murry CE, Jennings RB and Reimer KA (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74(5)**:1124-1136.

Murry CE, Richard VJ, Reimer KA, Jennings RB (1990) Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circulation Research* **66(4)**:913-931.

Mylroie H, Dumont O, Bauer A, Thornton CC, Mackey J, Calay D, Hamdulay SS, Choo JR, Boyle JJ, Samarel AM, Randi AM, Evans PC, Mason JC (2015) PKCε-CREB-Nrf2 signalling induces HO-1 in the vascular endothelium and enhances resistance to inflammation and apoptosis. *Cardiovascular Research* **106(3)**:509-519.

Mymrikov EV, Seit-Nebi AS, Gusev NB (2011) Large potentials of small heat shock proteins. *Physiological Reviews* **91(4)**:1123-1159.

Nadtochiy SM, Urciuoli W, Zhang J, Schafer X, Munger J, Brookes PS (2015) Metabolomic profiling of the heart during acute ischemic preconditioning reveals a role for SIRT1 in rapid cardioprotective metabolic adaptation. *Journal of Molecular and Cellular Cardiology* **88**:64-72.

Nagueh SF, Smiseth OA, Appleton CP, Byrd BF 3rd, Dokainish H, Edvardsen T, Flachskampf FA, Gillebert TC, Klein AL, Lancellotti P, Marino P, Oh JK, Popescu BA, Waggoner AD (2016) Recommendations for the Evaluation of Left Ventricular Diastolic Function by Echocardiography: An Update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *Journal of the American Society of Echocardiography* **29(4)**:277-314.

Najafi M (2014) Serum creatinine role in predicting outcome after cardiac surgery beyond acute kidney injury. *World Journal of Cardiology* **6(9)**:1006-1021.

Nakayama Y, Ueda S, Yamagishi S, Obara N, Taguchi K, Ando R, Kaida Y, Iwatani R, Kaifu K, Yokoro M, Toyonaga M, Kusumoto T, Fukami K,Okuda S (2014) Asymmetric dimethylarginine accumulates in the kidney during ischemia/reperfusion injury. *Kidney International* **85(3)**:570-578.

Nathan DF, Vos MH, Lindquist S (1997) *In vivo* functions of the Saccharomyces cerevisiae Hsp90 chaperone. *Proceedings of the National Academy of Sciences of the United States of America* **94(24)**:12949-12956.

Nazareth W, Yafei N, Crompton M (1991) Inhibition of anoxia-induced injury in heart myocytes by cyclosporin A. *Journal of Molecular and Cellular Cardiology* **23(12)**:1351-4.

Niedner MF, Foley JL, Riffenburgh RH, Bichell DP, Peterson BM, Rodarte A (2010) B-type natriuretic peptide: perioperative patterns in congenital heart disease. *Congenital Heart Disease* **5(3)**:243-255.

Nisemblat S, Yaniv O, Parnas A, Frolow F, Azem A (2015) Crystal structure of the human mitochondrial chaperonin symmetrical football complex. *Proceedings of the National Academy of Sciences of the United States of America* **112(19)**:6044-6049.

Noble EG, Milne KJ, Melling CW (2008) Heat shock proteins and exercise: a primer. *Applied Physiology, Nutrition, and Metabolism* **33(5)**:1050-1065.

Nora JJ (1968) Multifactorial inheritance hypothesis for the etiology of congenital heart diseases. The genetic-environmental interaction. *Circulation* **38(3)**:604-17.

Obal D, Dai S, Keith R, Dimova N, Kingery J, Zheng YT, Zweier J, Velayutham M, Prabhu SD, Li Q, Conklin D, Yang D, Bhatnagar A, Bolli R, Rokosh G (2012) Cardiomyocyte-restricted overexpression of extracellular superoxide dismutase increases nitric oxide bioavailability and reduces infarct size after ischemia/ reperfusion. *Basic Research in Cardiology* **107(6)**:305.

O'Dowd BF, Heiber M, Chan A, Heng HH, Tsui LC, Kennedy JL, Shi X, Petronis A, George SR, Nguyen T (1993) A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* **136(1-2)**:355-360.

O'Dwyer MJ, Dempsey F, Crowley V, Kelleher DP, McManus R, Ryan T (2006) Septic shock is correlated with asymmetrical dimethyl arginine levels, which may be influenced by a polymorphism in the dimethylarginine dimethylaminohydrolase II gene: a prospective observational study. *Critical Care* **10(5)**:R139.

Oikawa S, Mano A, Takahashi R, Kakinuma Y (2015) Remote ischemic preconditioning with a specialised protocol activates the non-neuronal cardiac cholinergic system and increases ATP content in the heart. *International Immunopharmacology* **29(1)**:181-184.

Olas B (2014) Carbon monoxide is not always a poison gas for human organism: Physiological and pharmacological features of CO. *Chemico-Biological Interactions* **222C**:37-43.

Olas B (2015) Hydrogen sulfide in signaling pathways. *Clinica Chimica Acta* **439**:212-218.

Onda K, Tong S, Nakahara A, Kondo M, Monchusho H, Hirano T, Kaitu'u-Lino T, Beard S, Binder N, Tuohey L, Brownfoot F, Hannan NJ (2015) Sofalcone upregulates the nuclear factor (erythroid-derived 2)-like 2/heme oxygenase-1 pathway, reduces soluble fms-like tyrosine kinase-1, and quenches endothelial dysfunction: potential therapeutic for preeclampsia. *Hypertension* **65(4)**:855-862.

Ong SG, Hausenloy DJ (2012) Hypoxia-inducible factor as a therapeutic target for cardioprotection. *Pharmacology & Therapeutics* **136(1)**:69-81.

Ong SG, Lee WH, Theodorou L, Kodo K, Lim SY, Shukla DH, Briston T, Kiriakidis S, Ashcroft M, Davidson SM,Maxwell PH, Yellon DM, Hausenloy DJ (2014) HIF-1 reduces ischaemia-reperfusion injury in the heart by targeting the mitochondrial permeability transition pore. *Cardiovascular Research* **104(1)**:24-36.

Ong SG, Lee WH, Theodorou L, Kodo K, Lim SY, Shukla DH, Briston T, Kiriakidis S, Ashcroft M, Davidson SM,Maxwell PH, Yellon DM, Hausenloy DJ (2014) HIF-1 reduces ischaemia-reperfusion injury in the heart by targeting the mitochondrial permeability transition pore. *Cardiovascular Research* **104(1)**:24-36.

Osipov RM, Robich MP, Feng J, Liu Y, Clements RT, Glazer HP, Sodha NR, Szabo C, Bianchi C, Sellke FW (2009) Effect of hydrogen sulfide in a porcine model of myocardial ischemia-reperfusion: comparison of different administration regimens and characterization of the cellular mechanisms of protection. *Journal of Cardiovascular Pharmacology* **54(4)**:287-297.

Oyama J, Blais C Jr, Liu X, Pu M, Kobzik L, Kelly RA, Bourcier T (2004) Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. *Circulation* **109(6)**:784-789.

Pągowska-Klimek I, Świerzko AS, Michalski M, Głowacka E, Szala-Poździej A, Sokołowska A, Moll M, Krajewski WR, Romak J, Cedzyński M (2015) Activation of the lectin pathway of complement by cardiopulmonary bypass contributes to the development of systemic inflammatory response syndrome after pediatric cardiac surgery. *Clinical and Experimental Immunology* **184(2)**:257-263.

Pągowska-Klimek I, Świerzko AS, Michalski M, Moll M, Szala-Poździej A, Sokołowska A, Krajewski WR, Cedzyński M (2016) Mannose-binding lectin (MBL) insufficiency protects against the development of systemic inflammatory response after pediatric cardiac surgery. *Immunobiology* **221(2)**:175-181.

Palazzo AJ, Jones SP, Girod WG, Anderson DC, Granger DN, Lefer DJ (1998) Myocardial ischemia-reperfusion injury in CD18- and ICAM-1-deficient mice. *The American Journal of Physiology* **275(6 Pt 2)**:H2300-2307.

Palma G, Giordano R, Russolillo V, Cioffi S, Palumbo S, Mucerino M, Poli V, Vosa C (2011) Sildenafil therapy for pulmonary hypertension before and after pediatric congenital heart surgery. *Texas Heart Institute Journal* **38(3)**:238-342.

Palomero Rodríguez MA, Cacharro Moras LM, Suárez Gonzalo L, Moreno Gómez-Limón I, Mollinedo F (2009) Plasma nitrate/nitrite (NOx) is not a useful biomarker to predict inherent cardiopulmonary bypass inflammatory response. *Journal of Cardiac Surgery* **24(3)**:357.

Pan Q, Qin X, Ma S, Wang H, Cheng K, Song X, Gao H, Wang Q, Tao R, Wang Y, Li X, Xiong L, Cao F (2014) Myocardial protective effect of extracellular superoxide dismutase gene modified bone marrow mesenchymal stromal cells on infarcted mice hearts. *Theranostics* **4(5)**:475-486

Pan TT, Neo KL, Hu LF, Yong QC, Bian JS (2008) H2S preconditioning-induced PKC activation regulates intracellular calcium handling in rat cardiomyocytes. *American Journal of Physiology. Cell Physiology* **294(1)**:C169-177.

Pasqua T, Filice E, Mazza R, Quintieri AM, Carmela Cerra M, Iannacone R, Melfi D, Indiveri C, Gattuso A, Angelone T (2015) Cardiac and hepatic role of r-AtHSP70: basal effects and protection against ischemic and sepsis conditions. *Journal of Cellular and Molecular Medicine* **19(7)**:1492-1503.

Patel B, Khaliq A, Jarvis-Evans J, Boulton M, Arrol S, Mackness M, McLeod D (1995) Hypoxia induces HSP 70 gene expression in human hepatoma (HEP G2) cells. *Biochemistry and Molecular Biology International* **36(4)**:907-912.

Patel HH, Moore J, Hsu AK, Gross GJ (2002) Cardioprotection at a distance: mesenteric artery occlusion protects the myocardium via an opioid sensitive mechanism. *Journal of Molecular and Cellular Cardiology* **34(10)**:1317-1323.

Pavione MA, Carmona F, de Castro M, Carlotti AP (2012) Late remote ischemic preconditioning in children undergoing cardiopulmonary bypass: a randomised controlled trial. *The Journal of Thoracic and Cardiovascular Surgery* **144(1)**:178-183.

Pedersen KR, Ravn HB, Povlsen JV, Schmidt MR, Erlandsen EJ, Hjortdal VE (2012) Failure of remote ischemic preconditioning to reduce the risk of postoperative acute kidney injury in children undergoing operation for complex congenital heart disease: a randomised single-center study. *Journal of Thoracic and Cardiovascular Surgery* **143(3)**:576-583.

Pei H, Song X, Peng C, Tan Y, Li Y, Li X, Ma S, Wang Q, Huang R, Yang D, Li D, Gao E, Yang Y (2015) TNF-α inhibitor protects against myocardial ischemia/reperfusion injury via Notch1-mediated suppression of oxidative/nitrative stress. *Free Radical Biology & Medicine* **82**:114-121.

Peng EW, McCaig D, Pollock JC, MacArthur K, Lyall F, Danton MH (2011) Myocardial expression of heat shock protein 70i protects early postoperative right ventricular function in cyanotic tetralogy of Fallot. *The Journal of Thoracic and Cardiovascular Surgery* **141(5)**:1184-91.

Peng TC, Jan WC, Tsai PS, Huang CJ (2011) Heme oxygenase-1 mediates the protective effects of ischemic preconditioning on mitigating lung injury induced by lower limb ischemia-reperfusion in rats. *The Journal of Surgical Research* **167(2)**:e245-253.

Pepe S, Liaw NY, Hepponstall M, Sheeran FL, Yong MS, d'Udekem Y, Cheung MM, Konstantinov IE (2013) Effect of remote ischemic preconditioning on phosphorylated protein signaling in children undergoing tetralogy of Fallot repair: a randomised controlled trial. *Journal of the American Heart Association* **2(3)**:e000095.

Pickering JW, James MT, Palmer SC (2015) Acute kidney injury and prognosis after cardiopulmonary bypass: a meta-analysis of cohort studies. *American Journal of Kidney Diseases* **65(2)**:283-293.

Pierpont ME, Basson CT, Benson DW Jr, Gelb BD, Giglia TM, Goldmuntz E, McGee G, Sable CA, Srivastava D, Webb CL; American Heart Association Congenital Cardiac Defects Committee, Council on Cardiovascular Disease in the Young (2007) Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American Heart Association Congenital Cardiac Defects Committee, Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. *Circulation* **115(23)**:3015-3038.

Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G,Finet G, André-Fouët X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, Ovize M (2008) Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *The New England Journal of Medicine* **359(5)**:473-481.

Pockley AG, Muthana M, Calderwood SK (2008) The dual immunoregulatory roles of stress proteins. *Trends in Biochemical Sciences* **33(2)**:71-79.

Pourrajab F, Yazdi MB, Zarch MB, Zarch MB, Hekmatimoghaddam S (2015) Cross talk of the first-line defense TLRs with PI3K/Akt pathway, in preconditioning therapeutic approach. *Molecular and Cellular Therapies* **3**:4.

Preissler S, Deuerling E (2012) Ribosome-associated chaperones as key players in proteostasis. *Trends in Biochemical Sciences* **37(7)**:274-283.

Priya S, Sharma SK, Goloubinoff P (2013) Molecular chaperones as enzymes that catalytically unfold misfolded polypeptides. *FEBS letters* **587(13)**:1981-1987.

Przyklenk K, Bauer B, Ovize M, Kloner RA and Whittaker P (1993) Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* **87(3)**:893-899.

Qi R, Sarbeng EB, Liu Q, Le KQ, Xu X, Xu H, Yang J, Wong JL, Vorvis C, Hendrickson WA, Zhou L, Liu Q (2013) Allosteric opening of the polypeptide-binding site when an Hsp70 binds ATP. *Nature Structural & Molecular Biology* **20(7)**:900-907.

Qian J, Ren X, Wang X, Zhang P, Jones WK, Molkentin JD, Fan GC, Kranias EG (2009) Blockade of Hsp20 phosphorylation exacerbates cardiac ischemia/reperfusion injury by suppressed autophagy and increased cell death. *Circulation Research* **105(12)**:1223-1231.

Quinn FA, Ambruster DA (2013) Abbott Architect Family of Analyzers, In The Immunoassay Handbook. Editor David Wild, 4th Ed, Elsevier, Amsterdam, pp 562-565.

Quintana FJ, Cohen IR (2011) The HSP60 immune system network. *Trends in Immunology* **32(2)**:89-95.

Radomski MW, Palmer RM, Moncada S (1987) Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **2(8567)**:1057-1058.

Rahman IA, Mascaro JG, Steeds RP, Frenneaux MP, Nightingale P, Gosling P, Townsend P, Townend JN, Green D, Bonser RS (2010) Remote ischemic preconditioning in human coronary artery bypass surgery: from promise to disappointment? *Circulation* **122(11 Suppl)**:S53-59.

Ran R, Zhou G, Lu A, Zhang L, Tang Y, Rigby AC, Sharp FR (2004) Hsp70 mutant proteins modulate additional apoptotic pathways and improve cell survival. *Cell Stress & Chaperones* **9(3)**:229-242.

Randolph KA, Myers LL (2013) Basic Statistics in Multivariate Analysis. Oxford University Press, Oxford. 119pp.

Rapp UK, Kaufmann SH (2004) DNA vaccination with gp96-peptide fusion proteins induces protection against an intracellular bacterial pathogen. *International Immunology* **16(4)**:597-605.

Rashed E, Lizano P, Dai H, Thomas A, Suzuki CK, Depre C, Qiu H (2015) Heat shock protein 22 (Hsp22) regulates oxidative phosphorylation upon its mitochondrial translocation with the inducible nitric oxide synthase in mammalian heart. *PLoS One* **10(3)**:e0119537.

Rassaf T, Totzeck M, Hendgen-Cotta UB, Shiva S, Heusch G, Kelm M (2014) Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning. *Circulation Research* **114(10)**:1601-1610.

Rastaldo R, Cappello S, Folino A, Berta GN, Sprio AE, Losano G, Samaja M, Pagliaro P (2011) Apelin-13 limits infarct size and improves cardiac postischemic mechanical recovery only if given after ischemia. *American Journal of Physiology. Heart and Circulaory Physiology* **300(6)**:H2308-2315.

Ratzke C, Mickler M, Hellenkamp B, Buchner J, Hugel T (2010) Dynamics of heat shock protein 90 C-terminal dimerization is an important part of its conformational cycle. *Proceedings of the National Academy of Sciences of the United States of America* **107(37)**:16101-16106.

Ren XP, Wu J, Wang X, Sartor MA, Qian J, Jones K, Nicolaou P, Pritchard TJ, Fan GC (2009) MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20. *Circulation* **119(17)**:2357-66.

Reutzel-Selke A, Pratschke J, Martins PN, Denecke C, Jurisch A, Kotsch K, Pascer A, Neuhaus P, Tullius SG (2008) Ischemic preconditioning produces systemic protective and adoptively transferable effects. *Kidney International* **74(5)**:622-630.

Richards AA, Garg V (2010) Genetics of Congenital Heart Disease. *Current Cardiology Reviews* **6(2)**: 91–97.

Ritossa F (1962) A new puffing pattern induced by temperature shock and DNP in Drosophila. *Experientia* **18**:571–573.

Rizvi A, Tang XL, Qiu Y, Xuan YT, Takano H, Jadoon AK, Bolli R (1999) Increased protein synthesis is necessary for the development of late preconditioning against myocardial stunning. *The American Journal of Physiology* **277(3 Pt 2)**:H874-884.

Robotham JL, Takata M, Berman M, Harasawa Y (1991) Ejection fraction revisited. *Anesthesiology* **74(1)**:172-183.

Rodríguez-Sinovas A, Abdallah Y, Piper HM, Garcia-Dorado D (2007) Reperfusion injury as a therapeutic challenge in patients with acute myocardial infarction. *Heart Failure Reviews* **12(3-4)**:207-216.

Roffi M, Patrono C, Collet JP, Mueller C, Valgimigli M, Andreotti F, Bax JJ, Borger MA, Brotons C, Chew DP, Gencer B, Hasenfuss G, Kjeldsen K, Lancellotti P,Landmesser U, Mehilli J, Mukherjee D, Storey RF, Windecker S, Baumgartner H, Gaemperli O, Achenbach S, Agewall S, Badimon L, Baigent C, Bueno H, Bugiardini R, Carerj S, Casselman F, Cuisset T, Erol Ç, Fitzsimons D, Halle M, Hamm C, Hildick-Smith D, Huber K, Iliodromitis E, James S,Lewis BS, Lip GY, Piepoli MF, Richter D, Rosemann T, Sechtem U, Steg PG, Vrints C, Luis Zamorano J (2015) 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). *European Heart Journal* **37(3)**:267-315.

Rosenfeld GE, Mercer EJ, Mason CE, Evans T (2013) Small heat shock proteins Hspb7 and Hspb12 regulate early steps of cardiac morphogenesis. *Developmental Biology* **381(2)**:389-400.

Rossen RD, Michael LH, Hawkins HK, Youker K, Dreyer WJ, Baughn RE, Entman ML (1994) Cardiolipin-protein complexes and initiation of complement activation after coronary artery occlusion. *Circulation Research* **75(3)**:546-555.

Ruiz-Meana M, Rodríguez-Sinovas A, Cabestrero A, Boengler K, Heusch G, Garcia-Dorado D (2008) Mitochondrial connexin43 as a new player in the pathophysiology of myocardial ischaemia-reperfusion injury. *Cardiiovascular Research* **77(2)**:325-333.

Saibil H (1996) The lid that shapes the pot: structure and function of the chaperonin GroES. *Structure* **4(1)**:1-4.

Saibil H (2013) Chaperonee machines for protein folding, unfolding and disaggregation. *Nature Reviews: Molecular Cell Biology* **14(10)**:630-642.

Sakata Y, Dong JW, Vallejo JG, Huang CH, Baker JS, Tracey KJ, Tacheuchi O, Akira S, Mann DL (2007) Toll-like receptor 2 modulates left ventricular function following ischemia-reperfusion injury. *American Journal of Physiology. Heart and Circulatory Physiology* **292(1)**:H503-509.

Sanli C, Oguz D, Olgunturk R, Tunaoglu FS, Kula S, Pasaoglu H, Gulbahar O, Cevik A (2012) Elevated homocysteine and asymmetric dimethyl arginine levels in pulmonary hypertension associated with congenital heart disease. *Pediatric Cardiology* **33(8)**:1323-1331.

Sarkar FH, Li Y, Wang Z, Kong D (2008) NF-kappaB signaling pathway and its therapeutic implications in human diseases. *International Reviews of Immunology* **27(5)**:293-319.

Sarkar K, Cai Z, Gupta R, Parajuli N, Fox-Talbot K, Darshan MS, Gonzalez FJ, Semenza GL (2012) Hypoxia-inducible factor 1 transcriptional activity in endothelial cells is required for acute phase cardioprotection induced by ischemic preconditioning. *Proceedings of the National Academy of Sciences of the United States of America* **109(26)**:10504-10509.

Sauer RT, Baker TA (2011) AAA+ proteases: ATP-fueled machines of protein destruction. *Annual Review of Biochemistry* **80**:587-612.

Saxena A, Khosraviani S, Noel S, Mohan D, Donner T, Hamad AR (2015) Interleukin-10 paradox: A potent immunoregulatory cytokine that has been difficult to harness for immunotherapy. *Cytokine* **74(1)**:27-34.

Schäfer C, Ladilov YV, Siegmund B, Piper hM (2000) Importance of bicarbonate transport for protection of cardiomyocytes against reoxygenation injury. American Journal of Physiology. *Heart and circulation Physiology* **278(5)**:H1457-1463.

Schlesinger M, Bendas G (2015) Vascular cell adhesion molecule-1 (VCAM-1)--an increasing insight into its role in tumorigenicity and metastasis. *International Journal of Cancer* **136(11)**:2504-2514.

Schmidt MR, Støttrup NB, Contractor H, Hyldebrandt JA, Johannsen M, Pedersen CM, Birkler R, Ashrafian H, Sørensen KE, Kharbanda RK,Redington AN, Bøtker HE (2014a) Remote ischemic preconditioning with --but not without--metabolic support protects the neonatal porcine heart against ischemia-reperfusion injury. *International Journal of Cardiology* **170(3)**:388-393.

Schmidt MR, Støttrup NB, Michelsen MM, Contractor H, Sørensen KE, Kharbanda RK, Redington AN, Bøtker HE (2014b) Remote ischemic preconditioning impairs ventricular function and increases infarct size after prolonged ischemia in the isolated neonatal rabbit heart. *The Journal of Thoracic and Cardiovascular Surgery* **147(3)**:1049-1055.

Schoemaker RG, van Heijningen CL (2000) Bradykinin mediates cardiac preconditioning at a distance. *American Journal of Physiology. Heart and Circulatory Physiology* **278(5)**:H1571-1576.

Schulte G, Sommerschild H, Yang J, Tokuno S, Goiny M, Lövdahl C, Johansson B, Fredholm BB, Valen G (2004) Adenosine A receptors are necessary for protection of the murine heart by remote, delayed adaptation to ischaemia. *Acta Physiologica Scandinavica* **182(2)**:133-143.

Schulz R, Gres P, Skyschally A, Duschin A, Belosjorow S, Konietzka I, Heusch G (2003) Ischemic preconditioning preserves connexin 43 phosphorylation during sustained ischemia in pig hearts *in vivo*. *FASEB Journal* **17(10)**:1355-1357.

Schulze-Neick I, Li J, Penny DJ, Redington AN (2001) Pulmonary vascular resistance after cardiopulmonary bypass in infants: effect on postoperative recovery. *The Journal of Thoracic and Cardiovascular Surgery* **121(6)**:1033-1039.

Segarra G, Medina P, Vila JM, Chuan P, Domenech C, Torondel B, Lluch A (2001) Inhibition of nitric oxide activity by arginine analogs in human renal arteries. *American Journal of Hypertension* **14(11 Pt 1)**:1142-1148.

Semen K, Yelisyeyeva O, Jarocka-Karpowicz I, Kaminskyy D, Solovey L, Skrzydlewska E, Yavorskyi O (2016) Sildenafil reduces signs of oxidative stress in pulmonary arterial hypertension: Evaluation by fatty acid composition, level of hydroxynonenal and heart rate variability. *Redox Biology* **7**:48-57.

Semenza GL (2012) Hypoxia-Inducible Factors in Physiology and Medicine. *Cell* **148(3)**: 399-408.

Sen N, Paul BD, Gadalla MM, Mustafa AK, Sen T, Xu R, Kim S, Snyder SH (2012) Hydrogen sulfide-linked sulfhydration of NF-κB mediates its antiapoptotic actions. Molecular Cell **45(1)**:13-24.

Sen R, Baltimore D (1986) Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* **46(5)**:705-716.

Sharp FR, Zhan X, Liu DZ (2013) Heat shock proteins in the brain: role of Hsp70, Hsp 27, and HO-1 (Hsp32) and their therapeutic potential. *Translational Stroke Research* **4(6)**:685-692.

Shemetov AA, Seit-Nebi AS, Gusev NB (2008) Structure, properties, and functions of the human small heat-shock protein HSP22 (HspB8, H11, E2IG1): a critical review. *Journal of Neuroscience Research* **86(2)**:264-269.

Shen Y, Shen Z, Miao L, Xin X, Lin S, Zhu Y, Guo W, Zhu YZ (2015) miRNA-30 family inhibition protects against cardiac ischemic injury by regulating cystathionine-γ-lyaseexpression. *Antioxidants & Redox Signaling* **22(3)**:224-240.

Sherman MY, Gabai VL (2015) Hsp70 in cancer: back to the future. *Oncogene* **34(32)**:4153-4161.

Shernan SK, Fitch JC, Nussmeier NA, Chen JC, Rollins SA, Mojcik CF, Malloy KJ, Todaro TG, Filloon T, Boyce SW, Gangahar DM, Goldberg M, Saidman LJ,Mangano DT; Pexelizumab Study Investigators (2004) Impact of pexelizumab, an anti-C5 complement antibody, on total mortality and adverse cardiovascular outcomes in cardiac surgical patients undergoing cardiopulmonary bypass. *The Annals of Thoracic Surgery* **77(3)**:942-949

Shimamoto A, Chong AJ, Yada M, Shomura S, Takayama H, Fleisig AJ, Agnew ML, Hampton CR, Rothnie CL, Spring DJ, Pohlman TH, Shimpo H, Verrier ED (2006) Inhibition of Toll-like receptor 4 with eritoran attenuates myocardial ischemia-reperfusion injury. *Circulation* **114(1 Suppl)**:I270-1274.

Shimizu M, Saxena P, Konstantinov IE, Cherepanov V, Cheung MM, Wearden P, Zhangdong H, Schmidt M, Downey GP, Redington AN (2010) Remote ischemic preconditioning decreases adhesion and selectively modifies functional responses of human neutrophils. *The Journal of Surgical Research* **158(1)**:155-61.

Shimizu M, Tropak M, Diaz RJ, Suto F, Surendra H, Kuzmin E, Li J, Gross G, Wilson GJ, Callahan J, Redington AN (2009) Transient limb ischaemia remotely preconditions through a humoral mechanism acting directly on themyocardium: evidence suggesting cross-species protection. *Clinical Science* **117(5)**:191-200.

Shinmura K, Bolli R, Liu SQ, Tang XL, Kodani E, Xuan YT, Srivastava S, Bhatnagar A (2002) Aldose reductase is an obligatory mediator of the late phase of ischemic preconditioning. *Circulation Research* **91(3)**:240-246.

Sibiude J, Mandelbrot L, Blanche S, Le Chenadec J, Boullag-Bonnet N, Faye A, Dollfus C, Tubiana R, Bonnet D, Lelong N, Khoshnood B,Warszawski J (2014) Association between prenatal exposure to antiretroviral therapy and birth defects: an analysis of the French perinatal cohort study (ANRS CO1/CO11). *PLoS Medicine* **11(4)**:e1001635.

Simkhovich BZ, Marjoram P, Poizat C, Kedes L, Kloner RA (2003) Brief episode of ischemia activates protective genetic program in rat heart: a gene chip study. *Cardiovascular Research* **59(2)**:450-459.

Simkhovich BZ, Przyklenk K, Kloner RA (2013) Role of protein kinase C in ischemic "conditioning": from first evidence to current perspectives. *Journal of Cardiovascular Pharmacology and Therapeutics* **18(6)**:525-532.

Simpkin JC, Yellon DM, Davidson SM, Lim SY, Wynne AM, Smith CC (2007) Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemia-reperfusion injury. *Basic Research in Cardiology* **102(6)**:518-528.

Sin YY, Martin TP, Wills L, Currie S, Baillie GS (2015) Small heat shock protein 20 (Hsp20) facilitates nuclear import of protein kinase D 1 (PKD1) during cardiachypertrophy. *Cell Communication and Signalling* **13:16.**

Skippen PW, Krahn GE (2005) Acute renal failure in children undergoing cardiopulmonary bypass. *Critical Care and Resuscitation* **7(4)**:286-291.

Slagsvold KH, Moreira JB, Rognmo O, Høydal M, Bye A, Wisløff U, Wahba A (2014) Remote ischemic preconditioning preserves mitochondrial function and activates pro-survival protein kinase Akt in the left ventricle during cardiac surgery: a randomised trial. *International Journal of Cardiology* **177(2)**:409-417.

Smith KA, Joziasse IC, Chocron S, van Dinther M, Guryev V, Verhoeven MC, Rehmann H, van der Smagt JJ, Doevendans PA, Cuppen E, Mulder BJ, Ten Dijke P and Bakkers J (2009) Dominant-negative ALK2 allele associates with congenital heart defects. *Circulation* **119(24)**:3062-3069.

Smith PK, Carrier M, Chen JC, Haverich A, Levy JH, Menasché P, Shernan SK, Van de Werf F, Adams PX, Todaro TG, Verrier E (2006) Effect of pexelizumab in coronary artery bypass graft surgery with extended aortic cross-clamp time. *The Annals of Thoracic Surgery* **82(3)**:781-788.

Spoelstra-de Man AM, Smit B, Oudemans-van Straaten HM, Smulders YM (2015) Cardiovascular effects of hyperoxia during and after cardiac surgery. *Anaesthesia* **70(11)**:1307-1319.

Stahl GL, Shernan SK, Smith PK, Levy JH (2012) Complement activation and cardiac surgery: a novel target for improving outcomes. *Anesthesia and Analgesia* **115(4)**:759-771.

Stanker LH, Hnasko RM (2015) ELISA: Methods and Protocols. Humana Press - Springer, New York. pp 70-71.

Stark K, Esslinger UB, Reinhard W, Petrov G, Winkler T, Komajda M, Isnard R, Charron P, Villard E, Cambien F, Tiret L, Aumont MC, Dubourg O, Trochu JN,Fauchier L, Degroote P, Richter A, Maisch B, Wichter T, Zollbrecht C, Grassl M, Schunkert H, Linsel-Nitschke P, Erdmann J, Baumert J, Illig T, Klopp N,Wichmann HE, Meisinger C, Koenig W, Lichtner P, Meitinger T, Schillert A, König IR, Hetzer R, Heid IM, Regitz-Zagrosek V, Hengstenberg C (2010) Genetic association study

identifies HSPB7 as a risk gene for idiopathic dilated cardiomyopathy. *PLoS Genetics* **6(10)**:e1001167.

Staron M, Yang Y, Liu B, Li J, Shen Y, Zúñiga-Pflücker JC, Aguila HL, Goldschneider I, Li Z (2010) gp96, an endoplasmic reticulum master chaperone for integrins and Toll-like receptors, selectively regulates early T and B lymphopoiesis. *Blood* **115(12)**:2380-2390.

Stein AB, Bolli R, Guo Y, Wang OL, Tan W, Wu WJ, Zhu X, Zhu Y, Xuan YT (2007) The late phase of ischemic preconditioning induces a prosurvival genetic program that results in marked attenuation of apoptosis. *Journal of Molecular and Cellular Cardiology* **42(6)**:1075-1085.

Stühlinger MC, Conci E, Haubner BJ, Stocker EM, Schwaighofer J, Cooke JP, Tsao PS, Pachinger O, Metzler B (2007) Asymmetric dimethyl L-arginine (ADMA) is a critical regulator of myocardial reperfusion injury. *Cardiovascular Research* **75(2)**:417-425.

Su X, Sykes JB, Ao L, Raeburn CD, Fullerton DA, Meng X (2010) Extracellular heat shock cognate protein 70 induces cardiac functional tolerance to endotoxin: differential effect on TNF-alpha and ICAM-1 levels in heart tissue. *Cytokine* **51(1)**:60-66.

Sui X, Li D, Qiu H, Gaussin V, Depre C (2009) Activation of the bone morphogenetic protein receptor by H11kinase/Hsp22 promotes cardiac cell growth and survival. *Circulation Research* **104(7)**:887-895.

Summers DW, Wolfe KJ, Ren HY, Cyr DM (2013) The Type II Hsp40 Sis1 cooperates with Hsp70 and the E3 ligase Ubr1 to promote degradation of terminally misfolded cytosolic protein. *PLoS One* **8(1)**:e52099.

Sun J, Chen X, Chen H, Ma Z, Zhou J (2015) Maternal Alcohol Consumption before and during Pregnancy and the Risks of Congenital Heart Defects in Offspring: A Systematic Review and Meta-analysis. *Congenital Heart Disease* **10(5)**:E216-224.

Sun WH, Liu F, Chen Y, Zhu YC (2012) Hydrogen sulfide decreases the levels of ROS by inhibiting mitochondrial complex IV and increasing SODactivities in cardiomyocytes under ischemia/reperfusion. *Biochemical and Biophysical Research Communications* **421(2)**:164-169.

Sun X, Fontaine JM, Rest JS, Shelden EA, Welsh MJ, Benndorf R (2004) Interaction of human HSP22 (HSPB8) with other small heat shock proteins. *The Journal of Biological Chemistry* **279(4)**:2394-2402.

Sun X, Welsh MJ, Benndorf R (2006) Conformational changes resulting from pseudophosphorylation on mammalian small heat shock proteins--a two-hybrid study. *Cell Stress & Chaperones* **11(1)**:61-70.

Taggart DP, Bakkenist CJ, Biddolph SC, Graham AK, McGee JO (1997) Induction of myocardial heat shock protein 70 during cardiac surgery. *The Journal of Pathology* **182(3)**:362-366.

Taipale M, Jarosz DF, Lindquist S (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature Reviews. Molecular Cell Biology* **11(7)**:515-528.

Takaki S, Shehabi Y, Pickering JW, Endre Z, Miyashita T, Goto T (2015) Perioperative change in creatinine following cardiac surgery with cardiopulmonary bypass is useful in predictingacute kidney injury: a single-centre retrospective cohort study. *Interactive Cardiovascular and Thoracic Surgery* **21(4)**:465-469.

Tang G, Wu L, Liang W, Wang R (2005) Direct stimulation of K(ATP) channels by exogenous and endogenous hydrogen sulfide in vascular smooth muscle cells. *Molecular Pharmacology* **68(6)**:1757-1764.

Tang S, Chen H, Cheng Y, Nasir MA, Kemper N, Bao E (2016) The interactive association between heat shock factor 1 and heat shock proteins in primary myocardial cells subjected to heat stress. *International Journal of Molecular* Medicine **37(1)**:56-62.

Tang WH, Tong W, Shrestha K, Wang Z, Levison BS, Delfraino B, Hu B, Troughton RW, Klein AL, Hazen SL (2008) Differential effects of arginine methylation on diastolic dysfunction and disease progression in patients with chronic systolic heart failure. *European Heart Journal* **29(20)**:2506-2513.

Tang XL, Qiu Y, Park SW, Sun JZ, Kalya A, Bolli R (1996) Time course of late preconditioning against myocardial stunning in conscious pigs. *Circulation Research* **79(3)**:424-434.

Tang YL, Tang Y, Zhang YC, Agarwal A, Kasahara H, Qian K, Shen L, Phillips MI (2005) A hypoxia-inducible vigilant vector system for activating therapeutic genes in ischemia. *Gene Therapy* **12(15)**:1163-1170.

Tang ZL, Dai W, Li YJ, Deng HW (1999) Involvement of capsaicin-sensitive sensory nerves in early and delayed cardioprotection induced by a brief ischaemia of the small intestine. *Naunyn-Schmiedeberg´s Archives of Pharmacology* **359(3)**:243-247.

Tani M, Neely JR (1989) Role of intracellular Na+ in Ca2+ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H+-Na+ and Na+-Ca2+ exchange. *Circulation Research* **65(4)**:1045-1056.

Tao J, Zhu W, Li Y, Xin P, Li J, Liu M, Li J, Redington AN, Wei M (2011) Apelin-13 protects the heart against ischemia-reperfusion injury through inhibition of ER-dependent apoptotic pathways in a time-dependent fashion. *American Journal of Physiology. Heart and Circulatory Physiology* **301(4)**:H1471-1486.

Tapuria N, Kumar Y, Habib MM, Abu Amara M, Seifalian AM and Davidson BR (2008) Remote ischemic preconditioning: a novel protective method from ischemia reperfusion injury - a review. *The Journal of Surgical Research* **150(2)**:304-330.

Tarkowska A, Furmaga-Jabłońska W (2012) The evaluation of diagnostic role of cardiac troponin T (cTnT) in newborns with heart defects. *Scientific World Journal* **2012**:682538.

Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, Kawamata Y, Fukusumi S, Hinuma S, Kitada C, Kurokawa T, Onda H, Fujino M (1998) Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochemical and Biophysical Research Communications* **251(2)**:471-476.

Taylor RP, Benjamin IJ (2005) Small heat shock proteins: a new classification scheme in mammals. *Journal of Molecular and Cellular Cardiology* **38(3)**:433-444.

Teng H, Wu B, Zhao K, Yang G, Wu L, Wang R (2013) Oxygen-sensitive mitochondrial accumulation of cystathionine β-synthase mediated by Lon protease. *Proceedings of the National Academy of Sciences of the United States of America* **110(31)**:12679-12684.

Tenhunen R, Marver HS, Schmid R (1968) The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proceedings of the National Academy of Sciences of the United States of America* **61(2)**:748-755.

Tennant PW, Pearce MS, Bythell M and Rankin J (2010) 20-year survival of children born with congenital anomalies: a population-based study. *Lancet* **375(9715)**:649-656.

Terui K, Haga S, Enosawa S, Ohnuma N, Ozaki M (2004) Hypoxia/re-oxygenation-induced, redox-dependent activation of STAT1 (signal transducer and activator of transcription 1) confers resistance to apoptotic cell death via hsp70 induction. *The Biochemical Journal* **380(Pt 1)**:203-209.

Testori A, Richards J, Whitman E, Mann GB, Lutzky J, Camacho L, Parmiani G, Tosti G, Kirkwood JM, Hoos A, Yuh L, Gupta R, Srivastava PK; C-100-21 Study Group (2008) Phase III comparison of vitespen, an autologous tumor- derived heat shock protein gp96 peptide complex vaccine, with physician's choice of treatment for stage IV melanoma: the C-100-21 Study Group. *Journal of Clinical Oncology* **26(6)**:955-62.

Than A, Tee WT, Chen P (2012) Apelin secretion and expression of apelin receptors in 3T3-L1 adipocytes are differentially regulated by angiotensin type 1 and type 2 receptors. *Molecular and Cellular Endocrinology* **351(2)**:296-305.

The National Institute for Cardiovascular Outcomes Research (NICOR) (2014) National Aggregate - 2014 to 15 - Summary Data. Surgical procedure data. https://nicor4.nicor.org.uk/CHD/an_paeds.nsf/0/4E70457601F7348F80257F8E0052CBF8?OpenDocument?Summary

Royal Hospital For Sick Children - 2014 to 15 - Summary Data. Surgical procedure data. https://nicor4.nicor.org.uk/CHD/an_paeds.nsf/0/8A725D44BFFE9A4F80257F8E0051F0C0?OpenDocument?Summary

Last accessed 27/04/2016

Thielmann M, Kottenberg E, Boengler K, Raffelsieper C, Neuhaeuser M, Peters J, Jakob H, Heusch G (2010) Remote ischemic preconditioning reduces myocardial injury after coronary artery bypass surgery withcrystalloid cardioplegic arrest. *Basic Research in Cardiology* **105(5)**:657-664.

Thielmann M, Kottenberg E, Kleinbongard P, Wendt D, Gedik N, Pasa S, Price V, Tsagakis K, Neuhäuser M, Peters J, Jakob H, Heusch G (2013) Cardioprotective and prognostic effects of remote ischaemic preconditioning in patients undergoing coronary artery bypass surgery: a single-centre randomised, double-blind, controlled trial. *Lancet* **382(9892)**:597-604.

Thornton J, Striplin S, Liu GS, Swafford A, Stanley AW, Van Winkle DM, Downey JM (1990) Inhibition of protein synthesis does not block myocardial protection afforded by preconditioning. *The American Journal of Physiology* **259(6 Pt 2)**:H1822-1825.

Tian J, Guo X, Liu XM, Liu L, Weng QF, Dong SJ, Knowlton AA, Yuan WJ, Lin L (2013) Extracellular HSP60 induces inflammation through activating and up-regulating TLRs in cardiomyocytes. *Cardiovascular Research* **98(3)**:391-401.

Tie HT, Luo MZ, Li ZH, Wang Q, Wu QC, Li Q, Zhang M (2015) Remote Ischemic Preconditioning Fails to Benefit Pediatric Patients Undergoing Congenital Cardiac Surgery: A Meta-Analysis of Randomised Controlled Trials. *Medicine* **94(43)**:e1895.

Tie HT, Luo MZ2, Li ZH3, Wang Q3, Wu QC (2014) Remote ischemic preconditioning for pediatric patients undergoing congenital cardiac surgery: a meta-analysis. *International Journal of Cardiology* **177(2)**:551-553.

Tilg H, Dinarello CA, Mier JW (1997) IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunology Today* **18(9)**:428-432.

Tissières A, Mitchell HK, Tracy UM (1974) Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. *Journal of Molecular Biology* **84(3)**:389-398.

Tornatore L, Thotakura AK, Bennett J, Moretti M, Franzoso G (2012) The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. *Trends in Cell Biology* **22(11)**:557-566.

Tornatore L, Thotakura AK, Bennett J, Moretti M, Franzoso G (2012) The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. *Trends in Cell Biology* **22(11)**:557-566.

Townsend N, Bhatnagar P, Wickramasinghe K, Williams J, Vujcich D, Rayner M (2013). Children and young people statistics 2013. British Heart Foundation: London.

Tranter M, Ren X, Forde T, Wilhide ME, Chen J, Sartor MA, Medvedovic M, Jones WK (2010) NF- kappaB driven cardioprotective gene programs; Hsp70.3 and cardioprotection after late ischemic preconditioning. *Journal of Molecular and Cellular Cardiology* **49(4)**:664-672.

Tsan MF, Gao B (2009) Heat shock proteins and immune system. *Journal of Leukocyte Biology* **85(6)**:905-910.

Tsao PS, Aoki N, Lefer DJ, Johnson G 3rd, Lefer AM (1990) Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. *Circulation* **82(4)**:1402-1412.

Turer AT, Hill JA (2010) Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. *The American Journal of Cardiology* **106(3)**:360-368.

Vakeva AP, Agah A, Rollins SA, Matis LA, Li L, Stahl GL (1998) Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy. *Circulation* **97(22)**:2259-2267.

Valentim L, Laurence KM, Townsend PA, Carroll CJ, Soond S, Scarabelli TM, Knight RA, Latchman DS, Stephanou A (2006) Urocortin inhibits Beclin1-mediated autophagic cell death in cardiac myocytes exposed to ischaemia/reperfusioninjury. *Journal of Molecular and Cellular Cardiology* **40(6)**:846-852.

Vallance P, Leone A, Calver A, Collier J, Moncada S (1992) Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* **339(8793)**:572-575.

Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM (1996) Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science* **274(5288)**:787-789.

Van der Heiden K, Cuhlmann S, Luong le A, Zakkar M, Evans PC (2010) Role of nuclear factor kappa B in cardiovascular health and disease. *Clinical Science* **118(10)**:593-605.

van der Linde D, Konings EE, Slager MA, Witsenburg M, Helbing WA, Takkenberg JJ, Roos-Hesselink JW (2011) Birth prevalence of congenital heart disease worldwide: a systematic review and meta-analysis. *Journal of the American College of Cardiology* **58(21)**:2241-2247.

van Roon J, van Eden W, van Roy J, Lafeber F, Bijlsma J (1997) Stimulation of Suppressive T Cell Responses by Human but not Bacterial 60-kD Heat-shock Protein in Synovial Fluid of Patients with Rheumatoid Arthritis *FASEB* **10**:A1312.

VanBenthuysen KM, McMurtry IF, Horwitz LD (1987) Reperfusion after acute coronary occlusion in dogs impairs endothelium-dependent relaxation to acetylcholine and augments contractile reactivity in vitro. *The Journal of Clinical Investigation* **79(1)**:265-274.

Varano Della Vergiliana JF, Lansley SM, Porcel JM, Bielsa S, Brown JS, Creaney J, Temple SE, Waterer GW, Lee YC (2013) Bacterial infection elicits heat shock protein 72 release from pleural mesothelial cells. *PLoS One* **8(5)**:e63873.

Vassalos A, Peng E, Young D, Walker S, Pollock J, Macarthur K, Lyall F, Danton MH (2011) Pre-operative sildenafil and pulmonary endothelial-related complications following cardiopulmonary bypass: a randomised trial in children undergoing cardiac surgery. *Anaesthesia* **66(6)**:472-480.

Vassalos A, Young D, MacArthur K, Pollock J, Lyall F, Danton MH (2011) Cystatin C: influence of perfusion and myocardial injury on early (<24 h) renal function after pediatric cardiac surgery. *Paediatric Anaesthesia* **21(12)**:1185-1191.

Vassalos A, Lilley S, Young D, Peng E, MacArthur K, Pollock J, Lyall F, Danton MH (2009) Tissue Doppler imaging following paediatric cardiac surgery: early patterns of change and relationship to outcome. *Interactive Cardiovascular and Thoracic Surgery* **9(2)**:173-177.

Veinot JP, Gattinger DA, Fliss H (1997) Early apoptosis in human myocardial infarcts. *Human Pathology* **28(4)**:485-492.

Ventura-Clapier R, Veksler V (1994) Myocardial ischemic contracture. Metabolites affect rigor tension development and stiffness. *Circulation Research* **74(5)**:920-929.

Venugopal V, Hausenloy DJ, Ludman A, Di Salvo C, Kolvekar S, Yap J, Lawrence D, Bognolo J, Yellon DM (2009) Remote ischaemic preconditioning reduces myocardial injury in patients undergoing cardiac surgery with cold-blood cardioplegia: a randomised controlled trial. *Heart* **95(19)**:1567-1571.

Verrier ED, Shernan SK, Taylor KM, Van de Werf F, Newman MF, Chen JC, Carrier M, Haverich A, Malloy KJ, Adams PX, Todaro TG, Mojcik CF, Rollins SA,Levy JH; PRIMO-CABG Investigators (2004) Terminal complement blockade with pexelizumab during coronary artery bypass graft surgery requiring cardiopulmonary bypass: a randomised trial. *JAMA* **291(19)**:2319-2327.

Viaro F, Baldo CF, Capellini VK, Celotto AC, Bassetto S, Rodrigues AJ, Evora PR (2008) Plasma nitrate/nitrite (NOx) is not a useful biomarker to predict inherent cardiopulmonary bypass inflammatory response. *Journal of Cardiac Surgery* **23(4)**:336-338.

Videm V, Albrigtsen M (2008) Soluble ICAM-1 and VCAM-1 as markers of endothelial activation. *Scandinavian Journal of Immunology* **67(5)**:523-531.

Vihervaara A, Sistonen L (2014) HSF1 at a glance. *Journal of Cell Science* **127(Pt 2)**:261-266.

Villard E, Perret C, Gary F, Proust C, Dilanian G, Hengstenberg C, Ruppert V, Arbustini E, Wichter T, Germain M, Dubourg O, Tavazzi L, Aumont MC, DeGroote P, Fauchier L, Trochu JN, Gibelin P, Aupetit JF, Stark K, Erdmann J, Hetzer R, Roberts AM, Barton PJ, Regitz-Zagrosek V; Cardiogenics Consortium, Aslam U,Duboscq-Bidot L, Meyborg M, Maisch B, Madeira H, Waldenström A, Galve E, Cleland JG, Dorent R, Roizes G, Zeller T, Blankenberg S, Goodall AH, Cook S,Tregouet DA, Tiret L, Isnard R, Komajda M, Charron P, Cambien F (2011) A genome-wide association study identifies two loci associated with heart failure due to dilated cardiomyopathy. *European Heart Journal* **32(9)**:1065-1076.

Voelkel T, Andresen C, Unger A, Just S, Rottbauer W, Linke WA (2013) Lysine methyltransferase Smyd2 regulates Hsp90-mediated protection of the sarcomeric titin springs andcardiac function. *Biochimica et Biophysica Acta* **1833(4)**:812-822.

Vos MJ, Kanon B, Kampinga HH (2009) HSPB7 is a SC35 speckle resident small heat shock protein. *Biochimica et Biophysica Acta* **1793(8)**:1343-1353.

Vos MJ, Zijlstra MP, Kanon B, van Waarde-Verhagen MA, Brunt ER, Oosterveld-Hut HM, Carra S, Sibon OC, Kampinga HH (2010) HSPB7 is the most potent polyQ aggregation suppressor within the HSPB family of molecular chaperones. *Human Molecular Genetics* **19(23)**:4677-4693.

Vulapalli SR, Chen Z, Chua BH, Wang T, Liang CS (2002) Cardioselective overexpression of HO-1 prevents I/R-induced cardiac dysfunction and apoptosis. *American Journal of Physiology. Heart and Circulatory Physiology* **283(2)**:H688-694.

Wagner R, Piler P, Bedanova H, Adamek P, Grodecka L, Freiberger T (2010) Myocardial injury is decreased by late remote ischaemic preconditioning and aggravated by tramadol in patients undergoing cardiac surgery: a randomised controlled trial. *Interactive Cardiovascular and Thoracic Surgery* **11(6)**:758-762.

Walker S (2013) Molecular mechanisms initiated within cyanotic and acyanotic infant myocardium during cardio-pulmonary bypass *in vivo* and ischemic-reperfusion injury in vitro. Unpublished PhD thesis. University of Glasgow.

Walker S, Danton MH, Lang AD, Lyall F (2014) Apelin receptor (APJ) expression during cardiopulmonary bypass in children undergoing surgical repair. *PLoS One* **9(9)**:e106262.

Walport MJ (2001) Complement. First of two parts. *The New England Journal of Medicine* **344(14)**:1058-1066.

Wan S, Izzat MB, Lee TW, Wan IY, Tang NL, Yim AP (1999) Avoiding cardiopulmonary bypass in multivessel CABG reduces cytokine response and myocardial injury. *The Annals of Thoracic Surgery* **68(1)**:52-57.

Wan S, LeClerc JL, Vincent JL (1997) Cytokine responses to cardiopulmonary bypass: lessons learned from cardiac transplantation. *The Annals of Thoracic Surgery* **63(1)**:269-276.

Wang E, Feng Y, Zhang M, Zou L, Li Y, Buys ES, Huang P, Brouckaert P, Chao W (2011) Toll-like receptor 4 signaling confers cardiac protection against ischemic injury via inducible nitric oxide synthase- and soluble guanylate cyclase-dependent mechanisms. *Anesthesiology* **114(3)**:603-613.

Wang E, Feng Y, Zhang M, Zou L, Li Y, Buys ES, Huang P, Brouckaert P, Chao W (2011) Toll- like receptor 4 signaling confers cardiac protection against ischemic injury via inducible nitric oxidesynthase- and soluble guanylate cyclase-dependent mechanisms. *Anesthesiology* **114(3)**:603-613.

Wang P, Chen H, Qin H, Sankarapandi S, Becher MW, Wong PC, Zweier JL (1998) Overexpression of human copper, zinc-superoxide dismutase (SOD1) prevents postischemic injury. *Proceedings of the National Academy of Sciences of the United States of America* **95(8)**:4556-4560.

Wang P, Isaak CK, Siow YL, O K (2014) Downregulation of cystathionine β-synthase and cystathionine γ-liase expression stimulates inflammation inkidney ischemia-reperfusion injury. *Physiological Reports* **2**(12).

Wang P, Wu P, Siegel MI, Egan RW, Billah MM (1995) Interleukin (IL)-10 inhibits nucleaf factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *The Journal of Biological Chemistry* **270(16)**:9558-9563.

Wang Y, Xu H, Mizoguchi K, Oe M, Maeta H (2001) Intestinal ischemia induces late preconditioning against myocardial infarction: a role for inducible nitric oxide synthase. *Cardiovascular Research* **49(2)**:391-398.

Warren OJ, Smith AJ, Alexiou C, Rogers PL, Jawad N, Vincent C, Darzi AW, Athanasiou T (2009) The inflammatory response to cardiopulmonary bypass: part 1--mechanisms of pathogenesis. *Journal of Cardiothoracic and Vascular Anesthesia* **23(2)**:223-31.

Webb GD, Lim LH, Oh VM, Yeo SB, Cheong YP, Ali MY, El Oakley R, Lee CN, Wong PS, Caleb MG, Salto-Tellez M, Bhatia M, Chan ES, Taylor EA, Moore PK (2008) Contractile and vasorelaxant effects of hydrogen sulfide and its biosynthesis in the human internal mammary artery. *The Journal of Pharmacology and Experimental Therapeutics* **324(2)**:876-882.

Weeks SD, Baranova EV1, Heirbaut M1, Beelen S1, Shkumatov AV1, Gusev NB2, Strelkov SV3 (2014) Molecular structure and dynamics of the dimeric human small heat shock protein HSPB6. *Journal of Structural Biology* **185(3)**:342-54.

Weinbrenner C, Nelles M, Herzog N, Sárváry L, Strasser RH (2002) Remote preconditioning by infrarenal occlusion of the aorta protects the heart from infarction: a newly identified non-neuronal but PKC-dependent pathway. *Cardiovascular Research* **55(3)**:590-601.

Weinhaus AJ, Roberts KP (2009) Anatomy of the human heart, In: Handbook of cardiac anatomy, physiology, and devices (ed Iaizzo PA), Springer, New York, pp: 59-74.

Wessels MW1, Willems PJ (2010) Genetic factors in non-syndromic congenital heart malformations. *Clinical Genetics* **78(2)**:103-123.

Wharton J, Strange JW, Møller GM, Growcott EJ, Ren X, Franklyn AP, Phillips SC, Wilkins MR (2005) Antiproliferative effects of phosphodiesterase type 5 inhibition in human pulmonary artery cells. *American Journal of Respiratory and Critical Care Medicine* **172(1)**:105-113.

Whiting D, Yuki K, DiNardo JA (2015) Cardiopulmonary bypass in the pediatric population. *Best Practice & Research. Clinical Anaesthesiology* **29(2)**:241-256.

Wild D (2013) Immunoassay for Begginners, In The Immunoassay Handbook. Editor David Wild, 4th Ed, Elsevier, Amsterdam, pp 8.

Wilhide ME, Tranter M, Ren X, Chen J, Sartor MA, Medvedovic M, Jones WK (2011) Identification of a NF-κB cardioprotective gene program: NF-κB regulation of Hsp70.1 contributes to cardioprotection after permanent coronary occlusion. *Journal of Molecular and Cellular Cardiology* **51(1)**:82-89.

Willeit P, Freitag DF, Laukkanen JA, Chowdhury S, Gobin R, Mayr M, Di Angelantonio E, Chowdhury R (2015) Asymmetric dimethylarginine and cardiovascular risk: systematic review and meta-analysis of 22 prospective studies. *Journal of the American Heart Association* **4(6)**:e001833.

Willmund F, del Alamo M, Pechmann S, Chen T, Albanèse V, Dammer EB, Peng J, Frydman J (2013) The cotranslational function of ribosome-associated Hsp70 in eukaryotic protein homeostasis. *Cell* **152(1-2)**:196-209.

Wolfrum S, Schneider K, Heidbreder M, Nienstedt J, Dominiak P, Dendorfer A (2002) Remote preconditioning protects the heart by activating myocardial PKCepsilon-isoform. *Cardiovascular Research* **55(3)**:583-589.

Wu K, Xu W, You Q, Guo R, Feng J, Zhang C, Wu W (2012) Increased expression of heat shock protein 90 under chemical hypoxic conditions protects cardiomyocytes against injury induced by serum and glucose deprivation. *International Journal of Molecular Medicine* **30(5)**:1138-1144.

Wu Q, Gui P, Wu J, Ding D, Purusram G, Dong N, Yao S (2011) Effect of limb ischemic preconditioning on myocardial injury in patients undergoing mitral valve replacement surgery. -A randomised controlled trial-. *Circulation Journal* **75(8)**:1885-1889.

Wu S, Hong F, Gewirth D, Guo B, Liu B, Li Z (2012) The molecular chaperone gp96/GRP94 interacts with Toll-like receptors and integrins via its C-terminal hydrophobic domain. *The Journal of Biological Chemistry* **287(9)**:6735-6742

Wu YN, Yu H, Zhu XH, Yuan HJ, Kang Y, Jiao JJ, Gao WZ, Liu YX, Lou JS (2011) Noninvasive delayed limb ischemic preconditioning attenuates myocardial ischemia-reperfusion injury reperfusion injury in rats by a mitochondrial K(ATP) channel-dependent mechanism. *Physiological Research* **60(2)**:271-279.

Wypij D, Newburger JW, Rappaport LA, duPlessis AJ, Jonas RA, Wernovsky G, Lin M, Bellinger DC (2003) The effect of duration of deep hypothermic circulatory arrest in infant heart surgery on late neurodevelopment: the Boston Circulatory Arrest Trial. *The Journal of Thoracic and Cardiovascular Surgery* **126(5)**:1397-1403.

Xiao L, Lu R, Hu CP, Deng HW, Li YJ (2001) Delayed cardioprotection by intestinal preconditioning is mediated by calcitonin gene-related peptide. *European Journal of Pharmacology* **427(2)**:131-135.

Xie JJ, Liao XL, Chen WG, Huang DD, Chang FJ, Chen W, Luo ZL, Wang ZP, Ou JS (2012) Remote ischaemic preconditioning reduces myocardial injury in patients undergoing heart valve surgery:randomised controlled trial. *Heart* **98(5)**:384-388.

Xing Y, Tang B, Zhu C, Li W, Li Z, Zhao J, Gong WD, Wu ZQ, Zhu CC, Zhang YQ (2016) N-myc downstream-regulated gene 4, up-regulated by tumor necrosis factor-α and nuclear factor kappa B, aggravates cardiac ischemia/reperfusion injury by inhibiting reperfusion injury salvage kinase pathway. *Basic Research in Cardiology* **111**(2):11.

Xu M, Wang Y, Ayub A, Ashraf M (2001) Mitochondrial K(ATP) channel activation reduces anoxic injury by restoring mitochondrial membrane potential. American Journal of Physiology. *Heart and Circulatory Physiology* **281(3)**:H1295-303.

Xu Z, Prathapasinghe G, Wu N, Hwang SY, Siow YL, O K (2009) Ischemia-reperfusion reduces cystathionine-beta-synthase-mediated hydrogen sulfide generation in the kidney. *American Journal of Physiology. Renal Physiology* **297(1)**:F27-35.

Xuan YT, Guo Y, Zhu Y, Han H, Langenbach R, Dawn B, Bolli R (2003) Mechanism of cyclooxygenase-2 upregulation in late preconditioning. *Journal of Molecular and Cellular Cardiology* **35(5)**:525-537.

Yamagata K, Tagawa C, Matsufuji H, Chino M (2012) Dietary apigenin regulates high glucose and hypoxic reoxygenation-induced reductions in apelin expression in human endothelial cells. *The Journal of Nutritional Biochemistry* **23(8)**:929-936.

Yan L, Vatner DE, Kim SJ, Ge H, Masurekar M, Massover WH, Yang G, Matsui Y, Sadoshima J, Vatner SF (2005) Autophagy in chronically ischemic myocardium. *Proceedings of the National Academy of Sciences of the United States of America* **102(39)**:13807-13812.

Yan X, Qiu W, Jia B, Zhong H, Li X, Chen Z (2013) Myocardial protection by interferon-γ late preconditioning during cardiopulmonary bypass-associated myocardial ischemia-reperfusion in pigs. *Oncology Reports* **30(5)**:2145-2152.

Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE Jr, Drazner MH, Fonarow GC, Geraci SA, Horwich T, Januzzi JL,Johnson MR, Kasper EK, Levy WC, Masoudi FA, McBride PE, McMurray JJ, Mitchell JE, Peterson PN, Riegel B, Sam F, Stevenson

LW, Tang WH, Tsai EJ,Wilkoff BL; American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines (2013) 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation* **128(16)**:e240-327.

Yang C, Talukder MA, Varadharaj S, Velayutham M, Zweier JL. (2013) Early ischaemic preconditioning requires Akt- and PKA-mediated activation of eNOS via serine1176 phosphorylation. *Cardiovascular Research* **97(1)**:33-43.

Yang G, Wu L, Wang R (2006) Pro-apoptotic effect of endogenous H2S on human aorta smooth muscle cells. *FASEB Journal* **20(3)**:553-555.

Yang J, Li Y, Hu C (2011) Ischemic preconditioning protects against myocardial ischemia-reperfusion injury through inhibiting toll-likereceptor 4/NF-κB signaling pathway in rats. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* **36(10)**:972-978.

Yang L, Wang G, Du Y, Ji B, Zheng Z (2014) Remote ischemic preconditioning reduces cardiac troponin I release in cardiac surgery: a meta-analysis. Journal of Cardiothoracic and Vascular Anesthesia **28(3)**:682-689.

Yang S, Li H, Tang L, Ge G1, Ma J, Qiao Z, Liu H, Fang W (2015) Apelin-13 protects the heart against ischemia-reperfusion injury through the RISK-GSK-3β-mPTP pathway. *Archives of Medical Science* **11(5)**:1065-1073.

Yang Y, Lang XB, Zhang P, Lv R, Wang YF, Chen JH (2014) Remote ischemic preconditioning for prevention of acute kidney injury: a meta-analysis of randomised controlled trials. *American Journal of Kidney Diseases* **64(4)**:574-583.

Yang Z, Yang C, Xiao L, Liao X, Lan A, Wang X, Guo R, Chen P, Hu C, Feng J (2011) Novel insights into the role of HSP90 in cytoprotection of H2S against chemical hypoxia-induced injury in H9c2cardiac myocytes. *International Journal of Molecular Medicine* **28(3)**:397-403.

Yang Z, Zingarelli B, Szabó C (2000) Crucial role of endogenous interleukin-10 production in myocardial ischemia/reperfusion injury. *Circulation* **101(9)**:1019-1026.

Yellon DM, Downey JM (2003) Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiological Reviews* **83(4)**:1113-1151.

Yellon DM, Alkhulaifi AM and Pugsley WB (1993) Preconditioning the human myocardium. *Lancet* **342(8866)**:276-277.

Yet SF, Tian R, Layne MD, Wang ZY, Maemura K, Solovyeva M, Ith B, Melo LG, Zhang L, Ingwall JS, Dzau VJ, Lee ME, Perrella MA (2001) Cardiac-specific expression of heme oxygenase-1 protects against ischemia and reperfusion injury in transgenic mice. *Circulation Research* **89(2)**:168-173.

Yıldırım A, Karaağaç AT, Güzelmeriç F, Çine N, Öner NC (2013) Importance of adhesion molecules for children with congenital heart disease. *Cardiology in the Young* **23(1)**:35-40.

Yoshida T, Maulik N, Ho YS, Alam J, Das DK (2001) H(mox-1) constitutes an adaptive response to effect antioxidant cardioprotection: A study with transgenic mice heterozygous for targeted disruption of the Heme oxygenase-1 gene. *Circulation* **103(12)**:1695-1701.

Young PJ, Dalley P, Garden A, Horrocks C, La Flamme A, Mahon B, Miller J, Pilcher J, Weatherall M, Williams J, Young W, Beasley R (2012) A pilot study investigating the effects of remote ischemic preconditioning in high-risk cardiac surgery using a

randomised controlled double-blind protocol. *Basic Research in Cardiology* **107(3)**:256.

Yu M, Wen N, Wenzhong Z, Yuanchang X, Xiaoming D, Yongjin L (2010) Effect of repeated ischaemic preconditioning on TLR4 and proinflammatory cytokines TNF-α and IL-1β in myocardial ischaemia-reperfusion injury in a rat model. *Archives of Medical Science: AMS* **6(6)**:843-847.

Yu XH, Cui LB, Wu K, Zheng XL, Cayabyab FS, Chen ZW, Tang CK (2014) Hydrogen sulfide as a potent cardiovascular protective agent. *Clinical Chimica Acta* **437**:78-87.

Yu XH, Tang ZB, Liu LJ, Qian H, Tang SL, Zhang DW, Tian GP, Tang CK (2014) Apelin and its receptor APJ in cardiovascular diseases. *Clinica Chimica Acta: International Journal of Clinical Chemistry* **428**:1-8.

Zakkar M, Guida G, Suleiman MS, Angelini GD (2015) Cardiopulmonary bypass and oxidative stress. *Oxidative Medicine and Cellular Longevity* **2015:**189863.

Zappitelli M, Greenberg JH, Coca SG, Krawczeski CD, Li S, Thiessen-Philbrook HR, Bennett MR, Devarajan P, Parikh CR; Translational Research Investigating Biomarker Endpoints in Acute Kidney Injury (TRIBE-AKI) Consortium (2015) Association of definition of acute kidney injury by cystatin C rise with biomarkers and clinical outcomes in children undergoing cardiac surgery. *JAMA Pediatrics* **169(6)**:583-591.

Zarbock A, Schmidt C, Van Aken H, Wempe C, Martens S, Zahn PK, Wolf B, Goebel U, Schwer CI, Rosenberger P, Haeberle H, Görlich D, Kellum JA, Meersch M; Renal RIPC Investigators (2015) Effect of remote ischemic ischemic preconditioning on kidney injury among high-risk patients undergoing cardiac surgery: a randomised clinical trial. *JAMA* **313(21)**:2133-2141.

Zeller M, Korandji C, Guilland JC, Sicard P, Vergely C, Lorgis L, Beer JC, Duvillard L, Lagrost AC, Moreau D, Gambert P, Cottin Y, Rochette L (2008) Impact of asymmetric dimethylarginine on mortality after acute myocardial infarction. *Arteriosclerosis, Thrombosis and Vascular Biology* **28(5)**:954-960.

Zeng J, He W, Qu Z, Tang Y, Zhou Q, Zhang B (2014) Cold blood versus crystalloid cardioplegia for myocardial protection in adult cardiac surgery: a meta-analysis of randomised controlled studies. *Journal of Cardiothoracic and Vascular Anesthesia* **28(3)**:674-681.

Zeng XJ, Zhang LK, Wang HX, Lu LQ, Ma LQ, Tang CS (2009) Apelin protects heart against ischemia/reperfusion injury in rat. *Peptides* **30(6)**:1144-1152.

Zeymer U, Suryapranata H, Monassier JP, Opolski G, Davies J, Rasmanis G, Linssen G, Tebbe U, Schröder R, Tiemann R, Machnig T, Neuhaus KL; ESCAMI Investigators (2001) The Na(+)/H(+) exchange inhibitor eniporide as an adjunct to early reperfusion therapy for acute myocardialinfarction. Results of the evaluation of the safety and cardioprotective effects of eniporide in acute myocardial infarction (ESCAMI) trial. *Journal of the American College of Cardiology* **38(6)**:1644-1650.

Zeynalov E, Shah ZA, Li RC, Doré S (2009) Heme oxygenase 1 is associated with ischemic preconditioning-induced protection against brain ischemia. *Neurobiology of Disease* **35(2)**:264-269.

Zhai Y, Ao L, Cleveland JC, Zeng Q, Reece TB, Fullerton DA, Meng X (2015) Toll-like receptor 4 mediates the inflammatory responses and matrix protein remodeling in remote non-ischemicmyocardium in a mouse model of myocardial ischemia and reperfusion. *PLoS One* **10(3)**:e0121853.

Zhang K, Ezemaduka AN, Wang Z, Hu H, Shi X, Liu C, Lu X, Fu X, Chang Z, Yin CC (2015) A novel mechanism for heat shock proteins to function as molecular chaperones. *Nature Scientific Reports* **5**:8811.

Zhang SZ, Wang NF, Xu J, Gao Q, Lin GH, Bruce IC, Xia Q (2006) Kappa-opioid receptors mediate cardioprotection by remote preconditioning. *Anesthesiology* **105(3)**:550-556.

Zhang X, Wang X, Zhu H, Kranias EG, Tang Y, Peng T, Chang J, Fan GC (2012) Hsp20 functions as a novel cardiokine in promoting angiogenesis via activation of VEGFR2. *PLoS One* **7(3)**:e32765.

Zhang XQ, Tang R, Li L, Szucsik A, Javan H, Saegusa N, Spitzer KW, Selzman CH (2013) Cardiomyocyte-specific p65 NF-κB deletion protects the injured heart by preservation of calcium handling. *American Journal of Physiology. Heart and Circulatory Physiology* **305(7)**:H1089-1097.

Zhang XQ, Tang R, Li L, Szucsik A, Javan H, Saegusa N, Spitzer KW, Selzman CH (2013) Cardiomyocyte-specific p65 NF-κB deletion protects the injured heart by preservation of calcium handling. *American Journal of Physiology. Heart and Circulatory Physiology* **305(7)**:H1089-1097.

Zhang Z, Huang H, Liu P, Tang C, Wang J (2007) Hydrogen sulfide contributes to cardioprotection during ischemia-reperfusion injury by opening K ATP channels. *Canadian Journal of Physiology and Pharmacology* **85(12)**:1248-1253.

Zhao P, Wang J, He L, Ma H, Zhang X, Zhu X, Dolence EK, Ren J, Li J (2009) Deficiency in TLR4 signal transduction ameliorates cardiac injury and cardiomyocyte contractile dysfunction during ischemia. *Journal of Cellular and Molecular Medicine* **13(8A)**:1513-1525.

Zhao S, Lin Q, Li H, He Y, Fang X, Chen F, Chen C, Huang Z (2014) Carbon monoxide releasing molecule-2 attenuated ischemia/reperfusion-induced apoptosis in cardiomyocytes via a mitochondrial pathway. *Molecular Medicine Reports* **9(2)**:754-762.

Zhao W, Zhang J, Lu Y, Wang R (2001) The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. *The EMBO Journal* **20(21)**:6008-60016.

Zhao X, Eghbali-Webb M (2002) Gender-related differences in basal and hypoxia-induced activation of signal transduction pathways controlling cell cycle progression and apoptosis, in cardiac fibroblasts. *Endocrine* **18(2)**:137-145.

Zhao X, Eghbali-Webb M (2002) Gender-related differences in basal and hypoxia-induced activation of signal transduction pathways controlling cell cycle progression and apoptosis, in cardiac fibroblasts. *Endocrine* **18(2)**:137-145.

Zhao Y, Vanhoutte PM, Leung SW (2015) Vascular nitric oxide: Beyond eNOS. *Journal of Pharmacological Sciences* **129(2)**:83-94.

Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J (2003) Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *American Journal of Physiology. Heart and Circulatory Physiology* **285(2):**H579-88.

Zhao ZQ, Morris CD, Budde JM, Wang NP, Muraki S, Sun HY, Guyton RA (2003) Inhibition of myocardial apoptosis reduces infarct size and improves regional contractile dysfunction duringreperfusion. *Cardiovascular Research* **59(1)**:132-142.

Zhao ZZ, Wang Z, Li GH, Wang R, Tan JM, Cao X, Suo R, Jiang ZS (2011) Hydrogen sulfide inhibits macrophage-derived foam cell formation. *Experimental Biology and Medicine (Maywood)* **236(2)**:169-176.

Zhou C, Liu Y, Yao Y, Zhou S, Fang N, Wang W, Li L (2013) β-blockers and volatile anesthetics may attenuate cardioprotection by remote preconditioning in adult cardiac surgery: a meta-analysis of 15 randomised trials. *Journal of Cardiothoracic and Vascular Anesthesia* **27(2)**:305-311.

Zhou T, Chuang CC, Zuo L (2015) Molecular Characterization of Reactive Oxygen Species in Myocardial Ischemia-Reperfusion Injury. *BioMed Research International* **2015**:864946.

Zhou W, Zeng D, Chen R, Liu J, Yang G, Liu P and Zhou X (2010) Limb ischemic preconditioning reduces heart and lung injury after an open heart operation in infants. *Pediatric Cardiology* **31(1)**:22-29.

Zhou X, Sheng X, Chen M3, Wang Z, Yu L, Jiang H (2015) Tumor necrosis factor-α inhibitor: A promising therapeutic approach for attenuating myocardial ischemia-reperfusion by antioxidant stress. *International Journal of Cardiology* **190**:282-283.

Zhu JC, Shao JL, Ma H, Wang JK (2008) Interaction between endogenous cystathionine synthase/hydrogen sulfide and heme oxygenase-1/carbon monoxide systems during myocardial ischemic-reperfusion: experiment with rats. *Zhonghua Yi Xue Za Zhi* **88(45)**:3222-3225.

Zhu J, Yin R, Wu H, Yi J, Luo L, Dong G, Jing H (2006) Cystatin C as a reliable marker of renal function following heart valve replacement surgery with cardiopulmonary bypass. *Clinica Chimica Acta* **374(1-2)**:116-121.

Zhu X, Liu B, Zhou S, Chen YR, Deng Y, Zweier JL, He G (2007) Ischemic preconditioning prevents *in vivo* hyperoxygenation in postischemic myocardium with preservation of mitochondrial oxygen consumption. *American Journal of Physiiology. Heart and Circulatory Physiology* **293(3)**:H1442-1450.

Zhuravleva A, Gierasch LM (2015) Substrate-binding domain conformational dynamics mediate Hsp70 allostery. *Proceedings of the National Academy of Sciences of the United States of America* **112(22)**:E2865-873.

Zimmerman RF, Ezeanuna PU, Kane JC, Cleland CD, Kempananjappa TJ, Lucas FL, Kramer RS (2011) Ischemic preconditioning at a remote site prevents acute kidney injury in patients following cardiac surgery. *Kidney International* **80(8)**:861-867.

Zitta K, Meybohm P, Bein B, Gruenewald M, Lauer F, Steinfath M, Cremer J, Zacharowski K, Albrecht M (2014) Activities of cardiac tissue matrix metalloproteinases 2 and 9 are reduced by remote ischemic preconditioning in cardiosurgical patients with cardiopulmonary bypass. *Journal of Translational Medicine* **12**:94.

Zonneveld-Huijssoon E, van Wijk F, Roord S, Delemarre E, Meerding J, de Jager W, Klein M, Raz E, Albani S, Kuis W, Boes M, Prakken BJ (2012) TLR9 agonist CpG enhances protective nasal HSP60 peptide vaccine efficacy in experimental autoimmune arthritis. *Annals of the Rheumatic Diseases* **71(10)**:1706-1715.

Zou N, Ao L, Cleveland JC Jr, Yang X, Su X, Cai GY, Banerjee A, Fullerton DA, Meng X (2008) Critical role of extracellular heat shock cognate protein 70 in the myocardial inflammatory response and cardiac dysfunction after global ischemia-reperfusion. *American Journal of Physiology. Heart and Circulatory Physiology* **294(6)**:H2805-2813.

Zuehlke AD, Beebe K, Neckers L, Prince T (2015) Regulation and function of the human HSP90AA1 gene. *Gene* **570(1)**:8-16.

Zweier JL, Kuppusamy P, Williams R, Rayburn BK, Smith D, Weisfeldt ML, Flaherty JT (1989) Measurement and characterization of postischemic free radical generation in the isolated perfused heart. *The Journal of Biological Chemistry* **264(32)**:18890-5.

Zweier JL, Talukder MA (2006) The role of oxidants and free radicals in reperfusion injury. *Cardiovascular Research* **70(2)**:181-90.

Appendices

Appendix 1. Ethical approval documents

Ethical approval documents removed due to confidentiality issues.

Appendix 2. Information sheets and Consent forms

Department of Cardiac Surgery
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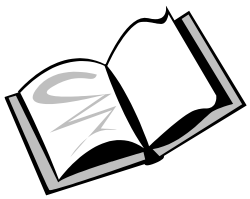
Children Information Sheet (7-11 years old)

Remote ischaemic preconditioning for cardiac surgery in children.

Full title: Effect of remote ischaemic preconditioning in cardiac dysfunction and end-organ injury following cardiac surgery with cardiopulmonary bypass in children: A translational approach investigating clinical outcome and myocardial molecular biology).



Please read this information sheet.

You can ask a parent or carer to help you.

What is this about?

This project will look at a way to protect the heart during surgery. We want to know if using a blood pressure cuff on your arm before the surgery is useful.

Why have I been asked to take part?

You have been invited because you need to have surgery on your heart.

Do I have to take part?

No. You and your parent or carer decide if you want to take part.

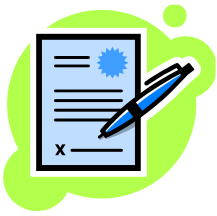
It is ok if you change your mind. It is your choice.

How do I let you know if I want to take part?

You can let you parent or carer know and they can tell me.

What will happen if I want to take part?

We will meet with you and your parent or carer.



We will ask you to sign a form to say you are happy to take part.

If you are unable to sign the form, you can tell me if you want to take part and choose somebody (such as your parent or carer) to sign the form for you.

A computer will decide if you get the blood pressure cuff or not.

If you do we will put the cuff on your arm and inflate it for 5 minutes, you will rest for 5 minutes and we will repeat this 3 times. Your parents or carer can be with you while we do this.

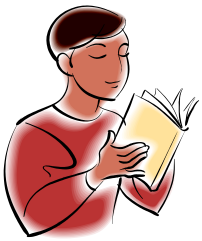
This will happen the day before your surgery and on the day of the surgery right before the doctors start when you will already be asleep.

You will have exams done on your blood but you won't need extra pricks because of it and an extra echo test will be done while you are still asleep.

What if I change my mind and do not want to take part?

You can change your mind or stop at any time. Nobody will be upset and you don't have to say why.

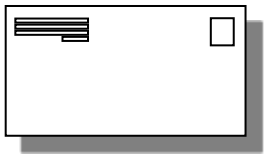
Will other people find out about this?



All the information we get will be private. Nothing will have your name on, so no one will know about it.

What happens to the information?

We will analyze all the information we get from you and other children and it will help us know if the intervention is useful or not. When the project is over we will publish what we found but nothing will have your name on it.



Will I be able to find out the results of the study?

Yes. Once the study has finished all the information will be available to you and your parent or carer.

You can ask us questions about this.

You can write to us or phone us to:

- Mr Mark Danton, Consultant Cardiac Surgeon, Yorkhill (0141 201 0251).
- Prof. Fiona Lyall, Professor of Foetal and Maternal health, University of Glasgow (0141 201 0657).
- Dr Maribel Verdesoto, PhD student, University of Glasgow (0141 201 0367).

Thank you for reading this.

Department of Cardiac Surgery
Yorkhill Hospital
G3 8SJ

Title: Potentially reducing injury to the heart muscle in children undergoing heart surgery by a prior application of a leg blood pressure cuff to stimulate the production of protective factors: a clinical evaluation.

(Title of Project: Remote ischaemic preconditioning in children undergoing cardiac surgery.

Full title: Effect of remote ischaemic preconditioning in cardiac dysfunction and end-organ injury following cardiac surgery with cardiopulmonary bypass in children: A translational approach investigating clinical outcome and myocardial molecular biology).

Young Person Information Sheet (12 – 16 years old)

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information.

Part 1 tells you the purpose of this study and what will happen during the study

Part 2 gives you more detailed information about the conduct of the study.

PART 1

Who is conducting the research?

Mr Mark Danton MD, consultant cardiac surgeon is the clinical supervisor for the research. Prof. Fiona Lyall, Professor of Maternal and Fetal Health - University of Glasgow will supervise the basic science. Dr Maribel Verdesoto will be the primary investigator and this study will lead to an educational PhD project by the University of Glasgow.

What is the purpose of the research project?

Remote ischaemic preconditioning (RIPC) is a procedure that could reduce injury to the heart during surgery. It consists of inflating a blood pressure cuff on your arm for three 5 minute cycles. This will reduce the blood flow in your arm, which will then activate the body´s own protective mechanisms and in that way reduce heart injury.

When you undergo cardiac surgery a system called cardiopulmonary bypass (CPB) is required. CPB substitutes the function of the heart and lungs during the surgery. While CPB is very safe and established process, it can cause injury both to the heart and/or other organs of the body. Typically the consequences of CPB are small and reversible with no obvious harm. The purpose of this study is to assess a therapy that could reduce any of the negative consequences of CPB, particularly to the heart.

Our study will investigate if this simple procedure (application of blood pressure cuff) protects against heart injury and injury to other organs in children going through cardiac surgery with CPB.

Why have I been chosen?

You have a congenital heart defect that needs surgery using cardiopulmonary bypass. We are hoping to evaluate children between 2 days to 16 years old.

Do I have to take part?

NO. This is voluntary. It is up to you to decide whether or not to take part. After discussing and considering the study you will be free to decide if you want to participate. You are free to stop at any time and without giving a reason. Your decision about this will not change, influence or affect the treatment or the standard of care you get. If you are happy to take part, and are satisfied with the explanations, you will be asked to sign a consent form. You will be given a copy of the signed consent form to keep for your records.

What do I have to do if I agree to take part?

This study will not change your treatment before, during or after the surgery. You will receive routine tests, which are normally done before the surgery, one day after surgery and before you are discharged from hospital. These include a test called an echo, which is a type of ultrasound that looks into the heart without using any radiation, similar to an ultrasound test which a mum gets during pregnancy. The echo exam will be more detailed and may take approximately 5-10 minutes longer than the usual exam.

You will be assigned to a treatment or control group by a computer. If you are in the treatment group you will get a blood pressure cuff placed on your arm and inflated for 5 minutes, then you will rest for 5 minutes and the process will be repeated for 2 additional times. This will happen two times: the day before surgery and during anaesthesia in the operating room just before the surgery starts. Your allocation to treatment or control groups will remain unknown for the researchers all through the study.

During the surgery an incision (cut) in the right atrium will be required; if so at that point a small portion of tissue will be removed (3-4 mm) right next to the cut. This has shown to be safe in previous studies.

If you require removal of heart muscle tissue as part of the standard surgical correction, this tissue will be kept for laboratory investigation. Such tissues are normally discarded but will be of use in this study.

Apart from routine blood sampling, additional tests will be performed for cardiac, lung and kidney analysis. You will not need an extra needle prick as all the samples will be obtained at the times when blood will routinely be taken from you for normal tests, three extra teaspoons of blood will be taken in total at over 48 hours.

You will be part of the study until the day of discharge (5 – 7 days) or up to 1 week following the surgery. An informative letter about your participation in the study will be sent to your GP.

What are the possible benefits of taking part?

A more detailed and more frequent echo exam of the heart function will be done for every participant.

The use of blood pressure cuffs as detailed in this study is safe as confirmed by previous studies. Excluding temporary discomfort (similar to having blood pressure measured) during the brief periods of cuff inflation we do not anticipate any potential complications apart of those expected of the normal surgery for your condition. This has been shown to be safe in other studies.

What happens when the research stops?

Your participation ends when the research stops. There is no further responsibility involved. It should be noted that the tissue taken from you may be kept for use in future research with your consent.

Who can I contact for further information?

If you require any further information on this study or would like to clarify any issues, please do not hesitate to contact us. Our contacts are as follows:
Mr Mark Danton, Consultant Cardiac Surgeon, Yorkhill (0141 201 0251).
Prof. Fiona Lyall, Professor of Foetal and Maternal health, University of Glasgow (0141 201 0657).
Dr Maribel Verdesoto, PhD student, University of Glasgow (0141 201 0367).

This completes Part 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART TWO

What if relevant new information becomes available?

Any new and relevant information that is obtained from this study will be available to the doctors who are responsible for your routine care.

What will happen if I don't want to carry on with the research?

You will still receive the standard, routine clinical care.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Department of Cardiac Surgery, Tel: 0141 201 0251 or 0141 201 0090).

If you remain unhappy and wish to complain formally, you can do this through the normal NHS Complaints Procedure that will be available to you by asking any hospital staff or by contacting:

Complaints Office - Yorkhill childrens' Hospital
Opening hours: 08:00 – 16:00, Monday-Friday
Complaints and patient liaison officer
Tel: 0141 201 9278

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs.

Will my taking part in the research project be kept confidential?

All personal information which is collected during the study will be kept strictly confidential. Your medical records and the data collected for the study will only be looked at by authorised persons. We will have a duty to protect your confidentiality as a research participant and nothing that could reveal your identity will be disclosed outside the research site. The data are held in accordance with the Data Protection Act, which means that we keep it safely and cannot reveal it to other people, without your permission.

What will happen to any samples I give?

The confidentiality of the sample will be protected. The heart muscle tissue removed during surgery will be analysed in the laboratory for levels of certain cellular proteins which are thought to give normal heart tissues protection against injury caused during heart surgery.

Will any genetic tests be done? No.

What will happen to the results of the research study?

The principal aim of the research is to improve our understanding of how the heart muscle in children with congenital heart disorders functions during corrective heart surgery and assess whether this procedure is effective against injury. We plan to publish our results in peer-reviewed journal as well as present the findings in local and international meetings. Your identity will be protected in any report.

Who is organising and funding the research?

This study received funding from the Yorkhill Hospital Children's Foundation for a 3 year PhD project conducted by the University of Glasgow.

Who has reviewed the study?

This study was given formal ethical approval by the West of Scotland Research Ethics Committee based in Western Infirmary, Glasgow.

Patients participating in this study will be given a copy of the information sheet and a signed consent form to keep.

Thank you for taking time to read this information sheet.

Department of Cardiac Surgery
Yorkhill Hospital
G3 8SJ

Title: Potentially reducing injury to the heart muscle in children undergoing heart surgery by a prior application of a leg blood pressure cuff to stimulate the production of protective factors: a clinical evaluation.

(Title of Project: Remote ischaemic preconditioning in children undergoing cardiac surgery. Full title: Effect of remote ischaemic preconditioning in cardiac dysfunction and end-organ injury following cardiac surgery with cardiopulmonary bypass in children: A translational approach investigating clinical outcome and myocardial molecular biology).

Parent/Guardian Information Sheet (0 – 6 years old)

Your child is being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information.

Part 1 tells you the purpose of this study and what will happen during the study

Part 2 gives you more detailed information about the conduct of the study.

PART 1

Who is conducting the research?

Mr Mark Danton MD, consultant cardiac surgeon is the clinical supervisor for the research. Prof. Fiona Lyall, Professor of Maternal and Fetal Health - University of Glasgow will supervise the basic science. Dr Maribel Verdesoto will be the primary investigator and this study will lead to an educational PhD project by the University of Glasgow.

What is the purpose of the research project?

Remote ischaemic preconditioning (RIPC) is a procedure that could potentially reduce injury to the heart during cardiac surgery. It consists of the inflation of a blood pressure cuff on the child's leg or arm for three 5 minute cycles. This briefly reduces blood flow to the leg or arm muscle, which will then activate the body´s own protective mechanisms and thereby reduce heart injury.

When children undergo cardiac surgery a system called cardiopulmonary bypass (CPB) is required. CPB substitutes the function of the heart and lungs temporally during surgery. During this process the patient´s blood flow is controlled by a bypass machine.

Whilst this is a very safe and established process, CPB can be associated with injury both to the heart and/or organs of the body. Typically the consequences of CPB are minor and reversible with no obvious harm to the patient. The purpose of this clinical study is to assess a potential therapy that could further reduce any of the negative consequences of CPB, particularly to the heart muscle.

Our study will investigate whether this simple mechanical procedure (application of blood pressure cuff) provides protection against heart injury and injury to other organs in children undergoing cardiac surgery with CPB.

This research we hope will allow us to understand and utilise the patient´s own protective mechanisms, reducing CPB-related injury.

Why has my child been chosen?

Your child has a congenital heart defect that requires surgical correction using cardiopulmonary bypass. We are hoping to evaluate children between 2 days to 16 years old.

Does my child have to take part?

NO. This is voluntary. It is up to you and your child (when possible) to decide whether or not to take part. Having discussed and considered the study you will be free to decide on participation. You are free to withdraw from the research at any time and without giving a reason. Your decision about this will not change, influence or affect the clinical treatment or the standard of care of the patient. If you are happy for your child to take part, and are satisfied with the explanations, you will be asked to sign a consent form. You will be given a copy of the signed consent form to keep for your records.

What does my child have to do if we agree to take part?

This study will not alter the clinical management of your child before, during or after the surgery. Your child will receive routine tests, which are normally performed prior to surgery, one day post-surgery and before discharge from hospital. These include a test called an echo, which is a type of ultrasound that looks into the heart without using any radiation, similar to an ultrasound test which a mum obtains during pregnancy. The echo will be more detailed and may take approximately 5-10 minutes longer than the usual examination.

Your child will be allocated to a treatment or control group through a computer randomisation process. If your child is in the treatment group a remote ischaemic preconditioning protocol will be applied as follows: a blood pressure cuff will be placed on the leg or arm of the child and inflated in order to reduce blood flow for 5 minutes, this process will be repeated for 2 additional times separated by 5 minutes of resting. This protocol will be performed at two phases: the day before surgery and during anaesthesia in the operating room just before the surgery starts. The allocation of the child to treatment or control groups will remain unknown for researchers all through the study.

During the surgery an incision (cut) in the right atrium will be required; if so at that point a small portion of tissue will be removed (3-4 mm) right next to the cut. This has shown to be safe in previous studies.

If your child requires removal of heart muscle tissue as part of the standard surgical correction, this tissue will be retained for laboratory investigation. Such myocardial tissues are normally discarded but will be of use in this study.

Additional blood tests will be performed for cardiac, lung and kidney analysis. The child will not need an additional needle prick as all the samples will be obtained during routine blood sampling; three extra teaspoons of blood will be taken in total at over 48 hours when the surgical/general patient care of your child takes place.

Your child will remain part of the study until the day of discharge (5 – 7 days) or up to 1 week following the surgery. An informative letter about the child´s participation in the study will be sent to the child´s GP.

What are the possible benefits of taking part?

A more detailed and more frequent echo assessment of the heart function will be performed for every participant.

The use of blood pressure cuffs to temporarily reduce blood flow for short periods is safe as confirmed by previous studies. Excluding temporary discomfort (similar to having blood pressure measured) during the brief periods of cuff inflation we do not anticipate any potential complications beyond that expected of the normal surgical treatment of your child's condition. This has been shown to be a safe procedure in other studies.

What happens when the research stops?

Your child participation ends when the research stops. There is no further responsibility involved. It should be noted that the tissue taken from your child may be kept for use in future research with your consent.

Who can I contact for further information?

If you require any further information on this study or would like to clarify any issues, please do not hesitate to contact us. Our contacts are as follows:
Mr Mark Danton, Consultant Cardiac Surgeon, Yorkhill (0141 201 0251).
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This completes Part 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART TWO

What if relevant new information becomes available?

Any new and relevant information that is obtained from this study will be made available to the clinicians who are responsible for your child's routine care.

What will happen if my child or I don't want to carry on with the research?

Your child will still receive the standard, routine clinical care.

What if there is a problem?

Any complaint about the way you or your child have been dealt with during the study or any possible harm you might suffer will be addressed. If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Department of Cardiac Surgery, Tel: 0141 201 0251 or 0141 201 0090).

If you remain unhappy and wish to complain formally, you can do this through the normal NHS Complaints Procedure that will be available to you by asking any hospital staff or by contacting:

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Tel: 0141 201 9278

In the event that something does go wrong and your child is harmed during the research study there are no special compensation arrangements. If your child is harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs.

Will my child's taking part in the research project be kept confidential?

All personal information which is collected during the study will be kept strictly confidential. Your child's medical records and the data collected for the study will only be looked at by authorised persons. We will have a duty to protect your child's confidentiality as a research participant and nothing that could reveal your child identity will be disclosed outside the research site. The data are held in accordance with the Data Protection Act, which means that we keep it safely and cannot reveal it to other people, without your permission.

What will happen to any samples my child gives?

The confidentiality of the sample will be protected. The heart muscle tissue removed during surgery will be analysed in the laboratory for levels of certain cellular proteins which are thought to give normal heart tissues protection against injury caused during heart surgery.

Will any genetic tests be done? No.

What will happen to the results of the research study?

The principal aim of the research is to improve our understanding of how the heart muscle in children with congenital heart disorders functions during corrective heart surgery and assess whether this procedure is effective against injury. We plan to publish our results in peer-reviewed journal as well as present the findings in local and international meetings. Your child's identity will be protected in any report.

Who is organising and funding the research?

This study received funding from the Yorkhill Hospital Children's Foundation for a 3 year PhD project conducted by the University of Glasgow.

Who has reviewed the study?

This study was given formal ethical approval by the West of Scotland Research Ethics Committee based in Western Infirmary, Glasgow.

Parents of children participating in this study will be given a copy of the information sheet and a signed consent form to keep.

Thank you for taking time to read this information sheet.

Department of Cardiac Surgery
Yorkhill Hospital
G3 8SJ

Patient Identification Number for this trial: _____

PARENTAL CONSENT FORM (0 – 6 YEARS OLD)

Title: Potentially reducing injury to the heart muscle in children undergoing heart surgery by a prior application of a leg blood pressure cuff to stimulate the production of protective factors: a clinical evaluation. *(Full title: Effect of remote ischaemic preconditining in cardiac dysfunction and end-organ injury following cardiac surgery with cardiopulmonary bypass in children: A translational approach investigating clinical outcome and myocardial molecular biology).*

Name of Researchers: Dr. Mark Danton (CI) and Miss Maribel Verdesoto (PI)

Please initial box

I confirm that I have read and understand the information sheet dated 12/08/2013 of the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

I understand that my child's participation is voluntary and that my child is free to withdraw at any time, without giving any reason, without his or her medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my child's medical notes and data collected during the study may be looked at by the research team and responsible individuals from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my child _____ taking part in the above study. ☐

5. I agree to my child´s tissue to be obtained, kept and used in future research. ☐

6. I agree to my child´s GP being contacted. ☐

_____	_____	_____
Name of Parent	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 copy for the patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Department of Cardiac Surgery
Yorkhill Hospital
G3 8SJ

Title of Project: Remote ischaemic preconditioning in children undergoing cardiac surgery.
(Full title: Effect of remote ischaemic preconditining in cardiac dysfunction and end-organ injury following cardiac surgery with cardiopulmonary bypass in children: A translational approach investigating clinical outcome and myocardial molecular biology).

Assent form for Children (7 – 11 years old)

To be completed by the child (or if unable, the parent on their behalf). Please circle your answers:

Have you read (or had read to you) about this project?	Yes/No
Has somebody else explained this project to you?	Yes/No
Do you understand what this project is about?	Yes/No
Have you asked all the questions you want?	Yes/No
Have you had your questions answered in a way that you understand?	Yes/No
Do you understand that it's OK to stop taking part at any time?	Yes/No
Are you happy to take part?	Yes/No

If <u>any</u> answers are 'no' or you don't want to take part, don't sign your name!

If you <u>do</u> want to take part, you can sign your name below

Your name _____

Signature _____

Date _____

The person who explained this project to you needs to sign too:

Print Name _____

Signature _____

Date _____

Thank you for your help

Department of Cardiac Surgery
Yorkhill Hospital
G3 8SJ

Patient Identification Number for this trial: _____

YOUNG PERSON CONSENT FORM (12 – 16 years old)

Title of Project: Remote ischaemic preconditioning in children undergoing cardiac surgery.

(Full title: Effect of remote ischaemic preconditining in cardiac dysfunction and end-organ injury following cardiac surgery with cardiopulmonary bypass in children: A translational approach investigating clinical outcome and myocardial molecular biology).

Name of Researchers: Dr. Mark Danton (CI) and Miss Maribel Verdesoto (PI)

Please initial box

1. I confirm that I have read and understand the information sheet dated 12/08/2013 of the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by the research team and responsible individuals from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐
5. I agree to my tissue to be obtained, kept and used in future research. ☐
6. I agree to my GP being contacted. ☐

_____ _	_____ _	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Parent	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 copy for the patient; 1 for researcher site file; 1 (original) to be kept in medical notes

If the child is deemed competent to sign the consent form, this should be accompanied by a parental signature of agreement to support their child's participation in the study.

Appendix 3. Descriptive statistics for PICU blood analyses

Variable	Group	PO1			PO2			PO3		
		N	Descriptive	P value	N	Descriptive	P value	N	Descriptive	P value
Urea & Electrolites										
Urea	RIPC	25	5.1 (4.2-33.0)	0.584	24	5.30 (4.35-20.35)	0.701	22	6.0 (0.0-16.3)	0.510
	Control	23	4.7 (3.8-24.0)		23	7.0 (4.1-34.0)		23	5.8 (0.0-52.0)	
Creatinine	RIPC	25	32.0 (27.0-39.0)	0.570	24	36.5 (18.5-49.5)	0.741	22	27.0 (0.0-41.0)	0.817
	Control	23	28.0 (22.0-41.0)		23	36.0 (22.0-44.0)		23	29.0 (0.0-37.0)	
Na+	RIPC	25	144.76±3.94	0.398	21	143.62±4.72	0.098	13	143.69±8.20	0.122
	Control	21	145.81±4.40		22	146.23±5.35		17	147.47±4.69	
K	RIPC	25	4.1(3.9-4.6)	0.748	21	4.3(4.0-4.6)	0.232	13	4.1(4.0-4.1)	0.263
	Control	21	4.1(3.7-4.6)		22	4.4(4.1-4.9)		17	4.3(4.0-4.5)	
Cl+	RIPC	25	114.44±4.42	0.482	21	113(109-117)	0.137	13	111(103-114)	0.053
	Control	21	115.43±5.05		21	116(113-122)		17	117(113-120)	
Ca	RIPC	23	2.3(2.2-2.3)	0.312	20	2.3(2.2-2.3)	0.574	12	2.2(2.2-2.3)	0.444
	Control	21	2.3(2.2-2.3)		21	2.3(2.2-2.3)		17	2.3(2.2-2.3)	
Phosphate	RIPC	23	1.9(1.4-2.4)	0.387	19	1.9(1.4-2.4)	0.320	12	1.50±0.68	0.969
	Control	20	2.1(1.8-2.4)		21	2.1(1.8-2.4)		17	1.49±0.51	
Mg+	RIPC	25	1.57±0.73	0.507	18	1.0(0.9-1.3)	0.349	12	1.0(0.8-1.1)	0.631
	Control	20	1.72±0.80		21	1.2(1.0-1.4)		16	1.0(0.8-1.0)	
Liver function tests										
AlkPhos	RIPC	25	110.48±50.63	0.193	21	115.05±48.84	0.081	13	145(100-194)	0.011
	Control	20	94.00±32.40		19	93.05±25.68		15	96(77-121)	
AST	RIPC	25	79(50-135)	0.492	20	77(46-146)	0.686	13	94(38-164)	0.786
	Control	19	78(42-121)		21	91(54-138)		15	82(68-128)	
ALT	RIPC	25	14(13-19)	0.622	20	19(13-36)	0.860	13	57(14-68)	0.650
	Control	20	14(12-19)		22	19(15-27)		16	47(23-125)	
Bilirrubin	RIPC	21	15.33±10.84	0.680	21	12(8-21)	0.357	13	10(9-15)	0.720
	Control	21	11.52±8.72		21	10(7-15)		14	11(8-14)	
Albumin	RIPC	25	37(35-41)	0.658	21	33.62±2.85	0.222	13	32.92±3.90	0.863
	Control	21	38(33-39)		22	34.73±2.995		17	32.65±4.60	
CRP	RIPC	24	3(3-8)	0.451	21	74.14±27.05	0.943	13	108(92-191)	0.779

Variable	Group	PO1			PO2			PO3		
		N	Descriptive	P value	N	Descriptive	P value	N	Descriptive	P value
	Control	21	3(3-3)		22	73.55±27.08		16	107(91-169)	
Whole blood count										
WBC	RIPC	25	10.6(7.2-15.2)	0.804	23	12.4(10.9-19.3)	0.489	13	13.5(11.9-23.3)	1.00
	Control	23	10.0(5.3-16.1)		22	15.9(12.5-18.5)		17	14.9(11.9-16.3)	
RBC	RIPC	25	11.51±6.49	0.992	23	4.13±0.67	0.4620	13	4.06±0.64	0.598
	Control	22	11.07±6.36		22	4.28±0.67		17	3.94±0.66	
Hb	RIPC	25	116.24±19.83	0.615	23	117(93-131)	0.088	13	117.62±16.37	0.904
	Control	23	119.57±25.55		22	127(111-140)		17	116.76±20.81	
HCT	RIPC	25	0.33(0.31-0.38)	0.940	23	0.36(0.29-0.39)	0.502	13	0.345±0.049	0.912
	Control	22	0.35(0.30-0.38)		22	0.37(0.32-0.41)		17	0.342±0.059	
MCV	RIPC	25	83.3(81.3-85.3)	0.135	23	84.3(81.2-86.1)	0.307	13	84.91±3.78	0.147
	Control	22	86.2(81.8-88.4)		22	86.6(82.5-88.6)		17	86.90±3.51	
MCH	RIPC	25	28.67±1.44	0.092	23	28.52±1.55	**0.049**	13	29.05±1.49	0.224
	Control	22	29.40±1.45		22	29.46±1.53		17	29.63±1.08	
Platelets	RIPC	23	159.35±73.49	0.594	21	173.33±60.56	0.214	13	165(126-188)	0.398
	Control	23	148.35±65.26		22	199.36±73.60		16	145(100-177)	
Neutro	RIPC	25	7.1(3.5-11.0)	0.565	23	9.1(6.8-14.6)	0.496	13	9.0(8.6-12.5)	0.837
	Control	22	5.2(3.5-10.6)		22	11.1(9.1-13.8)		17	10.7(7.9-11.3)	
Lympho	RIPC	25	2.65±1.13	0.207	22	1.8(1.2-4.0)	0.591	13	2.3(1.8-2.7)	0.496
	Control	21	2.25±0.99		19	1.6(1.1-2.5)		15	2.6(2.0-4.0)	
Monocyte	RIPC	25	0.7(0.4-1.0)	0.965	22	1.7(1.2-4.0)	0.675	13	1.6(1.4-2.8)	0.751
	Control	21	0.8(0.2-1.4)		19	1.5(1.3-2.3)		15	1.7(1.2-1.8)	
Eosinophil	RIPC	25	0.1(0.0-0.1)	0.157	22	0.0(0.0-0.0)	0.198	13	0.0(0.0-0.4)	0.650
	Control	21	0.0(0.0-0.1)		19	0.0(0.0-0.0)		15	0.0(0.0-0.1)	
Basophiles	RIPC	25	0.0(0.0-0.0)	1.00	22	0.0(0.0-0.0)	0.099	13	0.0(0.0-0.0)	0.786
	Control	21	0.0(0.0-0.0)		19	0.0(0.0-0.0)		15	0.0(0.0-0.0)	

Variable	Group	PO1			PO2			PO3		
		N	Descriptive	P value	N	Descriptive	P value	N	Descriptive	P value
Coagulation										
PT	RIPC	23	15(14-16)	0.235	19	16.63±2.09	0.197	10	17.60±2.91	0.523
	Control	23	16(14-19)		21	15.71±2.31		15	18.73±4.96	
APTT	RIPC	21	37(31-45)	0.472	14	39(31-55)	0.290	10	34(32-45)	0.738
	Control	20	38(30.5-54)		15	36(30-44)		13	36(32-38)	
Fibrinogen	RIPC	22	1.30(1.15-1.60)	0.503	14	2.60±0.69	0.260	10	3.75±0.97	0.081

Table 2. Descriptive statistics for PICU laboratory analysis according to RIPC or control allocation. Parameters were measured at PICU arrival (PO1), day 2 (PO2) and day 3 (PO3) after surgery. Abbreviations: AlkPhos: alkaline phosphatase; ALT: alanine aminotransferase; APTT: activated partial thromboplastin time; AST: aspartate aminotransferase; CRP: C reactive protein; Hb: haemoglobin; HCT: haematocrit; MCH: mean corpuscular haemoglobin; MCV: mean corpuscular volume; PICU: paediatric intensive care unit; PO: post-operative; PT: prothrombin time; RBC: red blood cells; WBC: white blood cells.

Appendix 4. Immediate post-operative echocardiographic data analysis.

An immediate post-operative echo was possible to be performed for a small number of patients; *Table 1* presents the descriptive statistics for echocardiographic measurements at the pre-operative (Pre-op), immediate post-operative (PO1) and next morning (PO2) analysis.

Variable	Time point	N	Mean – SD	Median – IQ range	P value
FS	Pre-op	12	31.75±9.353		0.701
	PO1		29.08±14.817		
	PO2		27.83±12.239		
EF	Pre-op	12		64(53-76)	0.393
	PO1			56(44-73)	
	PO2			61(48-68)	
MV LAT S	Pre-op	7	0.052±0.022		0.389
	PO1		0.052±0.015		
	PO2		0.061±0.021		
MV SEP S	Pre-op	5	0.498±0.018		0.683
	PO1		0.055±0.012		
	PO2		0.048±0.016		
LV IVRT	Pre-op	6	51.33±22.651		0.707
	PO1		53.50±7.868		
	PO2		48.50±12.145		
MV E/A	Pre-op	5	1.780±0.832		0.474
	PO1		1.499±0.116		
	PO2		1.810±0.415		
MV LAT E/E´	Pre-op	6	12.655±2.976		1.00
	PO1		12.598±2.976		
	PO2		12.625±4.821		
MV SEP E/E´	Pre-op	5	12.862±3.923		0.142
	PO1		13.966±3.813		
	PO2		15.797±2.996		
TV ANT S	Pre-op	7		0.090(0.053-0.100)	0.135
	PO1			0.046(0.040-0.076)	
	PO2			0.050(0.040-0.050)	

Table 1. Descriptive statistics for echocardiographic variables according to time point. Data is expressed as mean – SD or median – IQ range as appropriate. Abbreviations: EF: ejection fraction; FS: fractional shortening; LV IVRT: left ventricle isovolumetric relaxation time; MV E/A: mitral inflow pulsed wave Doppler E to A velocities ratio; MV LAT E/E´: lateral mitral annulus E to E' ratio; MV LAT S: lateral mitral annulus systolic velocity; MV SEP E/E´: septal mitral annulus E to E' ratio; MV SEP S: septal mitral annulus systolic velocity; PO: post-operative; TV ANT S: tricuspid annular systolic velocity.

Measurements of left ventricle systolic function (*Figure 1*), left ventricle diastolic function (*Figure 2*), and right ventricle systolic function (*Figure 3*) did not result in a significant repeated measure variation.

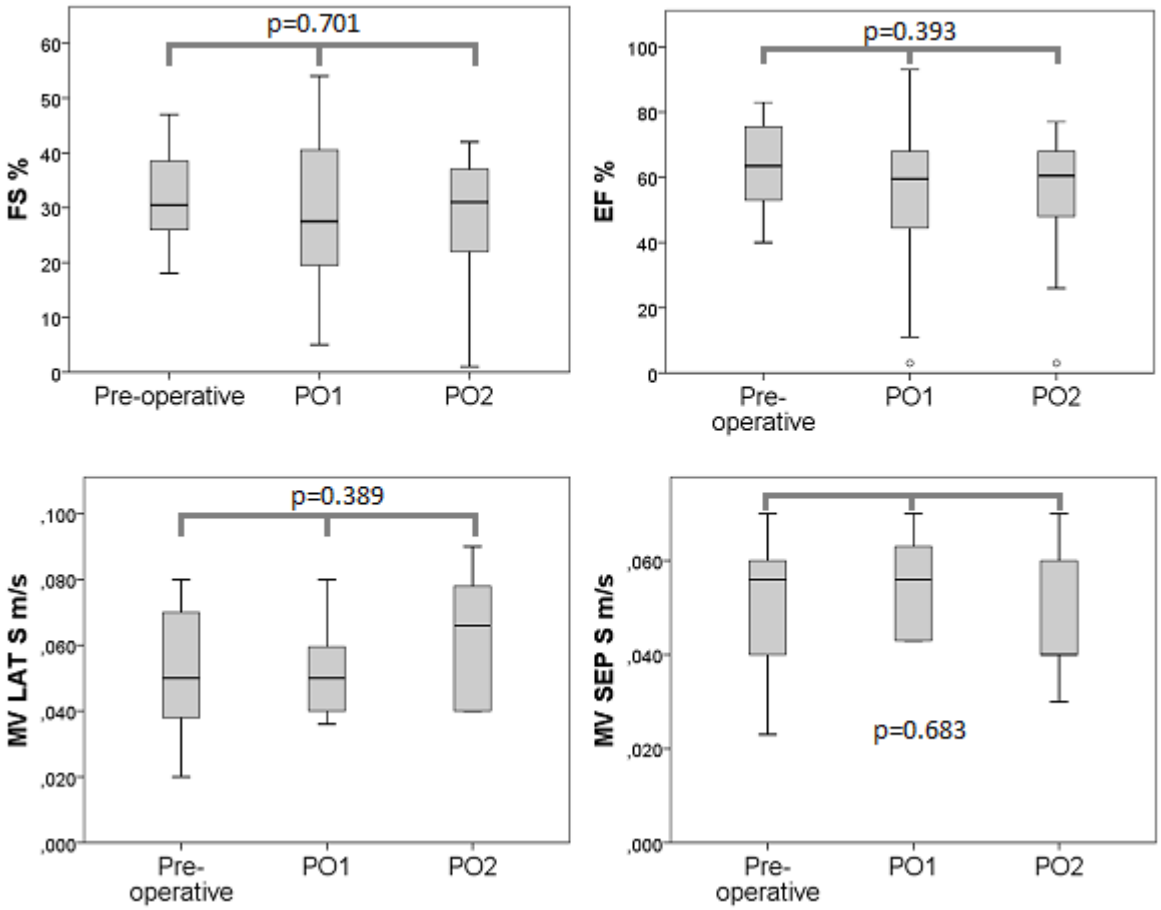

Figure 1. Echocardiographic measures of LV systolic function according to time point. Abbreviations: EF: ejection fraction; FS: fractional shortening; MV LAT S: lateral mitral annulus systolic velocity; MV SEP S: septal mitral annulus systolic velocity; PO: post-operative.

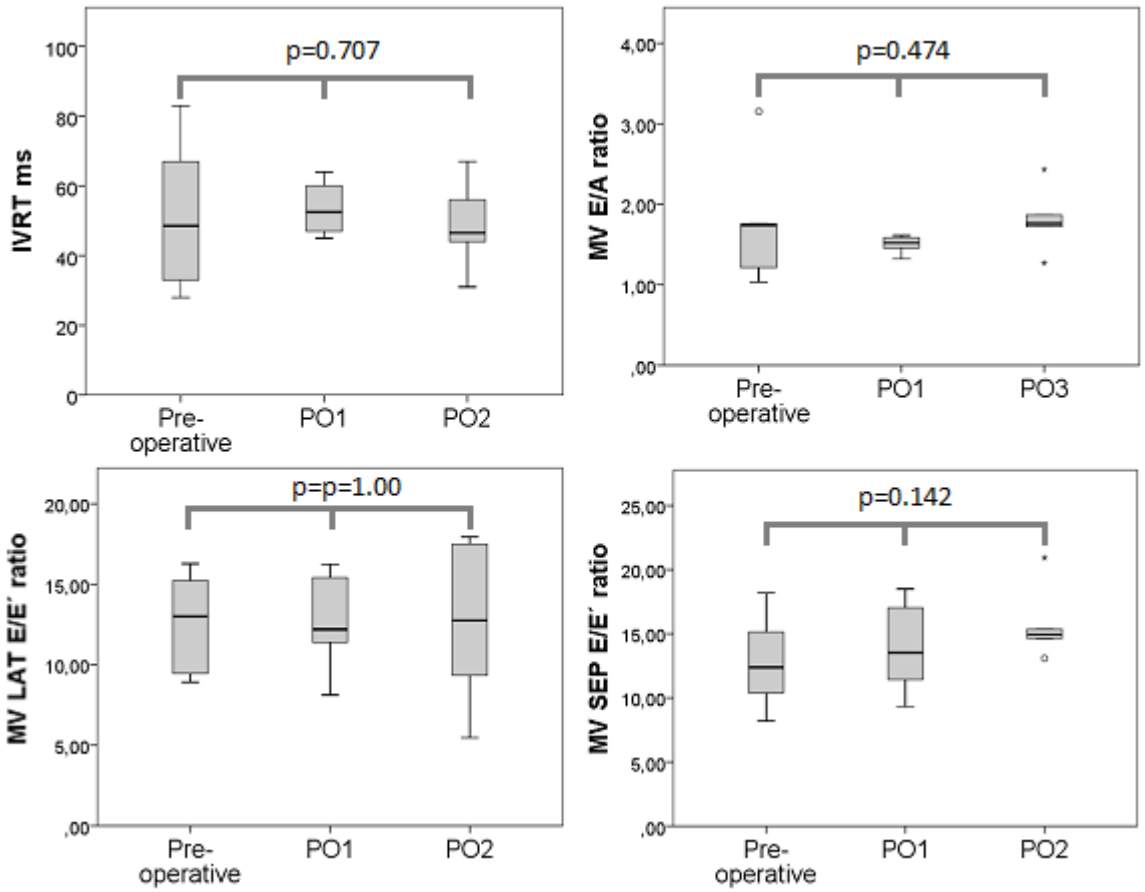

Figure 2. Echocardiographic measures of LV diastolic function according to time point. Abbreviations: IVRT: isovolumetric relaxation time; MV E/A: mitral inflow pulsed wave Doppler E to A velocities ratio; MV E/E´: lateral mitral annulus E to E' ratio; MV SEP E/E´: septal mitral annulus E to E' ratio; PO: post-operative.

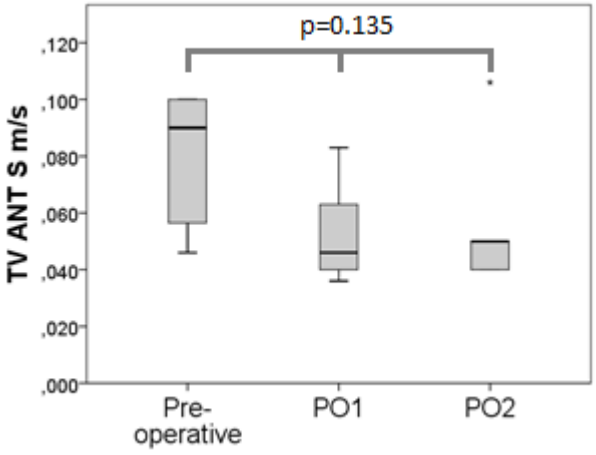

Figure 3. Echocardiographic measure of RV systolic function according to time point. Abbreviations: PO: post-operative; TV ANT S: tricuspid annular systolic velocity.