Title: Molecular mechanisms initiated within cyanotic and acyanotic infant myocardium during cardio-pulmonary bypass *in vivo* and ischemic-reperfusion injury *in vitro*.

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Abstract

Children with the congenital heart defect TOF require surgical repair. Their myocardium is exposed to chronic hypoxia due to insufficiently oxygenated blood shunted from the right to the left side of the heart through the VSD, resulting in cyanosis. Such corrective surgery often requires the use of CPB. During these procedures the aortic clamp is applied to allow access to the heart for the surgical correction creating an ischemic environment. When the clamp is removed and blood flow returned, the heart is reperfused. This exposes the myocardium to IR injury. Super-high oxygen (O_2) levels are often used in the bypass circuit after the cross-clamp is released. This means during reperfusion the myocardium is exposed to high levels of O_2. This is thought to be harmful, especially to children with cyanosis pre-operatively.

A growing body of evidence suggests that these high levels of O_2 may be damaging to the infant myocardium but the mechanisms are not that well understood. The first aim of this project was to investigate the molecular changes that occur within the infant myocardium in the context of congenital heart disease by comparing myocardium from the cyanotic patient group (TOF patients) and acyanotic patient group (patients with sub-aortic stenosis or truncus arteriosus i.e. disorders requiring myocardial resection but where the myocardium is acyanotic) during the ischemic phase. This was performed to reflect as near to pre-operative gene expression levels as possible. This thesis focused on the expression of inflammatory mediators (TLRs) and stress proteins (HSPs). It was found that TLR4 ($p=0.0303$), TLR2 ($p=0.0177$) and HSP27 (0.0303) mRNA expression were significantly higher in the myocardium from the cyanotic patient group compared to the acyanotic patient group. This could indicate a consequence of the hypoxic environment within such myocardium.

HSPs are stress proteins, which are induced upon cellular stress. The Aplein receptor (APJ) is upregulated in the heart during ischemia and IR injury and thought to be cardio-protective. Additional work was performed that investigated the protein expression of HSP27 and APJ during the ischemic phase in cyanotic and non-cyanotic TOF myocardium. This expression was compared with various measurements of cardiac function. HSP27 protein expression was significantly elevated in cyanotic myocardium pre-operatively. It was also associated with improved right ventricle function and systemic perfusion. This supports a cardio-protective effect of HSP27 in cyanotic TOF. APJ was found to be associated with

improved systemic perfusion when both cyanotic and non-cyanotic patient data were combined.

In order to determine if stress genes (HSPs) were inducible in the infant myocardium, they were exposed to classic heavy metal inducers sodium arsenite and cadmium chloride. Of the six HSPs investigated, all were shown to be present within the ischemic myocardium. Myocardial HSP32 ($p=0.0156$), HSP72 ($p=0.0156$) and HSP90 ($p=0.0156$) mRNA expression were significantly increased by exposure to heavy metals. Therefore, HSPs were shown to be inducible in infant myocardium. This was a good experimental control for the re-oxygenation experiment.

The expressions of the same genes were also examined *in vitro* during an attempt to mimic re-oxygenation of the ischemic myocardium when the aortic-clamp is removed. Tissue was removed during the ischemic phase from both cyanotic and acyanotic patients and exposed to differential O_2 levels. It was found exposure to 20% and 60% O_2 for 4 hours induced mRNA expression of HSP32 ($p=0.0391$ and $p=0.0078$ respectively) and HSP72 ($p=0.0078$ and $p=0.0078$ respectively). This could indicate cellular stress during re-oxygenation of infant myocardium during surgical correction. No difference was indicated between the cyanotic and acyanotic patient group.

The work of this thesis was focused on myocardial mRNA/ protein expression. Whether there is an alteration in circulating inflammatory mediators was not the focus of the present study but the results within this thesis highlighted the need for future studies to look at the effect of circulating factors on myocardial inflammation.

This project is novel because it allowed a better understanding of the molecular mechanisms at work within infant myocardium and how they are influenced by re-oxygenation injury. It also examined how they are affected by cyanosis. It has highlighted the best ways in which such investigations could be extended. It has contributed important molecular data to the area of research and could potentially help lead to an improvement in myocardial protection during cardiac surgery with CPB.

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Dedication

I would like to dedicate this thesis to my mum, dad and sister in return for all the wonderful support and help they have given over the last 4 years. All of this would not have been possible without them.

Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature _____

Printed name _____

Abbreviations and Definitions

ATF6 – activation of transcription factor 6
AT1 – angiotensin receptor
ADP – adenosine diphosphate
Apoptosis – programmed cell death where biological signals within the cell deliberately cause cell death.
APC – antigen presenting cells
APJ – Apelin receptor
APS – ammonium persulfate
ASD – atrial septal defect
ATP – adenosine triphosphate
AVP – vasopressin
BiP – binding immunoglobulin protein
Bp – base pair
BSA – bovine serum albumin
Ca^{2+} – calcium
CABG – coronary artery bypass grafting
$CdCl_2$ – cadmium chloride
cDNA – complementary DNA
CO_2 – carbon dioxide
CK – creatine kinase
CPB – cardio-pulmonary bypass
Chimeric – an organism that is made up of one or more different cells that are genetically distinct
DAMP – danger-associated molecular patterns
dH_2O – distilled water
DNA – deoxyribonucleic acid
dNTPs – deoxynucleotide triphosphates
Epicardium - outer protective layer of heart
EGR-1 – early growth response protein
E_m – resting membrane potential
ER – endoplasmic reticulum.
EOR – ER overload response
Eritoran – TLR4 antagonist that blocks activation of this receptor
FV – Fallot variant
GRP - glucose regulated proteins
Heat shock – heat stress that can induce HSP expression
Heterodimer – a protein made up of two different subunits
HIF-1 – hypoxia-inducible factor
Housekeeping genes – genes required for homeostasis of basic cell function
H - hydrogen
HO – heme-oxygenase
HMGB – high mobility group box
HSP –heat shock proteins
Hyperkalemic solutions – solution with high potassium content
iNOS – inducible nitric oxide synthase
gDNA – genomic DNA
GRP78 – 78 kDa glucose-regulated protein

GRP94 – 94 kDa glucose-regulated protein
IL – Interleukin
ICAM-1 – intercellular adhesion molecules
IFN-β – interferon -β
IRF3 – Interferon regulatory factor 3
IR injury – Ischemic-reperfusion injury
IVC – inferior vena cava
K – potassium
LA – left Atrium
Ligand – molecular that binds to a specific receptor to initiate its downstream signalling
LPS – lipopolysaccharides
LV – left ventricle
MAL – MYD88 adaptor-like protein
MHC – major histo-compatibility complex
MI – myocardial infarction
MCP-1 – monocyte chemotactic protein-1
mRNA – messenger RNA
MYD88 – myeloid differentiation factor-88
Myectomy – excision of a muscle
Myocardial hibernation – prolonged inactive contractile function of the heart that can be reversed
Myocardial stunning - contractile function stops briefly
N_2 – nitrogen
NaOH – sodium hydroxide
Na – sodium
$NaAsO_2$ – sodium arsenite
Necrosis – premature death of a healthy living cell caused by external factors such as infection, toxins or trauma. In contrast to apoptosis (see above)
NFkB – nuclear factor kappa B
NKC – natural killer cell
NO – nitric oxide
O_2 – oxygen
Oxidative phosphorylation – process that produces ATP (energy) within the mitochondria
PAMPs – pathogen associated molecular patterns
PCR – polymerase chain reaction
PaO_2 – arterial oxygen tension
POD1 – post-operative day one
PRR – pattern recognition receptors
PS – pulmonary stenosis
qPCR – quantitative polymerase chain reaction
rho – spearman rank correlation
RLT buffer – buffer provided with RNeasy Fibrous Tissue Midi Kit (Qiagen cat. no. 75742)
RNA – ribonucleic acid
RT – reverse transcription
RW1 buffer – buffer provided with RNeasy Fibrous Tissue Midi Kit (Qiagen cat. no. 75742)
ROD – relative optical density
ROS – reactive oxygen species
RA – right atrium
RQ – relative quantification

RV – right ventricle
RVOT – axis RV outflow tract
SaO_2 – systemic arterial oxygen saturation
SARM – sterile α- and armadillo-motif-containing protein
SAS – sub-aortic stenosis
sGC – soluble guanylate cyclase
SIRS – systemic inflammatory response syndrome
SDS - sodium dodecyl sulfate
SNPs – single-nucleotide polymorphisms
STD – stock standard
SVC – superior vena cava
S_vO_2 – mixed venous oxygen saturation
TA – truncus arteriosus
TEMED - Tetramethylethylenediamine
TGF-β – transforming growth factor beta
TIR – Toll/IL-1R/Resistance motif
TLR – Toll Like receptors
T_m – melting temperature of the primers
TNF-α – tumor necrosis factor-alpha
TOF – Tetralogy of Fallot
TRAM – TRIF-related adaptor molecule
TRIF – TIR domain-containing adaptor-inducing IFN-β
VCAM-1 – vascular cell adhesion molecule-1
VSD – ventricular septal defect
UPR – unfolded protein response.
Ubiquinone – part of the electron transport chain and is involved in aerobic respiration.
Ubisemiquinone – an electron donor during oxidative phosphorylation.
UV light – ultra-violet light

CHAPTER 1 – INTRODUCTION

1.1 The Heart

1.1.1 Anatomy

The heart is the main functioning part of the body's circulatory system and is responsible for taking deoxygenated blood from the systemic circulation, where it is re-oxygenated when it passes through the pulmonary circulation (lungs), and then transporting re-oxygenated blood back into the systemic circulation. Blood is transported throughout the body through various types of blood vessels, which include arteries and capillaries. This ensures that all the major organs of the body are provided with an adequate supply of oxygenated blood. Veins return the blood to the heart. The heart is positioned in the thorax, between the lungs and lies more to the left side of the body.

The heart consists of three layers of tissue the first of which is the outer layer called the epicardium, which consists of mainly connective tissue acting as a protective barrier. The second layer is the myocardium that makes up the muscular middle layer of the heart i.e. the cardiac muscle. The third layer is called the endocardium and is a thin, smooth continuous membrane lining the interior of the chambers of the heart and is the equivalent to the endothelium that lines blood vessels. The space within the endocardium is known as the ventricular cavity and is where the blood flows. Enclosing the heart (and its 3 layer) is a membrane known as the pericardium. This membrane is made up of two layers, an inner double layer of serous membrane and an outer fibrous layer. The space between the pericardium and the epicardium is known as the pericardial cavity.

The heart's interior (which is shown in figure 1-1) is separated into the left and right side of the heart by the septum (A). Each side of the heart is further divided by the valves of the heart, resulting in four chambers. These are the left (B) and right (C) atria and the left (D) and right (E) ventricles. The valve that separates the right atrium (RA) and right ventricle (RV) is the tricuspid valve (F) and consists of 3 leaflets. The valve that separates the left atrium (LA) and left ventricle (LV) is the mitral valve (G) and consists of 2 leaflets. Changing pressure in the chambers of the heart results in the opening and closing of the valves. When the pressure in the ventricles increases to a higher level than in the atria, the valves close thus preventing backflow of blood and ensuring the flow is one way through the heart. The closure of the valves is supported by the chordae tendineae, which are muscular cords connected to the

valves and which continue down into muscle projections that line the interior wall of the ventricles and that are covered by endothelium.

The flow of blood into the heart begins with de-oxygenated blood flowing into the RA from the superior and inferior venae cavae (H). The blood then travels from the RA through the tricuspid valve into the RV and then exits the heart entering the pulmonary circulation. This occurs via the pulmonary artery (I) (which contains the pulmonic valve (J)), which splits into the left and right pulmonary arteries. The pulmonary arteries carry blood to the lungs to be re-oxygenated. Re-oxygenated blood then re-enters the heart via the pulmonary veins (K), of which there are four, into the LA. Blood then goes through the mitral valve to the LV, where it exits the heart via the aorta (L) (which contains the aortic value (M)) which returns the oxygenated blood back to the systemic circulation.

The blood supply of the heart itself is provided by the coronary arteries, which branch off from the aorta as blood initially leaves the heart. The pressure at which blood flows within the LV is higher than in the RV because the systemic circulation has a higher resistance than the pulmonary circulation.

Figure 1-1. The internal structure of the heart

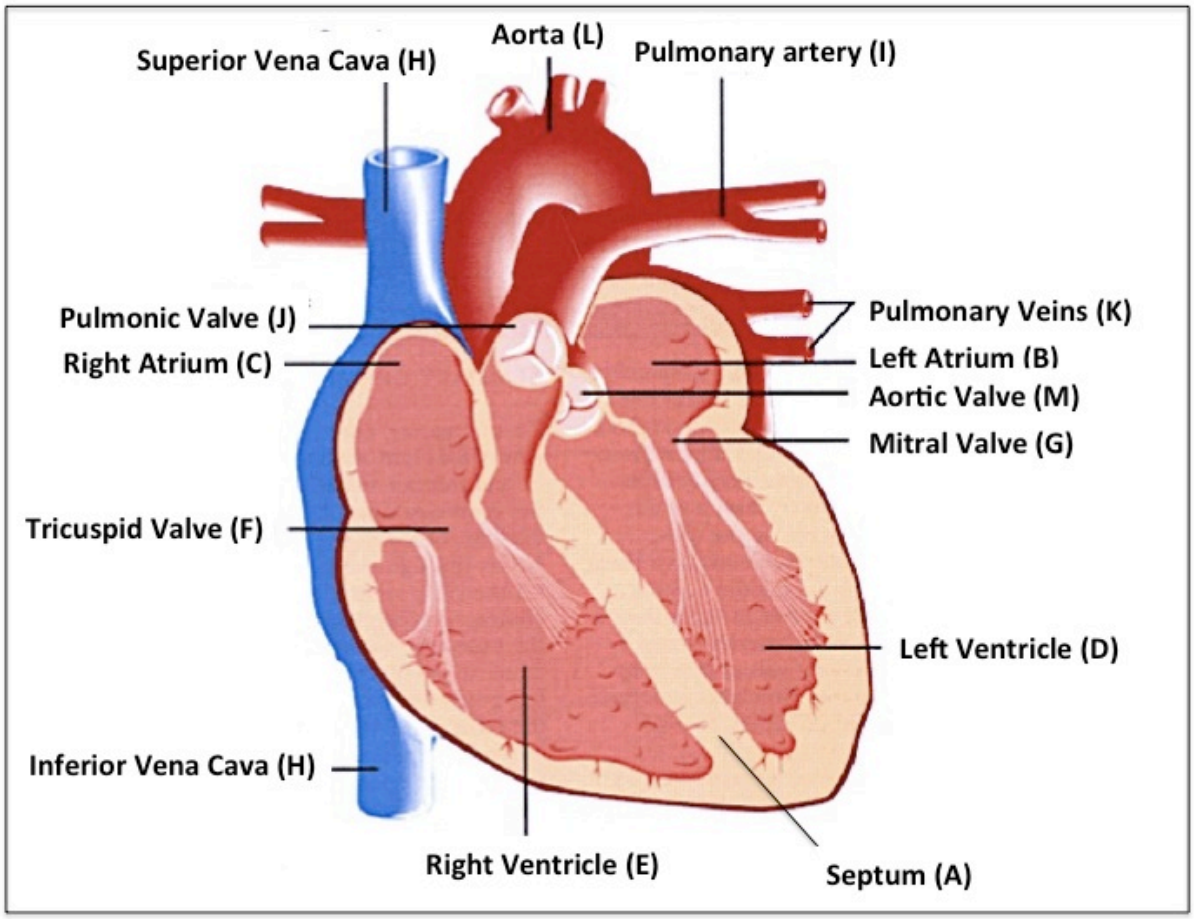


Figure shows the internal anatomy of the heart, including all the major chambers, blood vessels and valves (Modified from Chung and Rich, 1990).

1.1.2 Energy metabolism of the heart

The main function of the heart is to pump oxygenated blood from the heart to the rest of the body and this requires a lot of energy. The majority of energy utilised by the heart is provided by adenosine triphosphate (ATP) produced in the mitochondria by oxidative phosphorylation making cardiac cell function highly dependent on oxygen (O_2) availability (Ferrari, 1996). The importance of this is highlighted by the fact there is such a high number of mitochondria within cardiac tissue compared to other tissue types (Page, 1973). Also, the amount of energy used within the heart is tightly regulated therefore any interruption in blood supply would be highly detrimental (Grynberg *et al*., 1996).

1.1.3 Tetralogy of Fallot

Tetralogy of Fallot (TOF) is a congenital heart condition with an incidence of 1/3600 births and this makes up about 3.5% of babies born with cardiac abnormalities (Shinebourne *et al*., 2006). This condition was first recognised as early as 1671, but like the majority of other congenital cardiac abnormalities the aetiology is not yet defined (Apitz *et al*., 2009). This condition requires surgical repair and such surgical procedures require the use of cardio-pulmonary bypass (CPB).

TOF is characterised by four separate defects. These are: (1) pulmonary stenosis (PS) that causes restricted blood flow to the lungs and results in inadequate oxygenation of the blood; (2) a ventricular septal defect (VSD) where there is a hole in the septum separating both ventricles of the heart; (3) an over-riding aorta which is positioned just above the VSD; (4) a thickened right ventricle (hypertrophy) caused by the high pressure caused by the pulmonary stenosis (reviewed in Bailliard and Anderson, 2009).

Usually, the pressure within the LV is higher than within the RV. In the case of TOF, the pressure is actually equal in both the LV and RV. This is because the presence of the VSD causes the pressure within the two ventricles to equalise. Without the PS, this would make the volume of blood entering the pulmonary circulation too high causing pulmonary overcirculation and ultimately lead to heart failure. Thus the presence of the PS with the VSD helps maintains TOF patients until surgical repair can be performed.

Another consequence of the PS and VSD is that deoxygenated blood entering the heart is shunted from the RV to the LV through the VSD back into the systemic circulation. This recirculation of blood that has been poorly oxygenated results in an overall lower O_2 blood saturation level (~70-80%) with a typical arterial O_2 tension (PaO_2) of 40mmHg (normally would be 75-100mmHg). This means that the heart is chronically exposed to low levels of O_2, resulting in cyanotic damage (Apitz *et al*., 2009). This patient group is the primary source of cyanotic tissue used for experiments within this project.

1.1.4 Sub-aortic stenosis (control)

Sub-aortic stenosis (SAS) is a congenital heart defect that requires corrective heart surgery involving CPB. This condition is characterized by the presence of a fibrous ring in the left ventricular outflow tract located beneath the aortic valve (Gersony, 2001). The prevalence of this condition is 0.25 for every 1000 live births (Grech, 2001). This condition is not always apparent at birth and can also occur in conjunction or as a consequence of other congenital cardiac defects, for example, with a VSD (Gersony, 2001; Somerville *et al*., 1980).

When investigating the effect cyanosis on the myocardium and the consequences of ischemic-reperfusion (IR) injury, this is a good control group for the TOF patient group. This is because these patients experience no cyanotic damage but still require corrective cardiac surgery with CPB.

1.2 Cardiac surgery

There are a high number of cardiovascular pathological conditions involving both children and adults that can occur and require surgical intervention. The extent of the surgical procedure will vary depending on the nature of the defect. For example, in this study we have a specific interest in the surgical correction of congenital heart defects such as TOF and SAS, both of which require open-heart surgery with the use of a CPB machine (Starr and Hovaguimian, 1994; Carrascal Hinojal *et al*., 2006; Hraska *et al*., 2007; reviewed in Bailliard and Anderson, 2009).

1.2.1 Cardio-pulmonary Bypass (CPB)

The time period between the initial proposition and the final development of the CPB machine, where the circulating blood of the body could be diverted, properly oxygenated and

anti-coagulated, occurred over several decades. Its concept would not have been possible if not for the discovery of heparin, an anti-coagulant, which was necessary due to the fact that blood clotted when it came into contact with non-endothelial type surfaces (reviewed in Stammers, 1997). The first successful cardiac surgical procedure that used a CPB machine was one in 1953 where an atrial septal defect (ASD) was repaired in an 18-year patient (Gibbon, 1954). This itself was not followed by consistent success and the process underwent many advances in last 10-20 years. Open-heart surgery is now thought of as a relatively routine procedure (reviewed in Stammers, 1997).

The function of the CPB unit (which is highlighted in figure 1-2) is to temporarily replace and perform the basic functions of the heart and lungs i.e. to pump blood throughout the systemic circulation and to regulate both the levels of O_2 and carbon dioxide (CO_2) present within the bloodflow. It achieves this by diverting blood flow from the heart into the CPB unit (A), where gas exchange (B) occurs and energy of bloodflow (C) is provided via the pumps, then bloodflow returns to the arterial circulation (D). The coronary circulation within the heart is prevented and returned by the use of the cross-clamp (E) (reviewed in Paparella *et al*., 2002). To better describe the different stages of CPB and the effect it has on the heart, the different phases of the process are shown in table 1. The use of cardioplegia and the cross clamp, which is used to artificially arrest the heart during bypass, is described in the next section.

Figure 1-2. Cardio-pulmonary bypass unit

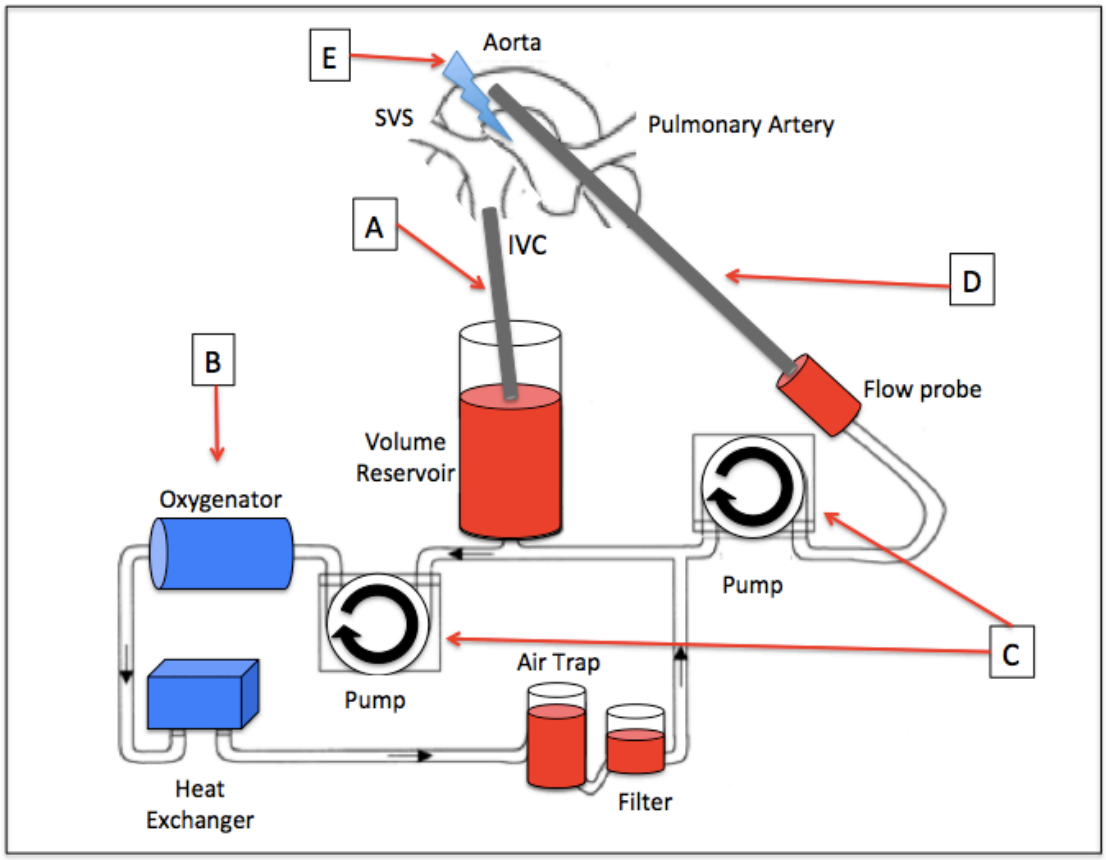


Figure shows the CPB circuit. (SVC – superior vena cava, IVC – inferior vena cava) (Modified from Dunbar *et al*., 2001) (A – systemic blood flow diverted from the heart into the CPB unit, B – gas exchange, C – movement of bloodflow via pumps, D – bloodflow returns to the arterial circulation, E – application of aortic clamp to stop coronary circulation).

Table 1. Phases of CPB

Phase	Conditon	Heart	Oxygen
1	In bed, room air		21% (ambient air)
2	Anesthetized, before bypass	Perfused Beating Pumping	50% (on ventilator)
3	On bypass	Perfused Beating Not pumping	High O_2
4	Cross-clamp and cardioplegia	Not perfused Arrested Not pumping	Ischemia (no coronary circulation)
5	Cross-clamp off	Reperfused Beating Not pumping	High O_2
6	Off bypass	Perfused Pumping	50% (on ventilator)

Period of intra-cardiac repair

During the use of CPB (see table 1), the heart and the systemic tissues of patients are exposed to a high level of O_2 primarily due to the re-oxygenation (B) of the blood within the extracorporeal unit of the CPB machine before it returns to the body. The level of O_2 used during both paediatric and adult cardiac surgery with CPB will often involve hyperoxic levels (Ihnken *et al*., 1998; reviewed in Allen *et al*., 2001). The systemic tissues are exposed to the highly oxygenated blood throughout the entire surgical procedure (on bypass) and the heart is exposed to this hyper-oxygenated blood before and after the application of the cross-clamp.

1.2.2 Protection of the myocardium from CPB

During repair of intra-cardiac defects (see figure 1-3 for an overview of the timeline of cardiac surgical repair with CPB) it is necessary to stop heart contraction. This is achieved by interrupting the myocardial blood flow (application of the cross-clamp) and infusing the heart with a myocardial paralysing solution (cardioplegia). This is done to prevent heart contraction in order reduce energy consumption and help prevent the exhaustion of metabolites. This is the mainstay of myocardial protection during cardiac surgery with CPB. Such protection is especially important for children with congenital heart defects such as TOF (see section 1.1.3) as they are thought to have more vulnerable myocardium (Merante *et al*., 1998; Najm *et al*., 2000; Corno *et al*., 2002).

1.2.2.1 Cardioplegia

Cardioplegia in its most basic form is a solution containing high potassium (K) content (20µMol/l) and the most common type is known as St. Thomas' Hospital cardioplegic solutions. These hyperkalemic (high K content) solutions result in depolarised cardiac arrest where the increased levels of K cause a change in the resting membrane potential (E_m) across the plasma membrane of the myocytes that will ultimately reach a point where mechanical contraction is terminated in diastole (reviewed in Chambers, 2003). It should be noted that the level at which K is elevated to should be tightly monitored because if such levels were to increase exponentially, the end result would be calcium (Ca^{2+}) overload causing potentially irreversible consequences (reviewed in Chambers, 2003). There are various types of cardioplegia, where it can be either crystalloid or blood based and where adenosine, instead of K, can be used as the paralysing agent. In whichever formulation, in the current era, essentially all paediatric cardiac surgery is conducting using cardioplegia.

As already stated, cardioplegia solution can be either crystalloid or blood based and over the years it had been found that blood-based cardioplegia has become the preferred type of transporter of hyperkalemic solution. Blood based cardioplegic solution was first suggested in 1979 as an alternative to crystalloid based hyperkalemic solution and there have been several studies to suggest it can provide better protection to the heart in comparison to crystalloid solutions but there is also data that shows there is no major clinical benefit of one over the other (Buckberg, 1979; Robinson *et al*., 1995; Mezzetti *et al*., 1995; Bolling *et al*., 1997; Young *et al*., 1997; Feindel *et al*., 1984; Ferreira *et al*., 2003; Guru *et al*., 2006; Jacob *et al*., 2008). In relation to paediatric surgery specifically, there is some of evidence that blood cardioplegia provides better myocardial protection compared with crystalloid cardioplegia but overall the literature findings are inconclusive (Caputo *et al*., 2002; Amark *et al*., 2005).

The type of cardioplegic hyperkalemic solution described so far is extracellular but there is an alternative intracellular solution known as cardioplegic solution HTK. It also causes depolarised arrest but does not cause it to the same degree and therefore could be a better alternative (Bretschneider, 1980; Krohn *et al*., 1989; reviewed in Fallouh *et al*., 2008).

Despite the St. Thomas' Hospital cardioplegic solutions being the most widely used, they may not provide the most optimal method of myocardial protection during cardiac surgery with CPB, with them causing potential complications of their own (reviewed in Chambers and Fallouh, 2010). In fact, there was a study performed with infant hypertrophied rat hearts and it was shown that St. Thomas' Hospital cardioplegic solution provided no enhanced protection against ischemic exposure (Karck *et al*., 1996). Alternatives to St. Thomas' Hospital cardioplegic solution include the use of polarised cardiac arrest instead, where the E_m across the membrane is kept closer to normal levels i.e. the resting potential. This can be done by various methods, including the use of sodium (Na) channel blockers and K channel openers (Snabaitis *et al*., 1997; Cohen *et al*., 1993; reviewed in Chambers, 2003; Dobson and Jones, 2004; reviewed in Fallouh and Chambers, 2008).

1.2.2.2 Temperature of cardioplegic delivery to the heart

Another consideration is the temperature at which the cardioplegic solution is delivered to the heart during cardiac surgery requiring CPB. Mild (32-34°C) and moderate (25°C) hypothermia is used fairly regularly in clinical practice (Belway *et al*., 2011; Stocker *et*

al., 2011). Deep hypothermia (<20°C) was used very early on during the first attempts at cardiac surgery. The heart and lungs can be protected at this temperature for about 15-20 minutes. Such procedures were used to repair very basic cardiac defects, such as ASDs, which lasted only a few minutes but carried significant risk. Therefore, the use of such procedures was quickly discontinued.

For both children and adults, there has been either slight or no difference found in post-operative outcome between the use of mild and moderate hypothermia during cardiac surgery with CPB (Nathan *et al*., 2007; Eggum *et al*., 2008; Stocker *et al*., 2011; Ali Aydemir *et al*., 2012; Leshnower *et al*., 2012). Despite the routine use of mild or moderate hypothermia, some evidence suggests that normothermic (37°C) cardiac surgery is as clinically beneficial (Durandy and Hulin, 2006; Durandy and Hulin, 2007; Poncelet *et al*., 2011; reviewed in Durandy *et al*., 2008). An interesting study was performed where several different methods of cardioplegia delivery were undertaken during surgical repair of congenital cardiac defects such as TOF. It was found that cold blood cardioplegia with hot shot (warm blood cardioplegia) was beneficial for cyanotic patients while for acyanotic paediatric patients it was found not to matter (Modi *et al*., 2004). These findings were confirmed by a later study by the same research group (Modi *et al*., 2006). This suggests that temperature is more of an issue for cyanotic patients over acyanotic patients, highlighting how much more vulnerable these patients could be to potential myocardial damage caused during heart surgery with CPB.

There are 2 main reasons that hypothermia is used during CPB. The first is that the lower temperature is thought to lower the body's metabolic rate and therefore reduce potential damage done by IR injury during cardiac surgery. In essence it is augmenting the protection given by cardioplegia, which artificially arrests the heart in order to help preserve metabolites within the heart needed for basic myocyte function. The second reason is the situation where bypass has to be temporarily delayed or stopped due to a complication; the lower temperature will provide some protection for the body, especially the brain, from the consequential low flow of blood.

1.2.2.3 Oxygen tension used during CPB

When on bypass, patients are often exposed to high levels of O_2 (see table 1). This primarily occurs when the blood passing through the CPB machine, which is oxygenated to a

high O_2 tension, is returned to the systemic circulation (Ihnken *et al*., 1998; reviewed in Allen *et al*., 2001). This ensures that all distal organs of the body, especially the brain, are properly protected during surgery by being adequately perfused. There are however some concerns about the use of such high levels of O_2 in such a clinical setting (see section 1.3).

1.2.2.4 Alternatives to surgery with CPB

The combination of using CPB and cardioplegic arrest during cardiac surgery and all that is involved can contribute to post-operative myocardial dysfunction and low cardiac output state. This can be caused by a wide range of factors that are described in more detail elsewhere (see section 1.3) (Kirklin *et al*., 1983; Menasche, 1995; reviewed in Kozik and Tweddell, 2006).

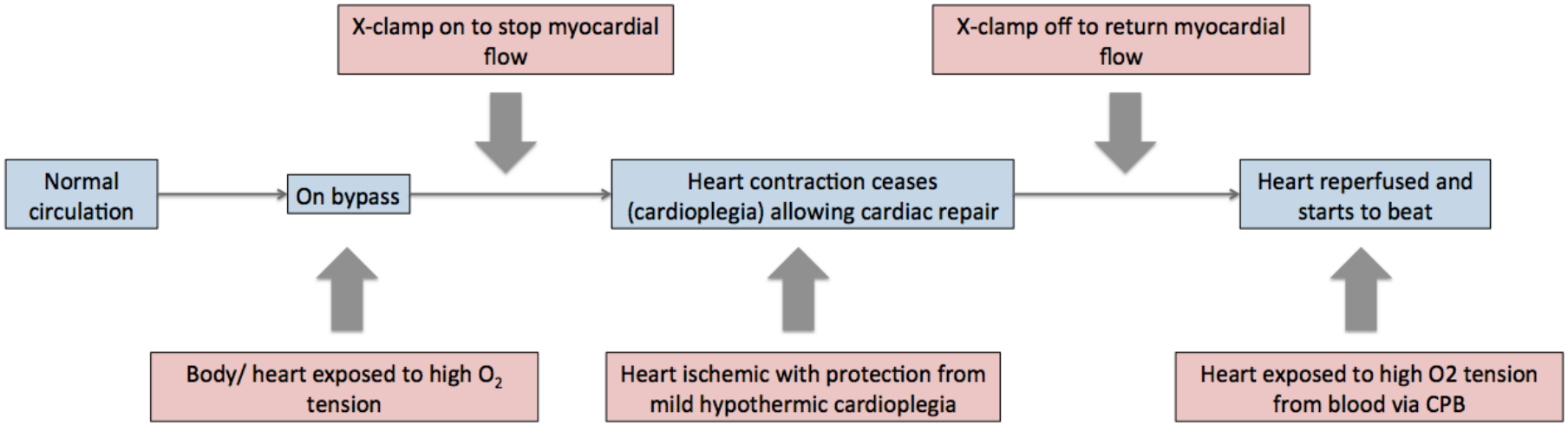
Alternatives to using cardiac surgery with CPB have been explored, such as the use of a minimal extracorporeal circulation system, which has been shown to reduce inflammation compared to use of more conventional CPB (Fromes *et al*., 2002). It has also been suggested that using 'off pump' surgery might be a viable option as it has been shown that there is less inflammation, oxidative stress and other post-operative damage than when compared with surgery using CPB (Brasil *et al*., 1998; Strüber *et al*., 1999; Matata *et al*., 2000). It should be noted that such a procedure would not be possible for the surgical repair of congenital cardiac defects.

1.2.2.5 Conclusion

Overall, the resulting gold standard of myocardial protection during open-heart surgery with CPB and the resultant ischemic period includes a combination of localised hypothermia and temporary paralysis of myocardial contraction by means of cardioplegia (crystalloid or blood), which will ensure the preservation of cardiac ATP reserves and help keep cardiac function normal post-operatively. This would not be possible without such measures, as the prolonged ischemia would cause severe myocardial damage (Bretschneider *et al*., 1975; reviewed in Reimer *et al*., 1983). Other important considerations are an adequate perfusion technique when the CPB is undertaken that often involves using hyperoxic levels of O_2 throughout surgery. Also, a good standard of surgical technique and proficiency in administration of anaesthetic drugs during the surgical procedure are important (reviewed in Durandy, 2008). Although such methods are adequate and widely used, alternative, better

methods of myocardial protection will always be beneficial as they would improve the success of surgery and enhance overall post-operative patient outcome. The question is: can investigating the molecular mechanisms involved in IR injury during paediatric cardiac surgery allow an improvement in current methods of myocardial protection?

Figure 1-3. Timeline of cardiac surgical repair with CPB



1.2.3 Surgical correction of TOF

One of the patient groups of interest in this study are patients suffering from the paediatric heart condition known as TOF (see section 1.1.3). Repair of this condition always involves open-heart surgery with CPB (reviewed in Kunes *et al*., 2007). The method of treatment depends on the severity of the defect. If the O_2 blood saturation level is below 70% when the baby is born, palliation (to reduce severity of symptoms) i.e. a shunt procedure is performed. Then total repair of the defect is performed as a secondary procedure when the baby is about 3-6 months. If the baby's blood saturation level is about or above 70% when born, then total repair is done as a primary procedure but not until the baby is 3-6 months.

The shunt procedure involves using a piece of synthetic material to divert blood leaving the heart back to the lungs. This provides an adequate flow of blood to the pulmonary circulation allowing sufficient re-oxygenation of enough blood that will alleviate the symptoms of TOF. Total cardiac surgical repair of TOF requires CPB and aortic-clamping. In this surgical procedure the VSD is closed by the use of a patch and the PS is widened when the obstruction is severe enough (reviewed in Bailliard and Anderson, 2009).

1.2.4 Surgical correction of sub-aortic stenosis (SAS)

Surgical correction of this condition (see section 1.1.4) requires cardiac surgery with CPB. The fibrous tissue causing the obstruction is removed once identified, with care being taken not to damage the aortic valve. In addition, a myectomy (excision of muscle) is often performed in order to make the ventricular outflow track larger and in order to help reduce the likely hood of the SAS from returning, which would require additional repair (Starr and Hovaguimian, 1994; Carrascal Hinojal *et al*., 2006; Hraska *et al*., 2007).

1.3 Consequences of open-heart surgery

Many congenital cardiac defects, including TOF and SAS (both previously defined in sections 1.1.3 and 1.1.4 respectively), require surgical correction. Such procedures usually involve aortic-clamping and the use of CPB (see section 1.2.1). Due to its invasive nature and the inevitable ischemic changes that occur, open-heart surgery will result in various forms of insult, which are described in more detail in the present section. The 3 different sources of such damage include direct physical trauma, myocardial IR injury and systemic inflammation

caused by CPB. Such damage needs to be minimized in order to help protect the myocardium and promote post-operative survival of patients undergoing such procedures. Current protective methods have already been described (see section 1.2.2).

1.3.1 Direct physical trauma

The first type of insult is the physical damage caused by the surgery itself and its associated inflammation (Gu *et al*., 1999; Tárnok *et al*., 1999; Prondzinsky *et al*., 2005). Such damage will result from tissue being cut, stitched or retracted during surgery. This damage will universally affect a number of different cell types within the heart, including cardiomyocytes, endothelial cells, vascular smooth muscle cells and connective tissue cells. It will often result in necrotic cell death where the cellular damage is beyond repair and can result in an inflammatory response (reviewed in de Jong *et al*., 2009).

1.3.2 Global myocardial IR injury

Global myocardial IR damage is caused by the application and subsequent release of the aortic-clamp and will cause an associated inflammatory response (Zahler *et al*., 1999; reviewed in Kunes *et al*., 2007). The heart will be exposed to ischemia upon application of the aortic-clamp due to the removal of the coronary blood flow through the heart, the period of which will vary depending on the type of surgical correction being performed (Attia *et al*., 1976; Nagashima, 1993; reviewed in de Jong *et al*., 2009). This will cause a reduction in the available O_2 and associated nutrients that are essential for proper heart function. The most important nutrient required for heart function is ATP, which is produced by oxidative phosphorylation within the mitochondria. This process requires O_2 therefore a deficiency of this will be severely detrimental to cardiac cell function (Ferrari, 1996). Therefore, during total ischemia, the process of oxidative phosphorylation will be inhibited, preventing the production of ATP via this pathway (Lesnefsky *et al*., 1997). This will cause a shift from aerobic to anaerobic glycolysis, which allows a boost in ATP production to occur in the absence of O_2 but with an adverse build up of lactic acid as well as hydrogen (H) ions (protons) from ATP hydrolysis (Allen and Orchard, 1987), a depletion of creatine kinase (CK) and an increase in inorganic phosphate levels (Allen *et al*., 1985). The majority of ATP produced from anaerobic glycolysis is used to preserve the functionality of ion pumps situated in the plasma membrane of cardiac cells (Weiss *et al*., 1985). This is in contrast to what happens during normoxic conditions within the heart where the majority of energy is used for

muscle cell contraction, with only a small amount being used for the maintenance of ion pumps (reviewed in Solaini and Harris, 2005).

Anaerobic glycolysis cannot be maintained for long due to the fact that the amount of ATP produced is insufficient to maintain cardiac cell function. Also, the accumulation of lactate and H ions, which causes a decrease in intracellular pH (acidosis) and an increase in the intracellular concentrations of Na^+ and Ca^{2+}, will also result in the inhibition of anaerobic glycolysis (Rovetto *et al*., 1975). This will cause an overall fall in available ATP, which combined with the other changes caused by anaerobic glycolysis will have the end result of inhibiting cardiac muscle contraction, which is the primary function of cardiac tissue (Hohl *et al*., 1982; Kingsley *et al*., 1991; reviewed in Doenst *et al*., 2008). This will eventually result in cardiac cell death if not reversed. During cardiac surgery, this problem is largely overcome by the use of cardioplegia and hypothermia to slow down cardiac cell metabolism in an effort to preserve ATP content (Opie *et al*., 1971). Although it has been shown that a fall in ATP is associated with loss of contraction in cardiac tissue during ischemia, it is not the sole cause (Kentish *et al*., 1986; Guth *et al*., 1993). Another cause of the ATP deficit is the continued functionality of mitochondrial ATPase during cardiac tissue ischemia that converts ATP to adenosine diphosphate (ADP) (Jennings *et al*., 1991).

The duration of the ischemic period is very important to the extent of the damage done to the cardiac tissue. The duration of safe ischemia (without cardioplegia) at normothermia is very short being only a few minutes. This would be extended to about 20 minutes if the tissue was at 20°C (deep hypothermia) or below. During cardiovascular disease, an occlusion of blood flow within the heart can cause the formation myocardial infarct (MI) if the period of ischemia is prolonged. Cell integrity in the cardiac tissue will become severely compromised, causing full contracture and final necrotic cell death. The affected areas will eventually form scar tissue known as a MI through action of the immune system (Jennings *et al*., 1957; Porter *et al*., 1893; Bretschneider *et al*., 1975; reviewed in Doenst *et al*., 2008). This highlights the potential adverse effects of prolonged ischemic exposure.

Ischemic exposure can result in contractile dysfunction within the heart. Specific consequences include a process known as myocardial stunning upon short-term ischemic exposure. This is where contractile function will stop briefly but can be reversed with no long

lasting side affects. Also, prolonged periods of partial ischemia or long term exposure to low blood flow can result in myocardial hibernation, where the inactive contractile function of the heart tissue is prolonged but can still be reversed (Lavallee *et al*., 1983; Darsee *et al*., 1981; Elsässer *et al*., 1997; reviewed in Doenst *et al*., 2008).

Ischemic exposure does not always result in necrotic cell death of cardiac cells. Necrotic cell death is when an exogenous factor, such as physical trauma or infection, causes the early death of a healthy living cell. The alternative is apoptosis, which is programmed cell death. This is where biological signals within the cell deliberately cause cell death and this is usually beneficial to the organism (Fliss and Gattinger, 1996; McCully *et al*., 2004; reviewed in Doenst *et al*., 2008). In a study that used a rat animal model of MI, it was shown that the dominant type of cell death seen in cardiac cells after MI was apoptosis. Necrotic cell death did occur but not to the same magnitude. Necrotic cell death continued after apoptotic cell death and contributed to a progressive loss of cells over time after the MI (Kajstura *et al*., 1996). This highlights that the avenue of cell death during ischemia is not straight forward. Another source of damage during ischemia is thought to be the production of reactive oxygen species (ROS) by the enzyme xanthine oxidase that is found in the cytoplasm of cardiac cells (Kevin *et al*., 2003; Pacher *et al*., 2006; reviewed in Suleiman *et al* 2008). Damage sustained by induced ischemia by the use of the aortic-clamp during cardiac surgery is protected against by several methods that have already been described (see section 1.2.2).

Once the surgical corrective procedure has been completed, the aortic-clamp is removed, which will return coronary blood flow to the ischemic heart. Reperfusion is the only method upon which damage done by ischemia can be reversed, but the extent to which this can be done will depend on the length and severity of the ischemic period and can itself cause damage to the already fragile cardiac tissue (Chandra, *et al*., 1976; reviewed in Suleiman *et al*., 2008). The heart is usually exposed to high levels of O_2 upon reperfusion (release of cross-clamp) during cardiac surgery (Ihnken *et al*., 1998; reviewed in Allen *et al*., 2001). Upon reperfusion of the ischemic heart, the tissue is exposed to O_2 and ATP synthesis by oxidative phosphorylation then resumes, as does normal ion channel function and therefore normal intracellular pH is restored and cardiac cell contraction is once again initialised. Despite this, there can still be further cell death by either necrosis or apoptosis (reviewed in Solaini and Harris, 2005).

Upon reperfusion of ischemic tissue, there is an even bigger rise in the intracellular Ca^{2+} concentration than that seen during the ischemic exposure of the tissue, with the end result being contractile impairment (Kusuoka *et al*. 1991; reviewed in Suleiman *et al*., 2001). This rise in intracellular Ca^{2+} can occur either via the myocardial Ca^{2+} channels (in the sarcolemma and the sarcoplasmic reticulum) or the reversal of the sarcolemmal Na^{+}/ Ca^{2+} exchanger (Smart *et al*., 1997). Examples of such contractile impairment include myocardial stunning (described previously) and ventricular fibrillation. These are examples of reversible impairment of cardiac contraction (Smart *et al*., 1997). In more detail, myocardial stunning is caused by an abnormality in the function of both the myocardial Ca^{2+} channels and the reversal of the Na^{+}/ Ca^{2+} exchanger. In comparison, ventricular fibrillation is caused via the abnormal function of the Na^{+}/ Ca^{2+} exchanger (Smart *et al*., 1997).

Another consequence of the large increase in intracellular Ca^{2+} during reperfusion is hypercontracture of the myocardium. This usually occurs after the reperfusion of tissue exposed to prolonged ischemia (Ladilov *et al*., 1995; Siegmund *et al*., 1997; Schäfer *et al*., 2001). There are 2 ways in which contracture occurs during reperfusion. This first is known as Ca^{2+} overload induced contracture. During ischemia there is an increase of intracellular Ca^{2+}. Upon reperfusion, ATP production via oxidative phosphorylation restarts. This allows cellular contraction and intracellular ion levels (including Ca^{2+} concentration) to normalise. The recovery of contraction is more immediate than that of ion levels. Therefore due to the high levels of Ca^{2+} within the cell, a Ca^{2+} driven contraction is caused resulting in hypercontracture. The other method is known as rigor induced contracture. This usually occurs after prolonged ischemia where reperfusion of the cardiac tissue is low and repletion of ATP within the tissue is slow (reviewed in Piper *et al*., 2004). Hypercontracture has been linked as a cause of necrotic cell death during the early phase of reperfusion of ischemic tissue and therefore could potentially cause an immune response (Fujiwara *et al*., 1989; Garcia-Dorado *et al*., 1992).

A rise in intracellular Ca^{2+} during reperfusion will also cause a noticeable rise in mitochondrial Ca^{2+} (Varadarajan *et al*., 2001). This has been linked to contributing to the cellular damage caused during reperfusion of ischemic tissue due to fact that when the influx of Ca^{2+} into the mitochondria is blocked, cellular damage is reduced, ATP production in

mitochondria is highly increased and contractile function that is impaired by the influx of mitochondrial Ca^{2+} is improved (Ferrari *et al*., 1982; Peng *et al*., 1980; Grover *et al*., 1990).

ROS production during reperfusion has been described as another major source of cellular damage, which is thought to occur via the mitochondrial respiratory chain through the action of the electron transport chain (Vanden Hoek *et al*., 1997; reviewed in Solaini and Harris, 2005). The most probable source of this is ubiquinone that is reduced during ischemia to ubisemiquinone. During reperfusion, this will react with O_2 to produce ROS (reviewed in Suleiman *et al*., 2001). It should be noted that xanthine oxidase has also been implicated in the production of ROS during reperfusion, but is thought less important in the heart during such injury (Thompson-Gorman *et al*., 1990; Xia *et al*., 1995; reviewed in Suleiman *et al*., 2001).

Under normal physiological conditions, the action of antioxidants easily regulates ROS production during IR injury but during reperfusion, such systems are unable to cope due to their impairment during ischemia (reviewed in Turer *et al*., 2010). This causes an excess of ROS, which can cause damage to many cellular components including nucleic acids, lipids and proteins. Also, the combined effect of the large intracellular/ mitochondrial influx of Ca^{2+} and the production of ROS via the mitochondrial respiratory chain that causes oxidative stress can result in the mitochondrial permeability transition pore being opened (Haworth and Hunter, 1979; Sedlic *et al*., 2010; Kim *et al*., 2006; reviewed in Halestrap, 2009). If this occurs chronically it will result in irreversible mitochondrial injury, leading to complications such as the release of ROS into the cytosol from within the mitochondria and ultimately result in necrotic cell death (Griffiths *et al*., 1995; reviewed in Suleiman *et al*., 2008). Another implicated source of ROS production during reperfusion is nitric oxide (NO). This is produced by the endothelium and its production is increased during early reperfusion, where it reacts with O_2 to create ROS that will cause tissue and cellular damage (Wang and Zweier, 1996).

1.3.2.1 Level of O_2 exposure during global myocardial IR injury

As already stated, during both paediatric and adult cardiac surgery with CPB, high levels of O_2 are often used (Ihnken *et al*., 1998; reviewed in Allen *et al*., 2001). This means that reperfusion of the heart during cardiac surgery will be at a high O_2 tension. There are some concerns that such hyperoxic exposure could be a potential source of damage during such procedures. There are several studies within the literature that support this. A study

compared the use of hyperoxic (O_2 tension (pO_2)= 5400 mmHg) CPB to normoxic (pO_2= 5140 mmHg) CPB in adults undergoing coronary artery bypass grafting (CABG). Several measurements were taken within each group, including the levels of polymorphonuclear leukocyte elastase (secreted by neutrophils during inflammation), CK, antioxidants and nitrate. All of these were higher in the group exposed to hyperoxic CPB. This indicated hyperoxic CPB resulted in more oxidative myocardial damage compared to normoxic CPB (Ihnken *et al*., 1998). A similar comparison was performed during surgical correction of cyanotic congenital heart defects. Patients either had controlled normoxic CPB (PO_2= 50–80 mmHg) or hyperoxic CPB (PO_2=150–180 mmHg). Troponin I (marker of myocardial damage), 8-isoprostane (marker of oxidative stress) and protein S100 (marker of inflammatory diseases) were all lower in the controlled normoxic CPB group. These findings along with other measurements taken indicated that hyperoxic CPB resulted in higher myocardial and oxidative stress than controlled CPB (Caputo *et al*., 2009). There is also some clinical evidence that the use of hyperoxic reperfusion after cardiac arrest was associated with higher in-hospital mortality when compared to the use of hypoxic or normoxic exposure (Kilgannon *et al*., 2010).

Surgical correction of congenital defects in patients with cyanosis is carried out on bypass at high levels of O_2 (300 to 400 mm Hg) (reviewed in Morita, 2012). There are several studies that used a pig animal model, which indicated cyanotic infant myocardium (PaO_2 of 25mmHg) was highly susceptible to re-oxygenation injury when exposed to high levels of O_2 (PaO_2 400mmHg) during reperfusion (Ihnken (i) *et al*., 1995; Ihnken (ii) *et al*., 1995; Morita (i) *et al*., 1995; Morita (ii) *et al*., 1995). This has also been shown in children with cyanotic damage undergoing corrective surgery on bypass for congenital heart defects (Modi *et al*., 2002; Bulutcu *et al*., 2002). There has also been evidence that controlling re-oxygenation during the use of CPB for patients that have congenital defects with pre-operative cyanotic damage will reduce post-operative injury. One such study recruited 31 children with cyanotic damage, who were put into 2 different groups. In the first group, bypass was initiated with very low O_2 levels that were gradually increased for 5 minutes until levelling out at about 200 to 300 mmHg. In the other group, bypass was initiated at very high levels of O_2 for 5 minutes then again lowered out to 200 to 300 mmHg. Patients that had controlled re-oxygenation experienced significantly lower levels of creatine-phosphokinase-MB (marker of cardiac injury) and ventilation time (Babu *et al*., 2012). This was shown in a similar study performed

by another research group (Zhu (i) *et al*., 2005). This is supporting evidence that hyperoxic O_2 exposure during CPB could be particularly harmful to the myocardium from children with cyanosis pre-operatively if not properly controlled. Therefore protecting such children during surgery on bypass is very important.

Hyperoxia is associated with the production of ROS; therefore during hyperoxic reperfusion the damage caused by ROS would be more exaggerated than normally seen at normoxic levels (Jamieson *et al*., 1986). In the case of paediatric surgery, the infant myocardium has been shown to be more susceptible to damage due to ROS during reperfusion (Otani *et al*., 1987). In relation to children with cyanosis before surgical correction on bypass, damage caused by free radicals (shown by measurement of hydroxy conjugated dienes) was observed during TOF surgical correction before and after aortic clamping and during reperfusion (Del Nido *et al*., 1987). Also, in a study using a pig animal model, it was shown that exposing infant myocardium to mechanically ventilated hyperoxia (PaO_2= 422 +/- 33 mmHg) induced the production of ROS in the infant myocardium. Also, the ability to increase production of anti-oxidants to provide protection from such damage was reduced (Bandali *et al*., 2004). Such studies highlight the potential danger of using hyperoxic levels of O_2 during cardiac surgery with CPB. Hyperoxic reperfusion has also been shown to exasperate impaired mitochondrial function within the heart. This could result in a detrimental post-operative clinical outcome as the loss of functional mitochondria would ultimately result in necrotic cell death (Angelos *et al*., 2011).

There are some studies that show that the use of hyperoxia does not actually cause additional damage. For example, in a study using a rat model of IR injury, the animals were exposed to 40 minutes of ischemia then either ventilated with normoxic levels of O_2 (40% O_2) or with hyperoxic levels of O_2 (95% O_2). The size of MI produced was unaffected by the level of O_2 exposure. This suggests that the level of O_2 used during CPB does not affect the amount of damage caused by IR injury (Mariero *et al*., 2012). A study that investigated the use of hyperoxic exposure (>95% O_2) before IR injury in a rat animal model showed that such pre-treatment resulted in a reduction in MI formation and a reduction in the amount of apoptotic cell death. This highlighted a cardio-protective role of hyperoxia (Foadoddini *et al*., 2011). Despite all these examples, it is inconclusive whether the use of hyperoxia levels of O_2 in the clinical setting is harmful enough to discontinue its use. This was shown in a retrospective

study of the use of hyperoxic exposure and its association to mortality after cardiac arrest. The study indicated that such a relationship was not highly supported and therefore cautioned preventing the use of hyperoxic exposure after cardiac arrest (Bellomo *et al*., 2011; Hoedemaekers and van der Hoeven, 2011).

During cardiac surgery with CPB, it is important to keep the brain protected by keeping it properly perfused. This is another reason why such high levels of O_2 are used during such procedures. It has been shown that hyperoxic exposure can cause apoptotic cell death within the white matter of the developing brain in a study using a rat animal model (Gerstner *et al*., 2008). The effect of hyperoxic reperfusion on cerebral outcome was investigated using a rat animal model of global cerebral ischemia. The rats were exposed to ischemia then resuscitated on a ventilator at either 21% O_2 for 1 hour or 100% O_2 for 1 hour. It was shown that hyperoxic (100% O_2) reperfusion resulted in increased hippocampal neuronal death and more inflammation when compared to normoxic (21% O_2) reperfusion (Hazelton *et al*., 2010). Another study using a canine cardiac arrest model illustrated that the use of hyperoxic reperfusion reduced hippocampal oxidative energy metabolism in the brain (Richards *et al*., 2007). Therefore cerebral damage could be a consequence of using high O_2 levels during CPB.

1.3.2.2 Myocardial IR injury in cardiovascular disease compared to cardiac surgery

It should be noted that there are some distinct differences in the IR injury characteristic of cardiovascular disease when compared to that seen during cardiac surgery, which is the situation that we are more concerned with. During cardiac surgery, the affect of IR injury (as described above) on the tissue is minimised due to the use of cardioplegic solution and hypothermia (refer to section 1.2.2). This combination slows down the metabolic rate of the heart tissue, conserving the stability of the cardiomyocytes during the ischemic period created by the application of the cross clamp (reviewed in Turer *et al*., 2010). Cardioplegia delivered at hypothermic temperature has been shown to improve the survival of the myocardium during cardiac surgery (Drescher *et al*., 2011). However, it has also been shown that the temperature used does not influence the extent of myocardial injury (Carrier *et al*., 1994). Reperfusion during cardiac surgery is caused when the aortic-clamp is released. The blood that is returned to the heart has gone through the CPB unit and is therefore artificially highly oxygenated and contains activated factors of the innate immune system due to the blood contact with the

extracorporeal circulation apparatus (described in section 1.3.1). Therefore the heart tissue in this case is exposed to a different version of IR injury than seen during cardiovascular disease, which lacks these factors.

The physical trauma and exposure to chemicals such as anaesthetics and cardioplegia solution are absent during the occurrence of cardiovascular disease therefore the effects of these chemicals are exclusive to the model of cardiac surgery (reviewed in Turer *et al*., 2010). In the case of anaesthetics, it has been shown that their use during cardiac surgery with CPB could possibly prevent an inflammatory response instead of being a potential cause (Hambsch *et al*., 2002). For example, sevoflurane has been shown to reduce the inflammatory response produced by damage done during cardiac surgery by reducing the expression of pro-inflammatory cytokines and inhibiting the number of activated neutrophils (Nader *et al*., 2004; Nader *et al*., 2006; Kawamura *et al*., 2006).

Another major distinction when comparing cardiac surgery and cardiovascular disease is, that a MI, which is a consequence of IR injury during cardiovascular disease, is a singular localised occurance. It usually occurs in a single blood vessel and is pre-dominantly an ischemic event. Tissue is not necessarily reperfused at all and if it is this usually occurs by surgical intervention (angioplasty or CABG) or by lysis of the clot causing the occlusion within the blood vessel to be removed. As such, any reperfusion that occurs will not be for quite a while after the ischemic event. This combined with all the other differences described above makes the approach to protecting the heart from damage during cardiac surgery definitively different from what would be needed during cardiovascular disease.

1.3.3 Localised myocardial inflammation

The types of cellular insults described so far that occur as a result of cardiac surgery, such as the physical trauma and IR injury, can cause certain aspects of the innate immune system to be activated, which themselves can result in their own sources of cellular damage via the activation of an inflammatory response within the heart (Franke *et al*., 2005; Prondzinsky *et al*., 2005; reviewed in Suleiman, 2008). Also, the use of CPB during open-heart surgery can contribute to the inflammation seen but this will be discussed in more detail later (see section 1.3.4) (Wan *et al*., 2004).

The activation of the innate immune system during cardiac surgery and its related insults is very much like what occurs when the body is infected by foreign pathogens such as Lipopolysaccharides (LPS), which are recognised by pattern recognition receptors (PRRs) such as Toll like receptors (TLRs) (reviewed in Akira *et al*., 2006). It is generally accepted that some type of cellular injury needs to occur in conjunction with activation of PRRs by foreign pathogens for the initiation of the immune system. However it has been shown that dendritic cells can be activated without any foreign pathogen detection but by cells dying due to viral infection or by necrosis. This shows that an immune reaction can occur without the presence of foreign pathogens (Gallucci *et al*., 1999).

The activation of the immune system due to cell injury/death in the heart has been thought to be because of certain intracellular protein mediators and other products of IR injury that are released upon damage/ cell death. These will then interact with PRRs, such as TLRs. This will result in downstream activation of transcription factors such as nuclear factor kappa B (NF-κB) promoting a pro-inflammatory response involving both protein and cellular components of the immune system. These mediators are known as danger-associated molecular patterns (DAMPs) (Gallucci *et al*., 1999; Shi *et al*., 2000) and examples include ROS, heat shock proteins (HSPs), and fibronectin (reviewed in Turer *et al*., 2010).

As already stated, during cardiac surgery a localised immune response in the heart is caused in part due to the physical injury of the procedure. This will cause unavoidable necrotic cell death when cells are damaged beyond repair. This will release intracellular mediators that will activate cellular and protein aspects of the immune system via receptors like TLRs. This will result in inflammation and an increase in pro-inflammatory cytokines (Gu *et al*., 1999; Tárnok *et al*., 1999; Prondzinsky *et al*., 2005).

During cardiac surgery with CPB, the application and release of the aortic-clamp exposes the myocardium to both ischemia and reperfusion respectively. Both ischemia and reperfusion can cause damage both separately and combined (refer to section 1.3). An inflammatory response is triggered by the ischemic damage via pathways controlled by transcription factors such as hypoxia-inducible factor (HIF-1) and early growth response protein (EGR-1). When the aortic-clamp is released blood is returned to the heart. This blood will have been passed through the CPB machine and will be highly oxygenated and will

contain a high level of primed cellular and protein components of the innate immune system. Myocardial exposure to IR injury will cause damage and cell death, activating inflammatory cascades via receptors such as TLRs. With the addition of the primed immune system components in the blood from the CPB machine any immune response begun in the heart by IR injury will be exaggerated (reviewed in Kunes *et al*., 2007).

Such activation of the immune system because of IR injury produces high levels of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-18 (IL-18) and tumor necrosis factor-alpha (TNF-α) (Sawa *et al*., 1998; Chandrasekar *et al*., 1999; Pomerantz *et al*., 2001; Bennermo *et al*., 2004; reviewed in Kunes *et al*., 2007) and also chemokines such as interleukin-8 (IL-8) (reviewed in Suleiman *et al*., 2008). This will increase the activation of adhesion molecules on the surface of the endothelium such as intercellular adhesion molecules (ICAM-1) and vascular cell adhesion molecules (VCAM-1), which will already have been activated to an extent by direct damage done to the endothelium by physical damage and IR injury (Kalawski *et al*., 1998). The expression of these adhesion molecules means that interactions between them and leukocytes within the blood that are activated both by localised immune system activation and also activation due to the passage of blood through the CPB machine (described later in this section) is promoted. Vascular permeability is then increased, allowing the migration of primed leukocytes such as neutrophils and monocytes into the area of the reperfused heart tissue (reviewed in Kunes *et al*., 2007). One of the main dangers that are presented here is the large production of ROS by the activated neutrophils that is in addition to ROS already produced during IR injury itself. This will cause damage to tissue already vulnerable due to previous insult and will increase the amount of cell death (necrosis and apoptosis) (Palatianos *et al*., 2004). Anti-inflammatory cytokines like interleukin-10 (IL-10) are also expressed during IR injury, which has been shown to protect the heart against such damage (Jones *et al*., 2001).

Other aspects of the immune system such as the complement system and the coagulation system are also activated due to IR injury and will contribute to the inflammatory response caused (Weiser, *et al*., 1996; Tanhehco *et al*., 1999; reviewed in Turer *et al*., 2010).

As already stated, TLRs have been implicated in initiating an inflammatory response upon their activation by intracellular mediators released after necrotic cell death. Toll like

receptor 2 (TLR2) and Toll like receptor 4 (TLR4) have been shown using animal models to be involved in the inflammatory response caused by IR injury in the myocardium (Chong *et al*., 2004; Shimamoto *et al*., 2006; Hua *et al*., 2007; Sakata *et al*., 2007; Cha *et al*., 2008; Arslan *et al*., 2009; Ha *et al*., 2010). Also HSPs, which are thought to have extracellular immunological functions once released from cellular confinement, have been shown to be involved in TLR signalling that initiates inflammation in response to cardiac IR injury (Dybdahl *et al*., 2002; Zou *et al*., 2008; Ao *et al*., 2009). HSPs have been further shown to be cardio-protective against IR injury in the heart, specifically HSP20 (Fan *et al*., 2005; Zhu (ii) *et al*., 2005; Qian *et al*., 2009), HSP27 (Kwon *et al*., 2007; Lu *et al*., 2008) and HSP72 (Nakamura *et al*., 2000; Kim *et al*., 2006; Liu *et al*., 2007).

1.3.4 Inflammatory response caused by CPB

The use of CPB during open-heart surgery is associated with the occurrence of a systemic inflammatory response that is in addition to the localised myocardial inflammation seen due to the other factors previously discussed (Butler *et al*., 1993). The CPB machine performs the functions of the heart and lungs during open-heart surgery (see section 1.2.1). This involves keeping the blood pumping around the body and keeping the blood sufficiently oxygenated (Warren *et al*, 2009). Although the CPB machine does its job adequately, the blood of the body is exposed to an artificial environment when it passes through the extracorporeal circulation apparatus of the CPB machine and the blood re-oxygenation is being controlled by a non-biological mechanism. The end result is the activation of the immune system, which will cause a systemic inflammatory response. This can have a multi-factorial affect on many organs of the body, especially the heart and lungs (Butler *et al*., 1993) and if severe enough this can manifest as systemic inflammatory response syndrome (SIRS) (Sablotzki *et al*., 2002).

The immune response that occurs due to IR injury during cardiac surgical repair has been described as a more localised response in the heart itself. The activation of components of the innate immune system by the use of CPB has been shown to occur more in the systemic tissues of the body. This is mostly caused via the primed components of the immune system in the blood that have come into contact with the extracorporeal unit of the CPB machine while the patient is on bypass (Tomic *et al*., 2005). Normally the blood of the body comes into contact only with the endothelium, which has many regulatory elements that will keep

components of the immune system in check. The surface of the extracorporeal circulation apparatus of the CPB machine lacks these endogenous protective mechanisms. When the blood encounters such foreign material this will activate both cellular and protein components of the immune system within the blood. This will cause systemic inflammation when the blood encounters endothelium in the systemic tissues while the patient is on bypass. This will also increase the localised inflammation seen in the heart when the coronary blood flow is returned upon the removal of the aortic-clamp (reviewed in Warren *et al*., 2009).

Cellular activation of immune components in the blood by CPB includes the activation of leukocytes, such as neutrophils and monocytes (Wachtfogel *et al*., 1987; Cameron *et al*., 1996; el Habbal *et al*., 1995; el Habbal *et al*., 1997; reviewed in Warren *et al*., 2009), the activation of platelets (Rinder, 1991; Rinder *et al*., 1994) and an increase in leukocyte–platelet co-aggregates (Rinder *et al*., 1992; Rinder *et al* 1994; Rinder *et al*., 1996). There is also an increase in the level of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 and also a resultant increase in the level of chemokines such as IL-8 and monocyte chemotactic protein-1 (MCP-1) (Wan *et al*., 1996; Zahler *et al*., 1999; Dreyer *et al*., 2000; Wan *et al*., 1999; Yamaguchi *et al*., 2005; Nesher *et al*., 2006; reviewed in de Jong *et al*., 2009). Activated monocytes are the source for pro-inflammatory cytokines like IL-6 and pro-inflammatory chemokines like IL-8 (reviewed in Warren *et al*., 2009). This increase in pro-inflammatory cytokines and chemokines causes the activation of the endothelium and results in an increase in the expression of adhesion molecules. This in turn will encourage the migration of leukocytes from the blood into the tissue, which itself can contribute to the systemic inflammation often associated with the use of CPB (Wei *et al*., 2003). Of all organs, in the context of cardiac surgery with CPB, the heart, lungs, kidney and the liver are normally the most severely affected by such systemic inflammation (Zahler *et al*., 1999; Hirai *et al*., 2001). It should also be noted that there is also an increase in the expression of anti-inflammatory cytokines, like IL-10, interleukin-4 (IL-4), interleukin-13 (IL-13) and transforming growth factor beta (TGF-β). This will usually correct and contain, via a negative feedback loop, the immediate increase in pro-inflammatory cytokines expression that occurs during an inflammatory response. During cardiac surgery with CPB, such regulation is not sufficient to regulate the expression of pro-inflammatory cytokines (McBride *et al*., 1996; Nathan *et al*., 2000; reviewed in Kunes *et al*., 2007).

Activation of protein components of the immune system due to cardiac surgery with CPB includes the complement system, which is activated via both the alternative and classical pathways (Chenoweth *et al*., 1981; Kirklin *et al*., 1983; Kirklin *et al*., 1986; Steinberg *et al*., 1993), the kallirenin-kinin system (Saatvedt *et al*., 1995; te Velthuis *et al*., 1997; Wachtfogel *et al*., 1998), the coagulation system that can function via the intrinsic and extrinsic coagulation pathways (Hunt *et al*., 1998; reviewed in Kunes *et al*., 2007) and the fibrinolytic system (Stibbe *et al*., 1984; Khalil *et al*., 2004).

The blood that has been through the CPB and returned to the systemic circulation will have been exposed to very high levels of O_2. Such exposure has also been shown to induce an inflammatory response. Such investigation usually involved studies that looked at damage caused within the lungs. A study was undertaken that involved patients undergoing cardiac surgery with CPB who underwent inter-operative exposure to either 50% or 100% O_2. It was shown that exposure to 100% O_2 resulted in an increase in the production of pro-inflammatory cytokines within the lungs (Pizov *et al*., 2000). Another study used both TLR4 null and wild type rats to investigate the effect of hyperoxia on the expression of pro-inflammatory mediators within in the lungs. The rats were exposed to 80% O_2 for either 48 hours or 96 hours. It was suggested that hyperoxic exposure caused inflammation within the lungs and that such inflammation was dependent on TLR4 expression and the activation of NFkB (Ogawa *et al*., 2007). TLR4 signalling was investigated in neutrophils in order to see if TLR4 activation was O_2 dependent. It was shown that LPS induced TLR4 signalling was inhibited in the presence of antioxidants. The downstream production of cytokines was also prevented. This highlighted that inflammation caused by TLR4 signalling was inhibited by the absence of ROS, showing it to be linked to O_2 exposure (Asehnoune *et al*., 2004).

1.3.5 Other known complications of CPB

Other known complications from the use of CPB during cardiac surgery include excessive bleeding, neurological complications, renal problems, pulmonary dysfunction or overall organ dysfunction (Khuri *et al*., 1992; Wessel *et al*., 1993; Bellinger *et al*., 1995; reviewed in Murphy *et al*., 2009).

1.3.6 Conclusion

The outcome of the localized cardiac inflammation and the systemic inflammation described will escalate myocardial dysfunction and/ or cell death already caused by all the different avenues described above. Such inflammation is the body's immune system trying to repair the damage caused. In the context of cardiac surgery, if the myocardial dysfunction caused occurs for an extensive period of time resulting in increased cell death this could have deleterious effects on the post ventricular function of patients who have undergone cardiac surgery with CPB. In children with congenital cardiac defects such as TOF, where the myocardium has already sustained some damage previous to the surgical correction due to the characteristic defects of the condition, it is even more important to minimize any potential inflammation that may occur during cardiac surgery. This would help minimize post-operative ventricular function complications once the surgical corrective procedure has been completed. Therefore, finding new and novel ways to protect the myocardium from such damage other than those used in the current clinical practice is an important avenue of investigation. In this project, observing the molecular mechanisms active within the myocardium and the expression of their key protein mediators during such damage was the focus in trying to illuminate such potential alternative protective mechanisms. Prime candidates include TLRs and HSPs, which have both been shown to be involved in the processes described above.

1.4 Toll like receptors (TLRs)

1.4.1 What are TLRs?

TLRs are a highly conserved family of type 1 trans-membrane glycoproteins that were first discovered in the *Drosophila* fly, where the homologous gene Toll was shown to be involved in adult fly development. In more detail, it was shown to be involved in the differentiation of various tissues along the dorsal-ventral axis, but also in their immune response against fungal infection. The latter was discovered by showing that mutations in the TLR gene dramatically reduced survival after such infection (Lemaitre *et al*., 1996). A human homologue of this *Drosophila* gene was then identified in a subsequent study, characterized later as TLR4. This was shown to be involved in the mammalian inflammatory response, specifically involving the activation of NFkB and the resulting activation of pro-inflammatory cytokines. TLR4 has now become one of the best characterized TLRs overall (Medzhitov *et al*., 1997). This role of TLRs in the innate immune response was also shown in a study where

mutant TLR4 mice were more prone to LPS infection due to their presumed inability to detect the pathogen in comparison with control mice, again showing the role of TLRs in innate immunity (Poltorak *et al*., 1998). TLRs were soon being recognised as primary initiating elements of the innate immune response.

TLRs are part of the Toll/interleukin-1 receptor superfamily. They all have similar methods of downstream signalling due to a universal Toll/IL-1R/Resistance (TIR) motif, which activate the same inflammatory signalling pathways (reviewed in Liew *et al*., 2005). So far 13 mammalian TLR have been identified (Nomura *et al*., 1994; Taguchi *et al*., 1995). Of these, TLR1-10 are functional in humans but TLR11-13 have been lost from the human genome. Twelve are functional in mice, except for TLR10, which has been rendered useless due to the insertion of a retrovirus (reviewed in Kawai and Akira, 2010).

1.4.2 What do TLRs do?

TLRs recognise their own unique ligands known as pathogen associated molecular patterns (PAMPs). These are summarised in table 2. This specificity of ligands for the different TLRs was originally highlighted by the fact that all responses of the mammalian immune system to LPS infection (pathogen) were affected by a mutation found at a single gene locus called *Lps.* This was later shown to encode the TLR4 protein (Watson and Riblet, 1974; reviewed in Beutler *et al*., 2004). This was also highlighted (in a study already mentioned) by the fact that mice with a mutant TLR4 gene were more susceptible to LPS infection when compared with control mice, again showing the specificity of TLR4 for the detection of LPS (Poltorak *et al*., 1998). Based on such studies, TLR4 and its interaction with LPS soon became one of the best characterization of the ligand specificity of TLRs. Due to the diversity in the PAMPs that can be detected by TLRs, which include molecules from protozoa, bacteria, fungi and viruses, TLRs provide a wide ranging system of detection that make them indispensable to the ability of the innate immune system to recognise foreign pathogens. Due to their ability to detect these PAMPs, TLRs are known as PRRs and TLRs are the best characterized overall.

Table 2 – Human TLR microbial, viral and HSP ligands

TLR	Ligand	References
1	Triacylated lipopeptides (with TLR2)	Jin *et al*., 2007
2	Bacterial lipopeptides, glycans, glycolipids, HSP70	Takeuchi *et al*., 1999; Gantner *et al*., 2003; Campos *et al*., 2001; Asea *et al*., 2002
3	Viral double-stranded RNA	Alexopoulou *et al*., 2001
4	LPS, Respiratory Syncytial Virus, HSP60,	Poltorak *et al*., 1998; Monick *et al*., 2003; Ohashi *et al*., 2000; Asea *et al*., 2002
5	Flagellin	Hayashi *et al*., 2001
6	Lipotechoic acid and diacylated lipopeptides (with TLR2)	Kang *et al*., 2009;
7	Viral single stranded RNA	Heil *et al*., 2004; Diebold *et al*., 2004; Lund *et al*., 2004
8	Viral single stranded RNA	Heil *et al*., 2004
9	Unmethylated DNA (CpG motifs), viral genomic DNA	Hemmi *et al*., 2000; Lund *et al*., 2003
10	Yet to be identified	

The recognition of PAMPs was very much based on the theory that the body's immune system operates solely on recognising 'self' from 'non self' (Janeway, 1989). This idea did not account for the initiation of the immune system by non-pathogenic insults such as ischemia or oxidative stress and so another explanation was proposed. It was thought that the innate immune system could recognise any threat to the body that would be characterized by danger signals produced upon cell or tissue injury/ death (Matzinger, 1994). In time some PPRs, including TLRs, were shown to recognise such danger signals that were endogenous ligands, which became known as DAMPs (Gallucci *et al*., 1999; Shi *et al*., 2000). For example, TLR4 can recognise heat shock protein 22 (HSP22), heat shock protein 60 (HSP60) and heat shock protein 72 (HSP70). All 3 are released extracellularly as a direct result of tissue damage and initiate downstream signalling resulting in NFkB activation and a pro-inflammatory response (Asea *et al*., 2002; Dybdahl *et al*., 2002; Ohashi *et al*., 2000; Roelofs *et al*., 2006; Vabulas *et al*., 2001; Vabulas *et al*., 2002; de Graaf *et al*., 2006; Galloway *et al*., 2008; Ao *et al*., 2009). TLR4 can also recognise fibrinogen and soluble heparan sulfate (Smiley *et al*., 2001; Johnson *et al*., 2002; Johnson *et al*., 2004). TLR2, another well-characterized TLR, can also recognise

HSP70, HSP60 but also hyaluronan fragments all of which will trigger an immune response (Asea *et al*., 2002; Vabulas *et al*., 2001; de Graaf *et al*., 2006; Galloway *et al*., 2008; Scheibner *et al*., 2006). HSPs are described elsewhere in more detail (see section 1.6) and they are known mainly for being induced by a cellular stress such as IR injury and their cardio-protective properties (Jayakumar *et al*., 2001; Kirchhoff *et al*., 2002; Okubo *et al*., 2001). Another more recently discovered DAMP is high mobility group box (HMGB) 1, which can activate leukocytes once released from cells via necrotic cell death and can interact with TLR2 and TLR4 (Scaffidi *et al*., 2002; Park *et al*., 2004). Based on this, TLRs are prime candidates of investigation for attenuating damage and inflammation caused during cardiac surgery with CPB. This is due to the fact that DAMPs released upon cellular injury can initiate TLR downstream signalling. The release of DAMPs has been thought to occur in a number of ways, which include the release from the cell upon necrotic cell death, cell secretion and release via enzymatic activity (reviewed in Gill *et al*., 2010).

1.4.3 The role of TLRs in the immune system

TLRs obviously play an integral role in the body's immune system, predominately in the innate response but with some interaction with the adaptive. This is highlighted by the fact that the majority of TLRs are expressed on many components of both the innate and the adaptive immune system. For example, in a study that investigated TLR expression in leukocytes, Toll like receptor 1 (TLR1) was expressed on monocytes, polymorphonuclear leukocytes, T cells, B cells, and natural killer cells. TLR2, 4, and Toll like receptor 5 (TLR5) were expressed on myelomonocytic cells and Toll like receptor 3 (TLR3) was only expressed on dendritic cells (Muzio *et al*., 2000). Another study showed the differential expression of TLR1-5 on monocytes as they matured into infant dendritic cells and a marked increased in TLR3 expression was observed while the rest tended to have a reduced expression during this transition (Visintin *et al*., 2001). In another study it was shown that TLR2 was expressed on infant dendritic cells via which lipopetides could initiate their maturation (Hertz *et al*., 2001) and also TLR2 expression was shown to occur on macrophages (Underhill *et al*., 2000). In other studies, TLR2 expression was shown on T cells and TLR4 expression was shown on B cells (Mokuno *et al*., 2000; Ogata *et al*., 2000).

TLRs are not just expressed on components of the immune system but are also expressed in epithelial cells, for example TLR2, 3 and 4 have been shown to be expressed in intestinal epithelial (Cario *et al*., 2000). Also TLR2 and 4 were activated when interacting with their cognate ligands in human epithelial cells (Faure *et al*., 2001). Another important fact about TLR expression is that they have 2 different intracellular localities. TLR3, 7, 8, and 9 are expressed in intracellular vesicles like endoplasmic reticulum (ER), endosomes and lysosomes and recognise mainly microbial nucleic acid. TLR1, 2, 4, 5, 6, and 10 are expressed on the plasma membrane where they usually detect, and are activated by, microbial membrane protein components (reviewed in Gill *et al*., 2010). The function and ligand detection of human TLR10 is still being investigated. In fact in a recent study it was shown to be able to dimerize with itself, TLR1 and TLR6. It also directly interacted with a TLR adapter protein called myeloid differentiation factor-88 (MYD88), described later, which is essential for TLR downstream signalling. It was also shown that there was no functional form of TLR10 in mice. This confirms previous finding, however a functional form was found in the rat genome where it was expressed on B cells and dendritic cells (Hasan *et al*., 2005). Another recent study showed that TLR10 directly interacted with TLR2. It was thought that this dimer's ligand would likely be triacylated lipopeptides and other microbial ligands. This resulted in the recruitment of MYD88 but did not involve the typical downstream signalling seen with other TLRs that would usually result in NFkB activation (Guan *et al*., 2010). Also, another study indicated that TLR10 expression was induced by both hypoxia and ROS. This then initiated the NFkB cascade pathway in human monocytes, highlighting a possible role of TLR10 in inflammation, but such data as present is limited (Kim *et al*., 2010). TLRs have also been shown to have a role in apoptosis (Ward *et al*., 2005; Goyal *et al*., 2002; Lombardo *et al*., 2007).

1.4.4 TLR signalling

Figure 1-4. TLR signalling of membrane bound TLRs

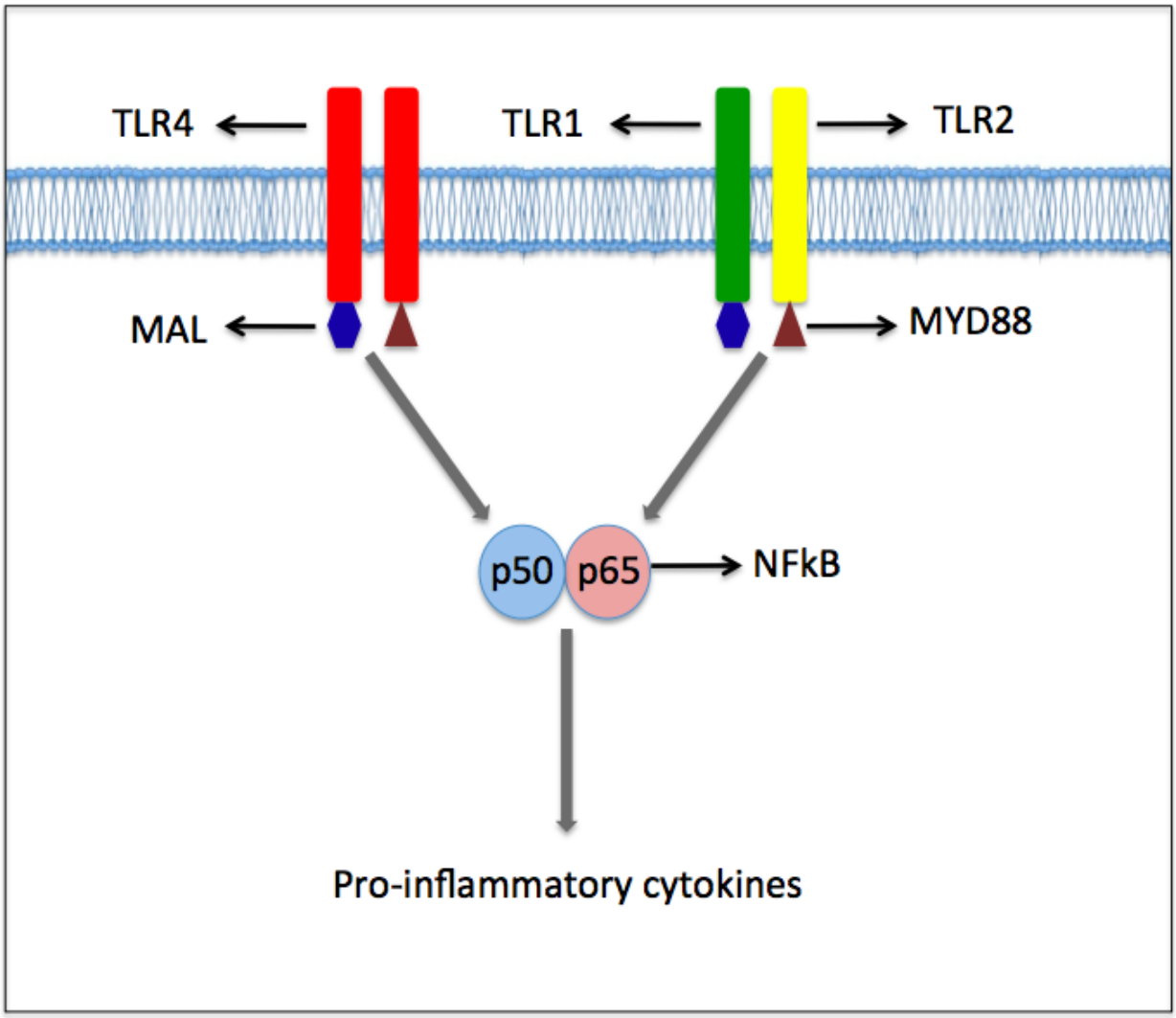


Figure showing the downstream signalling pathways of TLR1, 2 and 4, which involve the adapter proteins MYD88 and MAL. This results in the activation of NFkB signalling pathways. TLR1 and TLR2 form a heterodimer, while TLR4 forms a homodimer in order to initiate downstream signalling. This is the pathway relevant to work within this thesis.

TLR signalling (shown in figure 1-4) is generally accepted to occur by TLR protein dimerization, either with the formation of homodimers or heterodimers. It is known that TLR1 and 6 form heterodimers with TLR2 (Farhat *et al*., 2008). TLR4, 9, 3 and 5 are believed to form homodimers and recently TLR8 was shown to from heterodimers with TLR7 and 9 (Wang *et al*., 2006; reviewed in O'Neil and Bowie, 2007). All TLRs, except TLR3, signal via an adapter protein known as MYD88, which itself has the TIR motif already described allowing it to interact with the TLRs themselves and enable downstream signalling (reviewed in Liew *et al*., 2005). MYD88 was initially indicated to be involved in TLR signalling in a study that involved MYD88 null mice being exposed to LPS, the microbial ligand of TLR4. This resulted in the failure of LPS induced gene expression indicating MYD88 as a mediator of TLR4 expression. It was also noticed in this study that this lack of LPS gene induction was only partial, suggesting that there were other adapter proteins involved with TLR4 signalling (Kawai *et al*., 1999). Four other well known adapter proteins have been identified, which also have the TIR motif. These are known as MYD88 adaptor-like protein (MAL) or Tirap, TIR domain-containing adaptor-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). They are all involved in TLR signaling to some degree, allowing more specific activation of downstream signaling depending on the TLR activated (reviewed in Beutler *et al*., 2004).

The downstream signalling of TLR5, 7, 8, 9 and 11 solely involves the adapter protein MYD88 while MAL/ Tirap is required in the MYD88 signalling cascade produced by the activation of TLR1, 2, 6 and 4, which results in the activation of the transcription factor NFkB and the production of pro-inflammatory cytokines such as TNF and IL-6 (Libermann *et al*., 1990; Horng *et al*., 2002; Yamamoto (i) *et al*., 2002; reviewed in Beutler *et al*., 2004). It is of no surprise that TLR1, 2 and 6 share the same signalling pathway as they all form herterodimers with each other in order to initiate downstream signalling (Farhat *et al*., 2008)**.** TRIF is involved in both TLR3 and TLR4 signalling in a MYD88 independent signalling pathway, while TRAM is restricted to the TLR4 MYD88-independent signalling pathway and is in no way involved with TLR3 activation. The MYD88 independent signalling pathway causes the activation of *Interferon regulatory factor 3* (IRF3) and the resulting production of interferon-β (IFN-β) and its associated gene pathways **(**Fitzgerald *et al*., 2003; Yamamoto (ii) *et al*., 2002; reviewed in Chao *et al*., 2009). TLR4 therefore is shown to uniquely have two

different possible avenues of downstream signalling upon activation (reviewed in Beutler *et al*., 2004).

A fifth TLR adapter protein has also been identified that is known as sterile α- and armadillo-motif-containing protein **(**SARM). This protein can inhibit TRIF signalling of TLRs, which is the signalling cascade that occurs independently of MYD88 (Carty *et al*., 2006). This is a good example of how TLR signalling can be negatively regulated, which is very important in the case of the immune system, which if not tightly controlled can lead to the production of autoimmune and inflammatory conditions.

TLR downstream signalling initiated by either PAMPs or DAMPs use the same pathways but the distinction between the outcomes of activation by each of these initiators is generally different. For example, PAMP activation generally occurs in response to pathogenic infection therefore activation of the immune system is done in order to recruit immune system cellular components to destroy the invader. The activation by DAMPs, which is caused by a cellular insult such as ischemia and/ or reperfusion will usually result in the body trying to actively heal itself from the damage produced (reviewed in Gill *et al*., 2010). For the same signalling pathways to be used for such different outcomes, other proteins will come into play. For example, in studies involving TLR4 signalling initiated by either PAMPs or DAMPs, it was shown that a protein known as MD2 was involved in TLR4 signalling in response to LPS detection but was not involved in the detection of DAMPs (necrotic cell death) (Kim (ii) *et al*., 2007). Another protein known as CD14 was involved in the recognition of both PAMPs and DAMPs. Specifically CD14 initiated an inflammatory cascade in response to necrotic cell death (Chun *et al*., 2010). As such it is important to realise that there are many other proteins involved in such immune system gene signalling.

1.4.5 TLR and cardiac surgery: the role of immune system and resulting inflammation

There is much interest in the role of TLRs in the immune system and how they induce NFkB expression and the production of pro-inflammatory cytokines. One way in which this is highlighted is the current interest of TLR signalling in inflammatory and/ or autoimmune diseases, examples including irritable bowel disease (Hausmann *et al*., 2002), Behcet's disease (Kirino *et al*., 2008) and rheumatoid arthritis (Brentano *et al*., 2005; Kim (iii) *et al*., 2007).

Such examples shows what can happen when signal transduction in innate immune system cascades, which involve TLRs, are not kept in check. This highlights the role of TLRs within the inflammatory response. Consequently, TLRs are an attractive potential therapeutic tool that could help reduce potential damage caused by inflammation. The importance of such investigation can be shown by the fact TLR signalling is known to mediate NFkB gene signal transduction, which causes inflammation and the production of pro-inflammatory cytokines. Also, TLR signalling within in the heart has an affect on the amount of damage done by IR injury that can cause such inflammation (Jones *et al*., 2003; reviewed in Zhang and Ghosh, 2001; Faure *et al*., 2000; Sawa *et al*., 1997).

1.4.6 TLR response to cardiac surgery CPB

Damage done during cardiac surgery with CPB has already been described. This includes the physical damage, the myocardial IR injury caused and the innate immune response initiated either locally within the heart or systemically (see section 1.3). Such inflammation can cause more exaggerated cardiac dysfunction by causing additional damage and if severe enough can cause myocardial cell death. Any reduction in this type of damage would therefore be beneficial in order to promote the best post-operational outcome of such invasive surgical procedures.

As already stated there are two distinct sources of inflammation caused during cardiac surgery with CPB (see sections 1.3.3 and 1.3.4). These will contribute to either localised myocardial dysfunction and/ or cell death or in systemic organ dysfunction and/ or cell death due to the circulating inflammatory mediators activated in the blood that has been through the CPB machine. When this blood encounters the endothelial tissue within the systemic circulation, immune system cascades will be activated. This in turn will contribute to the localised myocardial inflammation already present and will also affect other multiple distal organs of the body. Therefore, there are two different avenues via which inflammation would need to be prevented. It should be noted that no matter the localisation or the reason for the inflammation induced, the downstream signalling cascades initiated would be the same.

It has been demonstrated that TLRs are expressed in human endothelial tissue and it was found that TLR4 expression was significantly higher than the expression of TLR2 (Faure *et al*., 2000; Faure *et al*., 2001; Fan *et al*., 2003; Mullick *et al*., 2008). TLRs are also expressed

on cells of the immune system, for example, they have been found on leukocytes, lymphocytes and antigen presenting cells (Muzio *et al*., 2000; Xu *et al*., 2001; Hoshino *et al*., 2002; Dasari *et al*., 2005)**.** Also, TLR expression, specifically TLR4, has been shown to be located in adventitial fibroblasts, which have been shown to be immunologically active by the fact that TLR4 signalling activation within these cells produced an inflammatory signalling cascade resulting in pro-inflammatory cytokine release (Vink *et al*., 2002). TLRs are also expressed in adult cardiac tissue, for example TLR2, TLR4 and TLR5 expression was shown in cardiomyocytes. When stimulated, this resulted in the activation of the transcription factor NFkB, resulting in a pro-inflammatory response (Boyd *et al*., 2006). TLR4 expression has also been characterized in both normal and failing human adult myocardium (Frantz *et al*., 1999).

TLR expression is usually activated by pathogenic invasion. Such expression can also be caused by cellular insults such as IR injury. The activation of an inflammatory response due IR injury via TLR4 signalling has been shown to occur in the heart (Chong *et al*., 2004; Feng *et al*., 2008). It is likely that the damage caused by IR injury will result in cardiac dysfunction and/ or cell death in the myocardium. This will likely cause the release of DAMPs such as HSPs, which will initiate TLR downstream signalling. This is supported by a study that showed the release of HSP72 in plasma taken from patients who had undergone adult cardiac surgery with CPB. An additional experiment from the same study showed that monocytes from TLR4 mutant mice were unable to produce cytokine release upon exposure to HSP72. Such cytokine release was possible in the wild type mice showing that TLR4 expression was required. The use of a TLR4 antibody prevented such cytokine release caused by HSP72 and therefore an interaction between TLR4 and HSP72 was shown (Dybdahl *et al*., 2002).

TLRs are expressed in the heart, in the endothelium and on cellular components of the immune system, which are all involved in the inflammation caused during cardiac surgery with CPB. The fact that TLR activation can be caused by IR injury means that the prevention of TLR signalling within the heart could potentially help stop the inflammatory cascades and the resulting cardiac dysfunction and cell death. This is supported by the fact that in TLR4 null mice, damage caused by IR injury was reduced, indicating preventing TLR4 activation and subsequent downstream signalling reduced damage caused by inflammation (Oyama *et al*., 2004). The main question is which of the TLRs are of the most relevant for investigation in

order to prevent or limit the damage and/ or cell death escalated by inflammation caused during cardiac surgery with CPB?

TLR2 and TLR4 are both the best characterized TLRs in the literature with regards to their expression in the heart and their role in inflammation. For example, it was shown in a study where both were expressed (along with TLR5) in adult human cardiomyocytes that upon their deliberate inducement a pro-inflammatory cascade was produced via NFkB. Therefore TLR4 and TL2 are good candidates for the investigation of how to reduce inflammation in the heart during IR injury caused by cardiac surgery (Boyd *et al*., 2006). Some more examples of studies that show the role played by TLR4 and TLR2 in the immune response and resultant inflammation caused in relation to IR injury and other insults are discussed below. MYD88, an adapter protein involved in the downstream signalling of both TLR4 and TLR2, will also be mentioned.

In relation to TLR4 expression in the heart, a study was performed using mutant TLR4 mice exposed to IR injury and a reduction in infarction size was observed in the TLR4 mutant mice when compared to wild type controls (Chong *et al*., 2004). Another study, already described, supported these findings (Oyama *et al*., 2004). A study that involved using the TLR4 inhibitor eritoran in a mouse animal model resulted in a reduced infarction size and a reduced inflammatory response in relation to IR injury (Shimamoto *et al*., 2006). In the same study, using TLR4 knockout mice it was shown that the reduction in infarct size was through a phosphoinositide 3-kinase-dependent pathway (Hua *et al*., 2007). A study conducted very recently showed that the pre-administration of LPS in mice before IR injury, which would induce TLR4 expression, could provide protection against damage. This protection was abolished in TLR4 knockout mice. This TLR4 signaling occurred via MYD88 and involved inducible nitric oxide synthase (iNOS) and soluble guanylate cyclase (sGC) (Wang *et al*., 2011). An interesting study investigated TLR4 expression during heart transplants performed between TLR4 knockout and wild type mice strains. The mice were exposed to a 2 hour period of hypothermic ischemia and were then reperfused. Serum samples were taken and the levels of cytokines were determined. The mutant group had lower levels of cytokines when compared to wild type (Kaczorowski *et al*., 2007). The same group investigated which other proteins were involved in such TLR4 signalling during hypothermic IR injury. It was found

that CD14, MYD88, TRIF, and HMGB1 were all involved in the inflammation caused (Kaczorowski *et al*., 2009).

In relation to cardiac function, a TLR4 expression study was performed using TLR4 knockout mice compared to wild type mice that were exposed to IR injury. It was found that despite the knockout mice having a reduction in MI formation and cytokine release, it was found that left-ventricular developed pressure was reduced in the TLR4 knockout mice compared to wild type (Kim (i) *et al*., 2007). This was contradicted in another study. When knockout TLR4 mice were exposed to IR injury, it was demonstrated that there was a reduction in the amount of cardiac dysfunction and a reduction in the production of pro-inflammatory cytokines caused by IR injury when compared to appropriate controls (Cha *et al*., 2008).

The majority of these animal studies indicate that a lack of TLR4 expression attenuates inflammation caused by IR injury. Most studies involve the use of TLR4 knockout mice, which usually results in a reduced MI or a decrease in the expression of pro-inflammatory cytokines. All except one study disagreed, where LPS induced TLR4 expression providing protection against IR injury (Wang *et al*., 2001). The fact that TLR4 expression was induced prior to IR injury could suggest a reason for the disagreement. Activation of the TLR4 signalling pathway may in some way provide protection against the subsequent insult of IR injury. Also lack of TLR4 has been shown to reduce the incidence of cardiac dysfunction (Cha *et al*., 2008). As before, there was a study that indicated the opposite where the lack of TLR4 expression did not prevent cardiac dysfunction (Kim (i) *et al*., 2007). Based on the evidence, TLR4 provides a good example of a gene worth investigating for its role in IR injury and inflammation. One distinction that should be made is that these animal studies do not involve exposure to activated components of the immune system that would be present within the systemic circulation during the use of CPB. Also, the mice used would not have undergone anaesthetics or cardioplegia. All these factors would likely effect TLR expression seen during cardiac surgery with CPB therefore the expression seen in these animal models may not be an appropriate comparison.

TLR4 is expressed in cardiac tissue as well as on cellular components of the immune system. It was shown using both wild type and TLR4 knockout mice, treated with LPS in order to induce TLR4 expression, that a lack of leukocyte TLR4 expression was responsible

for a reduction in cardiac dysfunction. This was not seen for TLR4 expressed on cardiac myocytes. This could indicate it is the activation of TLR4 signalling in the cells of the immune system present in the blood being circulated by CPB that is responsible for cardiac inflammation and resulting cardiac dyfunction caused during cardiac surgery (Tavener *et al*., 2004). These finding were contradicted in a study performed using TLR4 knockout, chimeric and wild type mice where it was indicated that it was cardiac myocardial TLR4 expression that caused dysfunction after MI rather than TLR4 expressed on cells of the immune system (Fallach *et al*., 2010). This role of cardiac TLR4 expression was again supported by a study that showed cardiac TLR4 expression induced by LPS was involved in impairment of cardiac function (Baumgarten *et al*., 2006). In another study it was found that removal of TLR4 expression in both cardiac tissue and on cellular components of the immune system contributed to reduced contractile dysfunction (Binck *et al*., 2005). All these studies suggests that inhibiting TLR4 expression could potentially limit the consequences of cardiac surgery with CPB that can often lead to such cardiac dysfunction. However, there was some confusion over whether it was cardiac TLR4 expression or TLR4 expression on aspects of the immune system responsible for this.

In relation to TLR2 expression it was recently shown that in TLR2 null mice that were exposed to IR injury, there was no difference in infarct size when compared to wild type controls. Despite this, the contractile ability of the wild type group after IR injury was sufficiently impaired when compared to the TLR2 null mice. This highlights the potential role of TLR2 in cardiac dysfunction that can be caused by IR injury during cardiac surgery (Sakata *et al*., 2007). In contradiction to this previous study, it was shown that the absence of TLR2 expression in TLR2 mutant mice reduced MI size as a result of IR injury. Also, the number of neutrophils within the myocardium in response to IR injury was reduced in TLR2 mutant mice. Lack of TLR2 expression also provided protection against endothelial dysfunction due to IR injury and also resulted in a reduced number ROS in the cardiac tissue (Favre *et al*., 2007). Using an antibody raised against circulating TLR2 that prevented its activation, it has been shown that infarction size in mice exposed to IR injury was reduced, again highlighting the role of TLR2 in myocardial IR injury and how the prevention of TLR2 induction could be beneficial in the setting of cardiac surgery (Arslan *et al*., 2010). In a study using wild type and TLR2 knockout mice, the activation of TLR2 signaling by administration of a TLR2 ligand before IR injury caused the activation of the phosphoinositide 3-kinase-dependent pathway.

This provided protection against IR injury. Such protection was lost in knockout TLR2 mice showing the phosphoinositide 3-kinase-dependent pathway was TLR2 dependent (Ha *et al*., 2010).

Again, all these animal studies that investigate TLR2 expression mostly involve the use of TLR2 knockout mice. Again, as with TLR4, most support the premise that a lack of TLR2 expression reduces MI formation and inflammation. There were again some studies that disagreed with this. One where TLR2 expression was induced prior to the IR injury suggested that the inducement of TLR2 expression prior to the IR insult provides protection against injury (Ha *et al*., 2010). This is similar to the study for TLR4 that disagreed with the majority of the other evidence for a similar reason (Wang *et al*., 2011). Also, there was one study that used TLR2 knockout mice that indicated that lack of functional TLR2 had no affect on MI formation (Sakata *et al*., 2007).

It has already been discussed that there was some disagreement about if it was circulating TLR4 expressed on cells on the immune system or if it was TLR4 expressed in cardiac tissue that was responsible for cardiac dysfunction. This was also raised in relation to TLR2 in a few studies. In one study, where bone marrow transplantation between wild type and TLR2 mutant mice was performed, it was indicated that it was circulating TLR2 responsible for a reduction in MI formation. This study was therefore more related to myocardial inflammation rather than cardiac dysfunction (Arslan *et al*., 2010). Another study that performed similar experiments while investigating endothelial dysfunction caused by IR injury suggested that both circulating TLR2 and TLR2 expressed within cardiac tissue was responsible for a reduction in damage done by IR injury (Favre *et al*., 2007). This again highlights the question of whether is it cardiac TLR expression or TLR expression on cells of the immune system that should be investigated.

MYD88, a key adapter protein in TLR signalling (apart from TLR3), could also be a potential target in order to reduce the incidence of inflammation in relation to IR injury. Experiments have been performed in rats in which MYD88 expression was blocked by transfection and then the animals were exposed to IR injury. Infarct size was significantly smaller in the knockout mice compared to controls. NFkB expression was also reduced compared to controls, as was apoptosis due to cardiac IR injury (Hua *et al*., 2005). Using

MYD88 null mice in comparison to wild type controls it was shown that upon exposure to IR injury, the MYD88 null mice sustained less damage and less inflammation compared to controls. This study illustrates that attenuating MYD88 expression could be a potential benefit as it may reduce inflammation caused by IR injury that can be caused during cardiac surgery (Feng *et al*., 2008). MYD88 is a downstream adapter protein involved in both TLR2 and TLR4 signalling. These animal studies show that a lack of functional MYD88 attenuates MI formation and inflammation caused by IR injury. Therefore, MYD88 is a good candidate to investigate in relation to inflammation caused by IR injury during cardiac surgery, just like TLR2 and TLR4.

In relation to evidence of TLR signalling during cardiac surgery, adult patients who underwent elective cardiac surgery with CPB had elevated circulating monocyte TLR2 and TLR4 expression post-operatively, with TLR4 being significantly increased (Dybdahl *et al*., 2002). Another study investigated TLR4 signalling during cardiac surgery with CPB using a TLR4 inhibitor known as eritoran (previously mentioned). Patients were infused with the inhibitor or a placebo (control) 1 hour prior to the initiation of CPB and there were no obvious benefits seen. Specifically, no difference in inflammation or organ dysfunction was seen when comparing patients infused with eritoran to the control patient group (Bennett-Guerrero *et al*., 2007).

Patients who undergo cardiac surgery are often exposed to hyperoxic levels of O_2 during surgical procedures (Ihnken *et al*., 1998; reviewed in Allen *et al*., 2001). Such exposure can cause the induction of TLR expression and activation of the related downstream signalling pathways that lead to inflammation. This is usually more problematic for the lungs. For example, a study was performed which involved cultured alveolar epithelial cells. The study investigated the effect of acute lung injury on TLR signalling. It was shown that hyperoxic exposure for 2 hours caused an increase in the production of ROS within the cells. This caused an increase in the expression of both TLR2 and TLR4 and an increase in the production of pro-inflammatory cytokines (Huang *et al*., 2011). Another study using TLR4 null mice showed that prolonged exposure to hyperoxia (100% O_2) caused a higher degree of damage to the lungs and a higher mortality rate in the null mice when compared to controls (Zhang *et al*., 2005). Another study investigated the effect of hyperoxic exposure (80% O_2 for either 48 hours or 96 hours) on both TLR4 null and wild type rats. Such exposure caused the activation

of the NFkB pathway within the lungs, which occurred via the receptor TLR4. Such activation was reduced in the mutant mice (Ogawa *et al*., 2007). Hyperoxic exposure can cause apoptosis and in a study involving TLR4 inducible transgenic mice, it was shown that when exposed to hyperoxia (100% O_2 for 72 hours), such mice that had TLR4 induced expression had a noticeable reduction in pulmonary apoptosis when compared with appropriate controls (Qureshi *et al*., 2006). Therefore, TLR4 has been shown to have a role in both inflammation and apoptotic cell death that is caused by exposure to hyperoxic levels of O_2.

Overall, there is an abundance of evidence involving animal studies that show that TLR4, TLR2 and MYD88 have an important role during IR injury and the resulting inflammation. There are a few examples of studies that promote TLR4 and TLR2 as being cardio-protective, but these studies suggested such protection was present due to the inducement of their expression prior to IR injury (Kim (i) *et al*., 2007; Wang *et al*., 2011). Also, there is some disagreement about whether it is cardiac TLR2/ 4 expression or the expression of TLR2/ 4 on cellular components of the immune system that should be focused on. Despite this, there is enough evidence cited that TLR4, TLR2 and MYD88 are valid indicators of inflammation and therefore valid genes to investigate in this project. Specifically, how their expression is affected by cardiac surgery with CPB and the resultant IR injury.

1.5 What is Nuclear factor kappa B?

TLRs have already been described in detail as PRRs that recognize PAMPs or DAMPs. Such activation of TLRs will facilitate downstream signalling that will result in the activation of the transcription factor NFkB (reviewed in Kawai and Akira, 2007).

NFkB was first discovered in 1986 where it was shown to be a nuclear factor that bound DNA, specifically the immunoglobulin κB light chain promoter in B lymphocytes (Sen and Baltimore, 1986). NFkB is part of a protein family that contains 5 different but structurally similar subunits that will combine to make different homo and hetero-dimers in order to become functional transcription factors. In fact, dimerization is a requirement for DNA binding overall for this protein family (Logeat *et la*, 1991; Bressler *et al*., 1993). The most common dimer formed within this protein family is p50-p65. This is what is often referred to as "NFkB" and is the one most abundant in the heart. This dimer has been shown to

have a DNA binding function (Urban (i) and Baeuerle, 1991; Urban (ii) *et al*., 1991; Ganchi *et al*., 1993; reviewed in de Winther *et al*., 2005).

1.5.1 NFkB and the heart

NFkB plays a part in many different roles with the cardiovascular system. Of all NFkB dimers present in the heart, p50-p65 seems to be the most dominantly expressed (Norman *et al*., 1998). In relation to TLRs, which are one of the main inflammatory mediators of interest in this project, NFkB can be activated by TLR signalling via both the MYD88 signalling pathway and also by the MYD88 (TRIF) independent signalling pathway. These have been described in more detail elsewhere but both result in the activation of NFkB and the production of pro-inflammatory cytokines, adhesion molecule activation and activation of leukocytes, all of which are aspects of an innate immune inflammatory response that can occur within the heart (reviewed in Kawai and Akira, 2007). Evidence that supports this is a study performed using knockout mice for MYD88 and TRIF. It showed that in mice that were deficient in both proteins, NFkB activity was completely stopped in relation to TLR4 activation. Also in TRIF deficient mice, the activity of NFkB was severely reduced (Yamamoto *et al*., 2003).

Evidence of NFkB expression in the heart includes a study that investigated myocardial infarction using p50 knockout mice. This study showed that when the subunit p50 of the NFkB dimer was absent, cardiac dysfunction was reduced, showing the role of NFkB in promoting a more severe outcome during heart failure (Frantz *et al*., 2006). This is supported by another study involving heart failure that showed NFkB expression was upregulated within cardiac tissue obtained from patients with this condition (Frantz *et al*., 2003).

In relation to damage done during cardiac surgery, such as IR injury, a study was performed using wild type and NFkB knockout mice. It was shown that the lack of the p50 subunit reduced the damage caused by IR injury compared with the wild type controls (Frantz *et al*., 2007). Specific examples showing the expression of NFkB during the use of cardiac surgery with CPB includes a study involving left ventricular biopsies obtained from the adult heart during cardiac surgery with CPB. It demonstrated that NFkB promoted an inflammatory response (Valen *et al*., 2001). Another study showed that CPB induced the expression of NFkB and TNF in patients undergoing elective cardiac surgery (Meldrum *et al*., 2003).

The interest of NFkB in this project is because of its role as a transcription factor. It can activate an inflammatory response, which includes the production of pro-inflammatory cytokines (Libermann and Baltimore, 1990). Many more examples of the pro-inflammatory nature of NFkB have already been mentioned in previous sections.

1.6 Heat Shock Proteins

1.6.1 What are HSPs?

In 1962, it was observed that exposing salivary gland cells of *Drosophila busckii* to heat shock caused the formation of visible "puffs" to form on the Drosophila's polytene chromosomes (Ritossa, 1962). This indicated heat induced gene expression had occurred at these chromosomal sites and the resulting proteins of this expression became known as heat shock proteins (HSPs) (Tissières *et al*., 1974). These HSP genes are highly conserved, being found in both eukaryotes and prokaryotes (reviewed in Feder and Hofmann, 1999). They are ubiquitously expressed in all cell types and are highly expressed within intracellular compartments. These include the cytosol of prokaryotes and the cytosol, mitochondria, endoplasmic reticulum and nucleus/nucleolus of eukaryotes (reviewed in Kiang and Tsokos, 1998; reviewed in Latchman, 2001; reviewed in Srivastava *et al*., 2002; reviewed in Pockley, 2003). As different HSPs were identified they were differentiated and named based upon their molecular weight (kD) (reviewed in de Jong *et al*., 2009). About 10 different families of HSPs have been identified. Although proteins from a single family were related in structure and function, there was little homology found when members of different families were compared (reviewed in Srivastava *et al*., 2002).

1.6.2 HSP families

Examples of the main classes of mammalian HSPs, as these are most relevant to the present discussion, include HSP110, HSP90, HSP70, HSP60, HSP40, HSP32 (heme oxygenase-1), small HSP (sHSP) (e.g. HSP20, HSP27 and αB-crystallin) and ubiquitin (protease) (reviewed in Latchman, 2001; reviewed in Pockley, 2003; reviewed in Noble *et al*., 2008). Only those families that have been the best characterised will be discussed in more detail.

The HSP90 superfamily makes up the majority of cellular protein content (Jakob *et al*., 1995). It is generally expressed in the nucleus and the cytoplasm and it functions as a

chaperone protein, helping intracellular proteins maintain their formation, especially during times of cellular stress (reviewed in Noble *et al*., 2008). It plays a key role in preventing the activation of HSP gene expression via its interaction with heat shock factor one (HSF1), the key regulator of HSP activation (Zou *et al*., 1998). It also influences the function of steroid receptors (reviewed in Pratt, 1998) and has been shown to be present in the extracellular space where it is thought to interact with the body's immune system (reviewed in Schmitt *et al*., 2007).

The HSP70 superfamily is very large with at least 12 members. It is highly conserved in evolution (Hunt and Morimoto, 1985) and is the best characterised of all HSP families (reviewed in Daugaard *et al*., 2007). Of these members, the inducible form of HSP70 (HSP72) and its constitutive form HSC70 (HSP73) are both localised in the cytoplasm (Brown *et al*., 1993) and the nucleus (reviewed in Noble *et al*., 2008). HSC70, unlike HSP70, is non inducible during cellular stress, with it undertaking important housekeeping functions, while HSP70 participates in cellular defense against insult (reviewed in Noble *et al*., 2008). HSP70/ HSC70 under normal conditions act as chaperones, assisting in protein folding and transport (Shi *et al*., 1992), but under stressful conditions, HSP70 is induced to help protect the cell against damage and also helps maintain cellular stability by being anti-apoptotic (reviewed in Schmitt *et al*., 2007). Other members of the family include HSP78 that is localised in the endoplasmic reticulum and HSP75 that is localised in the mitochondria. Both HSP78 and HSP75 function as chaperones (reviewed in Noble *et al*., 2008). As well as these intracellular roles, HSP70 has also been localised at the cellular membrane, specifically on mammalian tumour cells (Multhoff *et al*., 1995) and in the extracellular space where it is thought to interact with components of the immune system just like HSP90, which is described more in detail later (reviewed in Schmitt *et al*., 2007).

The HSP60 family, also known as chaperonins, are mainly localised in the mitochondria of mammalian cells (reviewed in Horvath *et al*., 2008) where they chaperone the protein folding and transport of mitochondrial proteins, and their proteolysis when they are beyond repair (reviewed in Garrido *et al*., 2001). HSP60 is also found in the cytoplasm, where it functions as an anti-apoptotic protein (reviewed in Arya *et al*., 2007). HSP60 is regulated by its co-chaperone HSP10 when performing its functions (Shan (i) *et al*., 2003; Shan (ii) *et al*., 2003). It was shown that both chaperonins functioned together to prevent cell death in the

mitochondrial membrane of rat cardiac myocytes (Lin *et al*., 2001). However, HSP60 can also be pro-apoptotic, as both HSP60 and HSP10 were shown to promote apoptosis in the mitochondria of Jurkat T cells. They did this by binding and promoting the function of caspase-3, which is activated by cytochrome C, resulting in the acceleration of apoptosis (Samali *et al*., 1999). HSP60 has been localised to the plasma membrane, been found in the plasma and also in the extracellular space where it is thought to interact with the immune system, all of which was shown in a study of heart failure using both rat and human models (Lin *et al*., 2007; reviewed in Horvath *et al*. 2008).

Hemeoxygenase (HO), which was first discovered by Tenhunen *et al* in 1968, is an enzyme that facilitates the degradation of heme. This reaction yields the by-products carbon monoxide, ferrous iron and biliverdin, which is then further converted into bilirubin by biliverdin reductase (Tenhunen *et al*., 1968; reviewed in Idriss *et al*., 2008). It exists in 3 isoforms, the first of which is HO-1, which is known to be induced by a number of factors that include heavy metals and ROS (reviewed in Idriss *et al*., 2008) and which is also known as heat shock protein 32 (HSP32). The second isoform is HO-2, and although it is non-inducible, it is constitutively expressed in various tissues throughout the body. Its specific function is thought to be maintaining the basic homeostatic balance of haem within the body's tissues (reviewed in Idriss *et al*., 2008). The third and final isoform HO-3 that was originally found in the rat is the least characterised within the literature and does not seem to have a major function. It has even been suggested that it is in fact a psudogene similar in structure to HO-2 (McCoubrey *et al.*, 1997; Hayashi *et al*., 2004). HO-1, which is also known as HSP32, is the one of greatest interest in relation to this project (Taketani *et al*., 1989).

HSP27 (HSP25) is a member of the small HSP superfamily, the members of which range between 15 and 30 kDa (reviewed in Schmitt *et al*., 2007). HSP27 is localised mainly in the cytoplasm and the nucleus (reviewed in Noble *et al*., 2008) where it acts as a chaperone, primarily preventing protein aggregation (Ehrnsperger *et al*., 1997). The induction of HSP27 expression is caused by heat shock and other types of cellular stress (Landry *et al*., 1989; reviewed in Salinthone *et al*., 2008). As well as other members of the sHSP family, HSP27 can undergo phosphorylation and form large oligomers, the extent of which depends on the extent of cellular stress (Garrido, 2002). Such phosphorylation, which causes a conformational change in the oligomers formed by HSP27, have been shown to improve the stability of the

cytoskeleton under heat shock stress in stable Chinese hamster cells which either constitutively expressed wild-type HSP27 or an HSP27 isoform that was not able to be phosphorylated (Lavoie *et al*., 1995). HSP27 has also been shown to protect the cytoskeleton in neonatal cardiac cells (van de Klundert *et al*., 1998). HSP27 is also an anti-apoptotic protein. Its interaction with cytochrome c upon its release from the mitochondria negatively regulates apoptotic cell death by preventing cytochrome c activating caspases in the cytosol (Bruey *et al*., 2000). Another well-characterised member of the sHSP family is αB-crystallin that is ubiquitously expressed, but has particularly high expression in the heart, eye lens and in muscles and like HSP27 is an anti-apoptotic protein (reviewed in Arya *et al*., 2007; reviewed in Noble *et al*., 2008). HSP27, just like HSP60, HSP70 and HSP90 has also been localised to the extracellular space (reviewed in Calderwood (i) *et al*., 2007).

Heat shock protein 20 (HSP20) was first discovered in 1994 when it was isolated from rat and human skeletal muscle (Kato *et al*., 1994). This protein, also known as HspB6, is part of the small heat shock protein family, which is made up of a further 9 other proteins (Kappé *et al*., 2003). Another member of note is HSP27, which has already been described. A possible function for HSP20 was shown in a study involving vascular smooth muscle. It was suggested that phosphorylated HSP20 was able to cause vasorelaxation, which could be beneficial if cellular stress within the heart causes vasoconstriction (Flynn *et al*., 2003). Another study that investigated the vasorelaxation function of HSP20 involved sildenafil, where the application of sildenafil was associated with a rise in phosphorylation of HSP20, which was thought to be essential for its function. This resulted in an increase in the vasorelaxation of a pre-contracted porcine coronary artery, suggesting that HSP20 played a role is this physiological process (Tessier *et al*., 2004).

1.6.3 Factors that induce HSP expression

After the initial experimentation with heat shock, several studies undertaken provided evidence of other stimuli that induced HSP expression. These include hypoxia (Patel *et al*., 1995), exercise (Locke and Noble, 1995), inflammation (reviewed in Horvath *et al*., 2008), hyperthermia (Slakey *et al.,* 1993), ischemia (reviewed in Richard *et al.,* 1996), viral infection (Collins and Hightower, 1982), bacterial infection (Zheng *et al.,* 2004), ROS (oxidative stress) (Kukreja *et al*., 1994), sodium arsenite (Li *et al*., 1983), steroid hormones (Norton and Latchman, 1989) and ethanol (Plesset *et al*., 1982). Consequently, this superfamily of proteins

is also known as the “stress proteins”, as they are expressed in response to such a range of stresses in order to reduce, repair or remove any cellular damage caused (Madden *et al*., 2008).

1.6.4 Functions of HSPs

1.6.4.1 Intra-cellular HSP

The best known function of these proteins is when they are acting as molecular chaperones, although it should be noted that not all HSPs fall into this category (reviewed in Horvath *et al*., 2008). Molecular chaperones, which are families of unrelated proteins, assist in the correct conformational assembly of newly produced proteins, fixing incorrectly folded proteins (protein disaggregation) and the subsequent translocation of correctly folded proteins to intracellular or extracellular destinations (reviewed in de Jong *et al*., 2009; reviewed in Horvath *et al*., 2008; reviewed in Liberek *et al*., 2008; reviewed in Latchman, 2001). Such functions are active even under normal physiological conditions, with constitutively expressed HSPs accounting for 5-10% of total cellular protein content (reviewed in Pockley, 2003). When a cell is under stress the expression and the action of HSPs are of upmost importance as they ensure proteins within the cell maintain their correct conformation and help facilitate the proteolysis of proteins denatured beyond repair (reviewed in Latchman, 2001). Table 3 highlights the intra-cellular roles of most well known HSPs

Table 3. Intracellular location and function of major mammalian HSP

Major Mammalian HSP families	**Intracellular Location**	**Intracellular function**
HSP 25/ 27 (Small HSP)	Cytoplasm/ nucleus	Molecular chaperone of anti- apoptotic death
HSP32 (HO-1)	Cytoplasm	Indirect anti-oxidant via haem degradation
HSP40	Cytoplasm/ nucleus	Co-chaperone with HSP70
HSP60 (chaperonins)	Mitochondria/ cytoplasm	Correct protein folding in mitochondria, anti/pro-apoptotic death, anti-inflammation.
HSP70: HSP70/72 (inducible) HSC70/ HSP73 (constitutive) HSP75	 Cytoplasm/ nucleus Cytoplasm/ nucleus Mitochondria	 Molecular chaperone, anti-inflammation. Molecular chaperone Mitochondrial chaperone of protein transport.
HSP90	Cytoplasm/ nucleus	Molecular chaperone (evolutionary modulator)
HSP110	Nucleolus/ cytoplasm	Molecular chaperone

(reviewed in Pockley, 2003; reviewed in Calderwood (i) *et al*., 2007; reviewed in Noble *et al*., 2008)

HSP are also involved in the regulation of apoptosis and through this helps maintain overall cell survival, especially in deleterious conditions. For example, the intrinsic (mitochondrial/ caspase-9) apoptotic pathway is inhibited by HSP27 (Bruey *et al*., 2000), HSP90 (Pandey *et al*., 2000) and HSP70 (Beere *et al*., 2000) but promoted by HSP10 and HSP60 (Samali *et al*., 1999). The extrinsic (receptor mediated) apoptotic pathway is inhibited by HSP70 (Park *et al*., 2001), HSP90 (Sato *et al.,* 2000) and HSP27 (Charrette *et al*., 2000). The fact that HSPs can regulate apoptosis means that they can help maintain cell stability, especially during times of cellular stress.

1.6.4.2 Extra-cellular HSPs

Originally, HSPs were believed to be solely intracellular, which is where their cytoprotective roles (maintenance of protein folding and regulation of apoptosis) are carried out. It has since been reported that some HSPs are not confined to the cytosol, with some also being extracellular. For example, HSP72 was expressed on the plasma membrane of human tumour cells (Botzler *et al*., 1998; Gastpar *et al*, 2004). Also, release of HSP70 was shown in neuronal cells (Robinson *et al*., 2005), human tumour cells (Barreto *et al*., 2003; Evdonin *et al*., 2004), systemic blood mononuclear cells (Hunter-Lavin *et al*., 2004) and vascular smooth muscle cells (Liao *et al*., 2000). HSP60 was observed at the mitochondrial plasma membrane (Gupta and Knowlton, 2002) and the plasma membrane of adult cardiac myocytes (Lin *et al*., 2007). It was also demonstrated that HSP60 was released from human neuroblastoma cells (Bassan *et al*., 1998). B cells have been shown to release HSP27, HSP70 and HSP90 (Clayton *et al*., 2005). HSP60 and HSP70 have also been detected in patient serum samples (Pockley *et al*., 1998; reviewed in Pockley *et al*, 2008). All this evidence supports the extracellular location of HSPs.

The method used by HSPs to become extracellular, with HSP70 being the most investigated, has long been a subject of controversy. The existence of extracellular HSPs has only been accepted in recent times (reviewed in Mambula *et al*., 2007). In order to investigate the method of HSP extracellular release, several studies were performed that involved HSP70. HSP70 secretion does not occur by the classical pathway, i.e. it does not encode a leader signal protein (reviewed in Calderwood (i) *et al*., 2007). There are several alternative pathways by which leaderless proteins can be secreted. The first is because of necrotic cell death, which was shown in a study where HSP70 was released from prostate carcinoma cells

when they become necrotic (Mambula (i) and Calrderwood, 2006). Another method of secretion was the release of proteins from the intracellular space via secretory vesicles, a method used for the release of Interlukin-1β (another leaderless protein) from activated monophages (MacKenzie *et al*., 2001). This method of release was shown to be available for the extracellular release of HSP90, HSP70, HSC70 and HSP27, which were excreted from heat shocked B-cells via exosomes (Clayton *et al*., 2005). Another pathway for the release of a leaderless protein such as HSP70 was shown to be via lysosomal endosomes that transfer intracellular proteins to the plasma membrane and release them into the extracellular space. This was shown to be the method by which HSP70 was released from tumour cells (Mambula and Calderwood, 2006).

1.6.4.3 HSPs and the immune system

The extracellular functions of HSPs have been extensively investigated and the majority of these functions include interaction with aspects of the immune system (table 4) (Calderwood (i) *et al*, 2007).

Table 4. Extracellular functions of mammalian HSPs

Major Mammalian HSP families	**Extracellular Location**	**Extracellular function**	**References**
HSP20 (small HSP)	Extracellular space	Anti-inflammatory	Wang *et al*., 2009
HSP25/ 27 (small HSP)	Extracellular space	Anti-inflammatory	Reviewed in Calderwood (i) et al., 2007)
HSP32 (HO-1)	Extracellular space	Anti-inflammatory	Yeh *et al*., 2009
HSP60 (chaperonins)	Plasma membrane Extracellular space	Pro-inflammatory	Lin *et al*., 2007 Bassan *et al*., 1998 Chen *et al*., 2000
HSP70/ 72 (inducible)	Plasma membrane Extracellular space	Antigen-presentation Pro-inflammatory Interacts with NKC	Botzler *et al*., 1998; Gastpar *et al*, 2004 Liao *et al*., 2000 Dybdahl et al., 2002 Noessner *et al*., 2002 Asea *et al*., 2000 Multhoff *et al*., 2001
HSP90	Extracellular space	Pro-immune	Reviewed in Calderwood (i) et al., 2007)

HSPs are thought to interact with aspects of the immune system in the extracellular space upon release (Basu *et al*., 2000; reviewed in Pockley *et al*., 2008). For example, HSP70, much like major histocompatibility complex (MHC) molecules, has been shown to bind antigenic peptides forming a HSP-peptide complex, which bind to receptors on the surface of antigen presenting cells (APC) and then are taken up by them (Noessner *et al*., 2002). This results in the major histocompatibility complex I (MHC I) presentation of these peptides on the surface of APC (antigen cross-presentation) (Arnold-Schild *et al.*, 1999; Singh-Jasuia *et al*., 2000). This causes the activation and proliferation of cytotoxic (CD8+) T lymphocytes (Blachere *et al*., 1997).

For HSP-peptide complexes to be taken up by APC, specific receptors need to be expressed on their surface (reviewed in Binder *et al*., 2004). Such examples include TLR2, TLR4 and the LPS receptor CD14 that are all specific for extracellular HSP70 (reviewed in Schmitt *et al*., 2007). Other examples of cell receptors that are thought to interact with extracellular HSPs and help facilitate their role in immunity include the oxidised LDL binding protein CD91/ LRP, the tumour necrosis factor CD40 and scavenger receptors (reviewed in Calderwood (ii) *et al*., 2007).

HSPs can also interact with APC receptors and cause an immune response without being bound to antigenic peptides when released into the extracellular space in response to trauma. For example, HSP70 can induce the expression of the pro-inflammatory cytokines tumour necrosis factor (TNF)-alpha, interleukin (IL)-1beta and IL-6 in human monocytes (APCs) by utilising the receptors TLR2 and TLR4 (Asea *et al*., 2000). HSP70 can also prime dendritic cells (Asea *et al*., 2002) and macrophages (Basu *et al*., 2002) to initiate the production of pro-inflammatory cytokines. HSP60 has also been shown to induce the production of pro-inflammatory cytokines like TNF-α from macrophages, also known APCs (Chen *et al*., 2000). It was shown in a study that macrophages of C3H/ HeJ mice, which are characterised by a mutant TLR4 gene, were unable to respond to HSP60, which is known to induce the production of pro-inflammatory cytokines. This indicated that HSP60 utilises TLR4 for interacting with macrophages (Ohashi *et al*., 2000). This release of pro-inflammatory cytokines is caused by the activation of the transcription factor NF-kB, whose downstream signaling is also responsible for increasing the production of enzymes such as nitric oxide

synthase (reviewed in Lee and Kim, 2007). All this evidence highlights the fact that HSPs are involved in causing a pro-inflammatory response by acting as DAMPs in the extracellular space. Therefore, they could be involved in the inflammation in the myocardium that occurs during cardiac surgery with CPB (reviewed in de Jong *et al*., 2009).

HSPs can also cause an anti-inflammatory response. For example, in a study where HO-1 (HSP32) was overexpressed in endothelial cells, it was shown that this over-expression inhibited inflammatory and cell death cascades, including a reduction of NFkB activation (Zabalgoitia *et al*., 2008). Another study using a rabbit model that investigated the affect HO-1 on damage done by CPB and cardioplegia showed that HO-1 was able to reduce the level of apoptosis of cardiomyocytes. There was also a reduction in the levels of pro-inflammatory cytokines produced by NFkB and transcription factor AP-1 activation during CPB and cardioplegia administration (Yeh *et al*., 2009). In relation to HSP20, its over-expression has been shown to be able to inhibit LPS induced NFkB expression, which results in the production of pro-inflammatory cytokines (Wang *et al*., 2009). HSP70 can be anti-inflammatory when it is intracellular, where it can inhibit the initiation of the NF-kB signaling cascade within cells of the immune system by blocking the NF-kB promoter (reviewed in de Jong *et al*., 2009). The fact that HSPs are shown to be anti-inflammatory highlights another way in which investigating their expression during myocardial IR injury could help limit damage during cardiac surgery.

Another important component of the innate immune system are natural killer cells (NKC), which have also been shown to interact with HSPs, in particular HSP70 via the NKC receptor CD94 (Gross *et al*., 2003). This interaction is thought to provide an additional activating signal for NKC, which is of particular interest in cancer therapy as HSP70 is highly expressed on several types of cancer cells (reviewed in Schmitt *et al*., 2007). HSP70 interaction with NKC can also cause them to have increased cytolytic activity (Multhoff *et al*., 2001) and cause an increase in its chemotaxis (Gastpar *et al*., 2004).

1.6.4.4 Pre-conditioning

A novel trait of HSP gene expression is that an initial cellular insult that causes HSP expression can enhance cellular defense against subsequent insult. This is known as pre-

conditioning. It was found that the level of cellular protection afforded was proportional to the level of HSP expression produced by the initial insult (reviewed in Latchman, 2001). This feature of HSPs became of particular interest for those in the cardiovascular field in relation to protection against IR injury, which is a consequence of cardiac surgery with CPB, a subject that has already been discussed (see section 1.3).

1.6.5 Regulation

Heat shock transcription factor 1 (HSF-1) is thought to be the overall regulator of stress-induced heat shock gene transcription in vertebrates (Sarge *et al.,* 1993; reviewed in Shamovsky and Nudler, 2008). Upon stress, HSF-1 is phosphorylated from a monomer (inactive) to a trimer (activated) and then translocated from the cytosol to the nucleus (Sarge *et al.,* 1993). This trimer has a high binding affinity for the promoter regions of HSP genes, known as heat shock elements. Once HSF-1 has undergone phosphorylation and the trimer binds, transcription of HSPs is activated (Sarge *et al.,* 1993; reviewed in Pockley, 2003). The efficiency of HSF-1 to carry out its function seems to be age dependent, with it becoming less affective as age increased. This was shown in a study where the response of HSF-1 was decreased in the myocardium of aged rats compared to that of rats that were younger (Locke and Tanguay, 1996).

1.6.6 HSPs, cardio-protection and cardiac surgery

A consequence of cardiac corrective surgery with CPB is IR injury of the myocardium (reviewed in de Jong *et al*., 2009). There are many physical methods employed during cardiac surgery that protect the heart against IR injury that have already been described (see section 1.2.2).

IR injury to the cardiac tissue during cardiac surgery using CPB is a type of cellular stress. Certain cardio-protective proteins are expressed under such stressful conditions. These would include HSPs, which are known molecular chaperones, whose function is to ensure that the proteins within the cytoplasm of a cell are maintained in the right conformation. They also help prevent apoptotic cell death and therefore helps maintain cellular stability. This is especially important during a stress such as IR injury that occurs during cardiac surgery. HSPs also provide subsequent protection against a subsequent insult in a process known as pre-conditioning (reviewed in de Jong *et al*., 2009).

HSP70 is highly expressed in myocardial tissue under assault and provides cardiac protection against stress, including against IR injury (reviewed in Latchman, 2001). The ability of HSP70 and other HSPs to provide such protection after IR injury in cardiac tissue has been shown in several studies, which are listed below. It should be noted that most of the following examples that highlight the protection provided by HSPs in myocardial tissue includes pre-conditioning (reviewed in Latchman, 2001). Examples also include HSP overexpression studies. These were performed because the studies involving HSPs being induced by pre-conditioning did not necessarily demonstrate that HSP expression was the sole source of protection against cardiac stress. Therefore they did not exclude other possible contributing factors produced during the initial stress factor (reviewed in Latchman, 2001).

1.6.6.1 HSPs cardiac expression studies using cardiac cells

Specific examples of studies involving cardiac cells include a study where stress induced expression of HSP72 by heat shock was shown to restrict the degree of myocardial damage caused by ischemia in myogenic heart cells (Mestril *et al*., 1994). Another study was performed using liposome-mediated gene transfer of HSP72 into rat myocardium cells where over-expression of HSP72 provided protection against IR injury, again highlighting its cardio-protective function (Liu *et al*., 2007). HSP27 has also been shown to provide protection against IR injury using cardiac cell studies. For example, HSP27 was transfected into rat myocytes, which provided protection against cellular stress (Kwon *et al*., 2007). Another study overexpressed HSP27 in rat cardiomyocytes that then underwent simulated IR injury. Such overexpression was shown to protect against insult (Lu *et al*., 2008). HSP20 has also been found to be cardio-protective in cardiac cells. There was a study performed using adult rat cardiomyocytes that were transfected with a mutant HSP20 gene. The cells were then exposed to IR injury and had reduced protective ability when compared to the rat cardiomyocytes containing the normal HSP20 gene (Nicolaou *et al*., 2008). A study using adult rat cardiomyocytes that overexpressed HSP20 showed that these cells were protected against apoptosis induced by a β-agonist, highlighting a potential cardio-protective role for HSP20 (Fan *et al*., 2004). All of these examples show that induction or the overexpression of HSPs can provide protection against IR injury, one of the main sources of damage to the myocardium during cardiac surgery with CPB.

1.6.6.2 HSPs cardiac expression studies using animal models

There have been several studies using animal models that have shown the cardio-protective effects of HSPs against IR injury. In one study, the induction of HSP27 and HSP70 by simulated MI reduced the amount of damage afflicted by IR injury (Pantos *et al*., 2007). Another study used mice with HSP72 null mutations that were exposed to IR injury. These knockout mice were shown to be more susceptible to damage caused by IR injury (Kim *et al*., 2006). When HSP27 was induced into rats using a protein delivery system, it was successfully expressed in the heart and resulted in a reduction in damage caused by IR injury (Kwon *et al*., 2007). Another study using a rat animal model showed that overexpression of HSP27 provided such mice with enhanced protection against damage caused by IR injury (Lu *et al*., 2008).

In relation to HSP20, this protein was over-expressed in a transgenic mouse model. These mice were exposed to IR injury and it was shown that this over-expression contributed to better contractile function after the insult. There was also a reduction in apoptosis and in MI formation (Fan *et al*., 2005). Interestingly, it was shown that it is the C-terminus of HSP20 that is responsible for its cardio-protective ability (Islamovic *et al*., 2007). A specific substitution mutation within the HSP20 gene completely knocked out this cardio-protective ability due the fact it reduced the degree of HSP20 phosphorylation (Nicolaou *et al*., 2008). This blockage of HSP20 phosphorylation has also been shown to stop the cardio-protective nature of HSP20 in the incidence of IR injury (Qian *et al*., 2009).

HO-1 (HSP32) was shown to be cardio-protective in a study performed using mice with impaired HO-1 gene expression that were exposed to IR injury in comparison to wild type controls. It was shown that mice with reduced HO-1 expression sustained more damage when compared to wild type controls (Yoshida *et al*., 2001). Such protection was also seen in a study using transgenic mice that had variable overexpression of HO-1 that were exposed to IR injury (Yet *et al*., 2001). Another study used a rat model where HO-1, conjugated into a viral vector, was introduced into the heart tissue prior to IR injury. It was found that there was a significant reduction in MI formation and inflammation when compared to wild type controls (Melo *et al*., 2002). A similar study involved a rat animal model of recurrent IR injury. An over-expression of HO-1 was induced by the use of viral vector delivery in the rat myocardium 5 weeks before exposure to the IR injury. This resulted in reduced inflammation, apoptosis and cardiac dysfunction (Pachori *et al*., 2006).

As with the studies involving the use of cardiac cell, all these studies using animal models again show that the induction and over-expression of HSPs can provide protection against IR injury. Also, as was often shown with the studies that investigated TLR expression, knockout mice were also used to investigate HSP expression. For both HSP72 and HSP32, a lack of functional protein seemed to make the animals more vulnerable to IR damage. All of this evidence again highlights the value of investigating the expression of HSPs in order to provide myocardial protection against IR injury.

1.6.6.3 HSPs expression studies during cardiac surgery

As well as the various studies described above to show the cardio-protective nature of HSPs in cardiac cells and animal models, other studies were carried out in order to show the benefit of HSPs in actual cases of cardiac surgical procedures. For example, a study was undertaken that investigated the expression of HSP27, HSP60, HSP70 and HSC70 in pediatric patients undergoing cardiac surgery with CPB. The levels of HSPs were measured in tissue from the RA removed before and after aortic clamping during the surgical corrective procedure. The use of cardioplegic arrest did not cause any significant differences in the expression of HSPs. However, it was shown that on average 40% of patients had an increase in HSP70 expression and 28% had an increase in the rest of the HSPs investigated after cardioplegic arrest. These patients were associated with a lower level of biomarkers that indicate the level of cardiac injury. This highlighted an association between HSP70 and other HSPs with a better post-operative outcome. Therefore HSPs were shown to be potentially cardio-protective (Giannessi *et al*., 2003). Another study was performed that investigated the expression of HSP72 in tissue from the RA from patients undergoing surgical repair of congenital heart defects before and after aortic clamping. There was no significant difference seen in the expression of HSP72 suggesting that ischemic insult alone does not induce an increase in HSP expression (Storti *et al*., 2001). Overall, all the evidence listed generally supports the fact that HSPs are cardio-protective, that they are present in the myocardium and that they are beneficial during and after cardiac surgery. Hence, investigating their expression could be beneficial for finding new methods of myocardial protection. There are other examples of HSP expression during cardiac surgery, which will be discussed later, that are related to extracellular HSPs (see section 1.6.6.5).

During cardiac surgery with CPB, patients are often exposed to hyperoxic levels of O_2. Such exposure can cause damage to the patients, in particular within the lungs. It has been shown that HSP expression can provide protection against such insult. For example, in a study where cultured human respiratory epithelial cells were exposed to hyperoxia (95% O_2), it was shown that overexpression of HSP70 resulted in increased cellular survival. This indicated that HSP expression was protective against oxidative damage sustained in the lungs during cardiac surgery with CPB (Wong *et al*., 1998). HSP27 expression has also been shown to be protective within the lungs. Lung epithelial cells were exposed to hyperoxia (95% O_2). Knockdown of HSP27 expression increased apoptotic cell death upon exposure to hyperoxia and overexpression of HSP27 decreased apoptotic cell death by exposure to hyperoxia. This study highlighted the protective role of HSP27 within the lungs when exposed to hyperoxia (Shao *et al*., 2009). Also, overexpression of HSP32 (HO-1) in human pulmonary epithelial cells was shown to provide enhance resistance against hyperoxic exposure (Lee *et al*., 1996).

1.6.6.4 HSPs expression and cardiac repair of congenital heart defects

Only a few studies have been carried out to investigate the expression of HSPs in relation to congenital heart defects such as TOF. For example, a study was undertaken where the level of HSP70 was investigated in muscle samples taken from the RV of patients that had undergone different types of corrective congenital heart surgery. HSP70 protein expression was demonstrated to some degree in all samples by the use of western blotting and immunohistochemistry. The cause of this expression was unclear and could have been caused by cellular stress, such as the trauma of the surgery itself, the hypoxic environment in the RV of such patients or by the hypertrophy seen in all samples used in the study (Nakamura *et al*., 2000). In a study which investigated HSP levels in hypertrophic cardiac muscle samples taken from the RV from patients undergoing congenital heart surgery, specifically the messenger RNA (mRNA) levels of HSP70 and HSP32, it was observed that all samples expressed significant levels of both. This upregulation of expression may be due to the trauma of the surgery itself, the hypoxic environment in the RV or by the hypertrophic nature of the tissue used (Takeuchi *et al*., 2003). Both these studies show the presence of HSP70, and some others, in the myocardium of children undergoing corrective cardiac surgery with CPB. Due to the fact that HSPs are known to be cardio-protective this is very important. This is because such children are especially vulnerable to the IR injury they are exposed to during corrective surgery. This is because they have cyanotic damage before surgery due to the chronic

exposure to hypoxia within the RV. Therefore, the investigation into the myocardial expression of HSPs in such vulnerable patients could potentially improve methods of myocardial protection employed during cardiac surgery.

1.6.6.5 Extra-cellular HSP expression during cardiac surgery

HSPs are beneficial due to their function as chaperones, which is their intracellular function. HSPs, as already stated, are also extracellular (Calderwood (i) *et al*, 2007). It has been shown that HSP70 and other HSPs are released into the extracellular space after cardiac surgery. For example, release of HSP70 was detected in the serum of patients who had undergone CABG surgery (with CPB) in adults (Dybdahl *et al*., 2002). During cardiac surgery with CPB and cardioplegic arrest in adult patients, a more exaggerated systemic release of HSP70 in patient serum was caused when compared to that seen during cardiac surgery without bypass (Dybdahl *et al*., 2004). This was most likely due to a higher degree of cellular damage caused during the cardiac surgery with CPB. Another study compared HSP70 expression in the plasma of adult patients undergoing surgery with and without CPB. HSP70 expression was significantly lower in patients that had not gone on bypass when compared to patients that had. This suggested that on pump surgery caused more stress and a more exaggerated increase in HSP72 expression. It also showed the extracellular location of HSP70 (Lin *et al*., 2010). An increase in HSP27, HSP60, HSP70 and HSP90α was found in the serum of adult patients undergoing cardiac surgery with CPB when compared to the levels seen in the serum from patients who had surgery without CPB. This highlighted the extracellular location of several HSPs during cardiac surgery and again demonstrated that the use of CPB increased the expression of HSPs (Szerafin *et al*., 2008). Overall, HSP70 and other HSPs have been demonstrated to be in the extracellular place following cardiac surgery with CPB. It has already been described (see section 1.6.4.2) that extracellular HSPs interact with aspects of the immune system. Therefore, HSPs could have a role in the myocardial and systemic inflammation caused during cardiac surgery with CPB and could therefore be used in order to find a way in which to prevent it.

1.6.7 Conclusion

Overall, it can be seen that IR injury is a complex process of response and protection to the insults, often mediated by the same proteins (e.g. HSP72). It can also be seen that the manipulation of these systems might help to reduce the initial injury as well as providing

protection from damage caused. This could have beneficial outcomes when used in the context of cardiac surgery in order to discover new or improved methods of cardio-protection during cardiac surgery with CPB. This is of even greater importance in pediatric heart surgery, as this often involves children with congenital heart defects causing them to have abnormal, cyanotic myocardium. This could involve the investigation of both intracellular and extracellular HSPs.

1.7 78 kDa glucose-regulated protein (GRP78)

78 kDa glucose-regulated protein (GRP78), which is also known as binding immunoglobulin protein (BiP) or heat shock 70 kDa protein 5 (HSPA5) is a stress protein that is expressed in response to cellular insult in order to either maintain or refold protein structures that have been disrupted due to damage caused (Shiu *et al*., 1977; Haas and Wabl, 1983; reviewed in Lee, 1992; reviewed in Lee, 2001). Stress proteins are generally known as either HSPs (see section 1.6) or glucose regulated proteins (GRP). Unlike HSPs, which are usually localised within the nucleus or the cytoplasm, GRPs are generally located within the endoplasmic reticulum (ER) (Munro and Pelham, 1987; reviewed in Lee, 1992; reviewed in Lee, 2001). The function of the ER within the cell is very diverse and includes being the site of protein folding and protein post translational modification, the maintenance of intracellular Ca^{2+} homeostasis, lipid synthesis and a site for the initiation of apoptotic pathways (Bush and Sze, 1986; Helenius *et al*., 1992; Cox *et al*., 1997; Wu *et al*., 2000; reviewed in Breckenridge *et al*., 2003).

The GRPs were initially discovered by experiments carried out involving glucose starvation, specifically, a cell culture experiment involving chick embryo fibroblasts was carried out that showed the induction of these genes in response to a deficit of glucose. The genes identified within this study were then named based on their apparent function and molecular weight and were therefore named GRP78 and GRP94 (Shiu *et al*., 1977). GRP78 is the one of interest in this project.

When cells undergo stress such as nutrient starvation, chronic exposure to ethanol, prevention of glycosylation or a reduction in intracellular Ca^{2+}, it can result in the upregulation of ER stress chaperones such as GRP78 due to the accumulation of misfolded proteins within the ER (Shiu *et al*., 1977; Li *et al*., 1993; Miles *et al*., 1994; reviewed in Lee, 2001). This process is known as the unfolded protein response (UPR) and is one of the ways in which the

cell returns protein homeostasis within the ER back to normal (Patil and Walter, 2001; Ma and Hendershot, 2001). In fact, measurements of GRP78 expression have been used as an indicator of ER stress (Mao *et al*., 2004; reviewed in Lee, 2005). An alternative to the UPR pathway is the ER overload response (EOR) pathway, where the accumulation of proteins (not misfolded) within the ER induces the expression of the transcription factor NFkB that will result in an inflammatory response (Pahl and Baeuerle, 1995; Pahl *et al*., 1996; Pahl and Baeuerle, 1997). The EOR pathway does not induce the expression of GRP ER chaperones (reviewed in Rapp and Kaufmann, 2003).

GRP78 is expressed in the heart. This was demonstrated in a study using a mouse model that showed that GRP78 is expressed at high levels within the heart during early stages of embryonic development, with GRP78 expression being mainly localized within cardiac myocytes (Barnes and Smoak, 2000). GRP78 expression within these cells was also increased after a 24 hour period of hypoglycaemia again highlighting how nutrient starvation can induce the expression of these stress proteins (Barnes and Smoak, 2000).

Studies involving cellular stress within the heart that investigated the expression of GRP78 include a study that used cultured rat ventricular myocytes to determine if 16 hours of hypoxia could induce the UPR and the induction of GRP78. This insult successfully caused an increase in GRP78 expression suggesting hypoxia is detrimental to the stability of proteins within the ER (Thuerauf *et al*., 2006). This increase in GRP78 expression was not observed during reoxygenation after the hypoxic insult (Thuerauf *et al*., 2006). Another study investigated the role of GRP78 in apoptosis caused by ischemic insult in cardiac cells. Specifically, GRP78 was expressed on the surface of cardiac myocytes. When these cells were exposed to ischemic insult, GRP78 expression was increased, as was the incidence of apoptosis (Hardy and Raiter, 2010). The introduction of a GRP78 specific peptide reduced the amount of apoptosis caused by the ischemic insult by binding GRP78. This highlighted GRP78 as a potential target for reducing damage done by hypoxia, which is a cellular insult that occurs during cardiac surgery with CPB (Hardy and Raiter, 2010). In a pig animal model of chronic myocardial ischemia that resulted in heart failure, it was shown that both mRNA and protein GRP78 expression was increased in the myocardial tissue from these animals as were apoptotic activation pathways, again indicating that GRP78 expression is induced by ischemic insult with the heart (Xin *et al*., 2011).

Another study investigated hypertension by using a rat animal model with diastolic heart failure. It showed that the mRNA and protein expression of GRP78 was increased in these animals, along with capsase-12, indicating that in a model of cardiac stress, GRP78 expression is increased along with the incidence of apoptosis (Sun *et al*., 2008). In relation to IR injury, a study using mouse hearts that were exposed to IR injury showed that the expression of GRP78 and GRP94 and therefore the UPR were increased due to this type of injury (Martindale *et al*., 2006). This same study used a transgenic mouse model that had specific cardiac expression of a protein called Activation of Transcription Factor 6 (ATF6). This expression was only activated upon exposure to tamoxifen. It showed that the UPR and the expression of GRP78 and GRP94 were higher in mice with tamoxifen induced ATF6 expression when compared to appropriate controls, showing that ATF6 was involved in activating the UFR and GRP78 expression during IR injury (Martindale *et al*., 2006). The mice with activated ATF6 expression also sustained less damage due to IR injury suggesting both ATF6 and GRP78 as having a protective role in the heart against IR injury (Martindale *et al*., 2006). All of these studies show that GRP78 is a potential candidate for investigation into how to protect the myocardium from injury during cardiac surgery with CPB other than the present methods used.

1.8 Apelin receptor (APJ) and it endogenous ligand Apelin

Apelin is a peptide that was discovered in 1998 (Tatemoto *et al*., 1998) as the endogenous peptide ligand of the G protein-coupled APJ receptor, the previously orphaned receptor isolated in 1993 (O'Dowd *et al*., 1993). Apelin is the only identified ligand for the APJ receptor. Apelin exists as several biologically active forms; Apelin-36, 17, 16, 13 and 12 have been found and are named by the number of amino acids in the COOH-terminal coding region (Berry *et al*., 2004). These different forms of the ligand are present due to the multiple cleavage sites on the precursor molecule, preproApelin (Habata *et al*., 1999). In 2009 it was found that [Pyr^{1}] Apelin-13 was the predominant form of the peptide but showed comparative functional status to the other two main isoforms, Apelin-13 and Apelin-36 (Maguire *et al*,. 2009).

The APJ receptor shares 31% of its structural sequence to that of the angiotensin II type-1A receptor (O'Dowd *et al*., 1993) and has been shown to counteract damaging effects of

angiotensin II (Siddiquee *et al*., 2011), suggesting a role of Apelin as a complementary mechanism to the effects of angiotensin II.

1.8.1 Function of Apelin/ APJ

The APJ receptor is expressed in endothelial cells, vascular smooth muscle cells and cardiomyocytes (Kleinz *et al*., 2005) and the primary function of Apelin-APJ is thought to be in the cardiovascular system (reviewed in Japp and Newby, 2008). However, Apelin also has a role in many different processes in several systems throughout the body.

Apelin is classed as an adipokine as it is produced by adipocytes and regulates glucose utilization in insulin resistant mice (Dray *et al*., 2008). Injection of the animals with Apelin reduced plasma levels of glucose, revealing Apelin as an endocrine regulator of glucose metabolism.

Apelin also plays a role in fluid homeostasis by counteracting the action of vasopressin (Galanth *et al*., 2012). Apelin, expressed in the supraoptic and the paraventricular nuclei, inhibits release of arginine vasopressin (AVP) by the AVP neuron (De Mota *et al*., 2004). This displays how Apelin interacts with AVP to act as a diuretic.

The main source of Apelin expression, however, is in the heart (Chandrasekaran *et al*., 2010), and its primary effect is thought to be in the cardiovascular system. Within the myocardium itself, Apelin-APJ activation causes an increase in contractility of the myocardiocytes and is one of the most powerful positive inotropes known (Farkasfalvi *et al.*, 2007). Apelin acts as an inotrope through modulation of Na^+/ H^+ and Na^+/ Ca^{2+} transporters (Szokodi *et al*., 2002). This potent inotropic effect is thought to be through both autocrine and paracrine signalling, due to the expression of Apelin both in the endocardium and myocardium (reviewed in Falcão-Pires *et al*., 2010). This was found to be the case as Apelin-APJ stimulation still occurs after the removal of the endocardium (the main source of Apelin) *in vitro* (Charo *et al*., 2009). Apelin is also expressed in the smooth muscle cells of the vasculature throughout the body (Chandrasekaran *et al*., 2010). Apelin was found to function as a potent vasodilator in both arterial and venous vessels from studies in rodent models (Charo *et al*., 2009) and in clinical trials (Japp *et al*., 2010). It elicits this effect by inducing NO dependent pathways of vasodilatation (Tatemoto *et al*., 2001). Thus, by reducing both afterload and preload, Apelin function in the vasculature has protective effects on the heart.

The overall effect of Apelin-APJ action is therefore potentially particularly beneficial in heart failure; when considering it functions as an inotrope, a vasodilator and a diuretic (Japp and Newby, 2008). As a result of the theoretical advantageous effects of Apelin-APJ function, much of the research into Apelin has been focussing on heart failure. It is known that expression of Apelin and APJ in the myocardium is increased in response to heart failure in rats (Atluri *et al*., 2007) and it is thought that this may be part of a compensatory mechanism in an attempt to restore normal cardiac output. Also, evidence has suggested that Apelin protects the heart against fibrotic remodelling (Pchejetski *et al*., 2012), an important process in the pathogenesis of heart failure. There have been positive results from one of the few human studies as infusion of Apelin to heart failure patients improved cardiac output and caused peripheral and coronary vasodilation (Japp *et al*., 2010). Also, as heart failure progresses and ventricular dysfunction worsens, myocardial Apelin production is reduced (Chandrasekaran *et al*., 2010).

1.8.2 Protection against ischemia

The Apelin-APJ axis has been shown to reduce tissue damage in times of ischemic stress. Apelin infusion in rodents reduced the infarct size after coronary artery occlusion in rodents (Kleinz and Baxter, 2008). APJ is expressed in higher levels in mature coronary vessels after prolonged periods of ischemia (Tycinska *et al*., 2012) suggesting that the APJ receptor is produced and expressed in higher levels in response to hypoxia. Apelin production is also mediated through hypoxia; expression of the ligand is increased in times of hypoxia through the hypoxia-inducible factor 1α pathway (Ronkainen *et al*., 2007). Apelin has been shown to directly counteract the damaging effects of ischemia on the myocardium (Kleinz and Baxter, 2008). Rodent models have shown direct protection against IR injury by Apelin administration (Simpkin *et al*., 2007; Zeng *et al*., 2009) and it has been proven that Apelin reduces this damage by reducing ER stress and therefore attenuating ER-dependent apoptosis (Tao *et al*., 2011).

The function of Apelin-APJ may therefore be beneficial during surgical procedures involving a CPB machine, which results in a motionless bloodless field and leaves the heart in ischemic conditions.

1.8.3 The characterization of Apelin-APJ

TOF is a common cyanotic congenital heart defect (see section 1.1.3) that requires surgical correction with CPB. Complete surgical repair of TOF is usually performed in the first year of life with a mortality rate of <2% (Starr, 2010). Although surgical repair is the best intervention option, patients are often left with side effects as a result of the procedure, most notably a deficiency in RV function as a result of CPB (Allen *et al*., 1997). This adverse effect has been attributed to the insult of IR injury (reviewed in Murphy and Steenbergen, 2008), which is a known adverse effect of this surgical procedure and studies have been undertaken to assess ways in which this damage can be avoided, such as ischemic post-conditioning, which was shown to provide some protection for the myocardium during surgery (Ji *et al*., 2011). Remote pre-conditioning is another more commonly studied method which attempts to reduce intra-operative myocardial damage and has been shown to decrease the effect of IR injury in a rodent study (Chiu *et al*., 2012) which involves pre-conditioning with HSPs. HSPs are peptides which are induced during times of ischemia to be produced as part of a cardio-protective mechanism (see section 1.6). It has also been found that HSPs can protect the myocardium of patients undergoing surgical repair of TOF (Peng *et al*., 2011). The role of Apelin-APJ function has not been investigated in this area. This is therefore a field with much promise for Apelin-APJ, as HSPs have been found to be beneficial in cardio-protection during paediatric cardiac surgery and the functions of Apelin and HSPs are very similar. To date, Apelin and the APJ receptor have never been identified in the infant myocardium. Therefore, looking at the presence of the APJ receptors and therefore Apelin activity in the infant heart would be an intriguing line of investigation

1.9 Aims and hypothesis

1) Experiment: Screening of differential gene expression in ventricular myocardium excised from cyanotic and control (acyanotic) patient groups.
    a. Aims: investigate the differential mRNA expression of inflammatory mediators and stress proteins within the myocardium from cyanotic and acyanotic patient groups and see how they differ.
    b. Hypothesis: Cyanotic patients have a different mRNA expression profile than acyanotic patients pre-operatively.
2) Experiment: Induction of HSP gene expression in excised ventricular myocardium: mRNA and protein level analysis of HSP gene expression induced by heavy metal chemical inducers.
    a. Aims: to determine the effect of exposure to heavy metals on the expression (mRNA and protein) of genes of interest within infant myocardium.
    b. Hypothesis: HSP expression (mRNA and protein) can be induced in infant myocardium using classic heavy metal inducers.
3) Experiment: Differential gene expression in RV myocardium excised from TOF and control patient groups exposed to differential oxygen levels.
    a. Aims: to expose ischemic infant myocardium to hyperoxic levels of O_2 to see its effect on the differential mRNA expression of genes of interest within such tissue.
    b. Hypothesis: reperfusion of ischemic infant myocardial tissue *in vitro* will cause an inflammatory/ stress response, which will be elevated in cyanotic patients who are more vulnerable to injury.
4) Experiment: The differential mRNA expression of GRP78 and APJ within infant myocardium
    a. Aims: to determine the differential expression of both genes within cyanotic patient myocardium compared to the acyanotic patient myocardium and how such expression is effected by re-oxygenation injury.
    b. Hypothesis: Cyanotic patients have a different mRNA expression profile than cyanotic patients pre-operatively and also after exposure of the infant myocardium to re-oxygenation injury.
5) Experiment: Protein expression of HSP27 and APJ within cyanotic and acyanotic TOF myocardium over ischemic time during cardiac surgery in comparison to cardiac output and contraction.
    a. Aims: to investigate the differential protein expression of both genes within cyanotic and acyanotic TOF myocardium over ischemic time and compare this to cardiac clinical data.
    b. Hypothesis: The expression of both genes will increase over ischemic time.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Consenting of patients

2.1.1 Identifying suitable patients

Children who underwent elective cardiac surgery for a Fallot type physiology (cyanotic) were identified as potential participants. Potential participants were identified by review of the cardiac surgical waiting list and clinical presentations at the multi-disciplinary weekly team meeting. Those attending included the health care team and Mr Mark Danton (consulting surgeon) who had overall responsibility co-ordinating the clinical team process and identifying potential patients. The control patient group, which includes children undergoing cardiac surgery in order to repair SAS (acyanotic), were identified by the same method as described above.

2.1.2 Patient recruitment

Parents were approached when they were attending the outpatient clinic. The consultant surgeon in charge would meet the parents and child and introduce the study for their consideration. If the parents were interested in participating in the study, Mr Danton would discuss the study and answer any questions the parents might have. The parents were also given an information sheet (as shown in appendix 1.1) and given appropriate time to read it and consider their child's participation before consenting. There was a period of approximately 1-2 weeks from the first introduction of the project until written consent was required. If the parents agreed for the patient to take part in the study, they were asked to sign a study consent form (as shown in appendix 2) on the day their child was admitted to hospital, usually 24/ 48 hours prior to surgery. All consent forms were filed in the clinical notes of the patient and a copy was kept in the study file. It was made very clear that the parents still held the right to withdraw from the study at any point, even when written consent had been given. Control group participants were approached in the same way (for information sheet for control patient goup see appendix 1.2).

The only instance when this method of approach would vary was for families who were unable to attend the outpatient clinic. In such cases, the child (patient) was admitted to hospital at least 48 hours prior to surgery and it was then that the parents of these children were approached for their potential participation in this study. If these parents were interested in their child participating in the study, Mr Danton would discuss the study with the parents

and answer any questions they might have. The parents were also given an information sheet and given an appropriate amount of time to read it to allow them to be able to consider their child's participation before consenting. If the parents agreed, they were asked to sign a study consent form on the day their child underwent surgery.

2.1.3 Ethical approval

Full ethical approval (see appendix 3.2) for this project was given by the Greater Glasgow and Clyde Health Board with the relevant R and D approval (shown in appendix 3.1). This process from start to finish took several months to complete.

2.2 Collection and processing of heart tissue frozen immediately following resection for protein and mRNA analysis

2.2.1 Tissue collected for protein analysis

All equipment used for processing the tissue was cleaned with 70% ethanol to sterilise them before use. A number of 1.5ml eppendorf tubes equal to the number of samples to be collected were put on dry ice to ensure they were at a low temperature. This is because they would be used for storage of snap frozen tissue samples. Liquid nitrogen (N_2) was collected in a suitable container and as it is very corrosive correct safety attire was worn.

Tissue was collected in theatre and the time point at which each sample was collected was noted. The hospital number (anonymous identifier), time of aortic-clamping, age and gender of the patient were also recorded. Tissue samples were collected in plastic galley pots then immediately snap frozen in liquid N_2. Snap frozen tissue was then brought back to the laboratory and then put into the pre-chilled 1.5ml eppendorf tubes that were kept on dry ice to ensure they remained cold. The tissue was then kept in -80°C storage until needed.

2.2.2 Tissue collected for molecular analysis

The method of tissue collection is as described in 2.1.1 except for the following differences. No liquid N_2 or dry ice was used. Using sterile barrier tips and Gilson pipettes, 1.2ml of RNA*later*® solution (Sigma-Aldrich, cat. no. R0901) was put into each pre-collected

1.5ml eppendorf tube (10µl of RNA*later*® solution is required per 1mg of tissue). Most pieces of tissue collected were approximately 100mg therefore 1.2ml was an adequate volume.

Tissue collected in theatre was immediately put into the 1.5ml eppendorfs containing the RNA*later*® solution. All eppendorf tubes were labelled appropriately. Once completed, the tissue samples in RNA*later*® solution were brought back to the laboratory and stored in the fridge, at least overnight, before being used for RNA extraction.

2.3 Reperfusion Experiments

Two sets of experiments were undertaken in order to investigate the effect of exposing ischemic myocardium from both the cyanotic and acyanotic patient groups to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2. This was done in order to recreate reperfusion of the myocardium that occurs in the clinical setting of paediatric open-heart surgery with CPB and to investigate the effect this had on the differential gene expression of genes of interest within myocardium from the acyanotic and cyanotic patient groups.

The first experimental model undertaken (figure 2-1) involved exposing tissue to 2% (hypoxia) and 20% (normoxia) O_2. It was initially not possible to expose tissue to 60% (hyperoxia) O_2 as such a high O_2 concentration required an incubator which could allow O_2 levels of 60%. The incubator purchased from Biospherix for this purpose was faulty and not fit for purpose. Thus a second incubator was sourced (New Brunswick® 48R carbon dioxide (CO_2) incubator). Upon receiving this incubator a second experimental model (figure 2-2) was designed and the tissue from each patient group was then exposed to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2. Both experiments are outlined in figure 2-1 and figure 2-2. The steps 1 to 5 in the diagrams correspond with steps 1 to 5 described within the text.

2.3.1 Experiment 1: exposing myocardial tissue to 2% (hypoxia) and 20% (normoxia) O_2

Step 1: two NuAire® incubators were set to 2% (hypoxia) and 20% (normoxia) O_2 (figure 2-1). 10ml of supplemented media (see appendix 4) was added to two sterile T5 culture flasks and 1ml of supplemented media was added to 6 wells of two sterile 24 well culture plates (6ml in total). One of each was put into each incubator that was set to the desired O_2% for at least 24 hours prior to tissue collection.

Step 2: When tissue was ready for processing, the T5 culture flasks were removed from the incubators. The first piece of tissue removed was put into RNA*later*® solution that would act as the baseline reference (A) (figure 2-1). For the rest of the tissue collected, each piece was cut in half and then each half was put into the 2% or 20% O_2 T5 culture flasks.

Step 3: The tissue was transported back to the laboratory in the 2% and 20% O_2 T5 culture flasks.

Step 4: Once back in the laboratory, the tissue was quickly removed from each of the T5 culture flasks (each done separately). All tissue was cut up into equal sized pieces and a small amount of tissue was removed from each flask to give the next reference points i.e. tissue collected in the 2% O_2 flask (B) and tissue collected in the 20% O_2 flask (C).

Step 5: After this, for each flask (separately), the 24 well culture plates were removed from their relevant incubators and the tissue was distributed into an equal number of wells in each plate that contained supplemented media. Once done, both 24 well culture plates were then put back into their relevant incubators and left for 1.5 hours. 1.5 hours was chosen as this is this length of time the myocardium is exposed to re-oxygenation in the clinical setting of such surgery i.e. upon removal of the aortic clamp. After the 1.5 hour incubation, the culture plates were removed and tissue in each 24 well culture plate was removed and put into RNA*later*® solution, representing the final two tissue collection points i.e. tissue incubated at 2% O_2 (D) and tissue incubated at 20% O_2 (E) (figure 2-1).

RNA was then extracted from the tissue (see section 2.5) that had been stabilised in RNA*later*® solution and this was later reverse transcribed into complementary DNA (cDNA) (see section 2.6), which was used in quantitative real time polymerase chain reactions (qPCR) using primers designed for the noted genes of interest (see section 2.8.3). Any observed differences in gene expression was analysed.

Figure 2-1. Diagram showing the first reperfusion experimental model

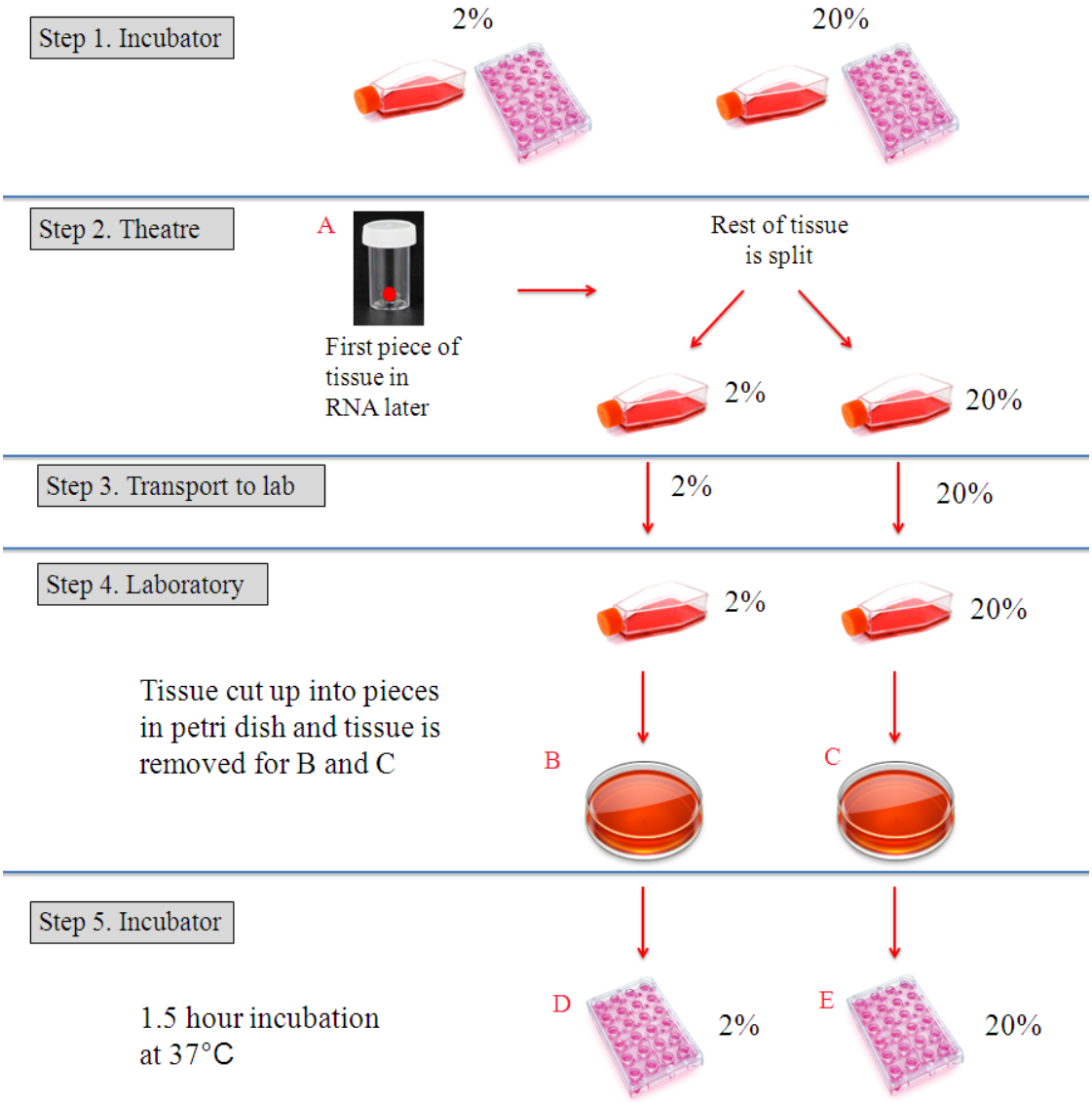


Figure showing the time course of experiment 1. Labelling on the diagrams refers to the following: A – collection of first reference samples, B – collection in flask containing 2% O_2, C – collection in flask containing 20% O_2, D – after 2% incubation and E – after 20% incubation.

2.3.2 Experiment 2: exposing myocardial tissue to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2

This set of experiments incorporated the new incubator, which could reach hyperoxic O_2 concentrations (60%). The set up for experiment 2 (figure 2-2) was the same as experiment 1 except for the following differences.

Step 1: The New Brunswick® 48R incubator was set up at 60% (hyperoxia) O_2 in additional to the other 2 incubators which were set up as before. Three 25 well culture plates and only one T5 culture flask were used during the experiment. The three 24 well culture plates were required as three different O_2 concentrations were being used instead of only two. Only one T5 culture flask was required as all tissue collected in theatre was being put into media incubated at 2% O_2 for at least 24 hours prior to tissue collection in order to keep the tissue in hypoxic conditions for transport over to the laboratory.

Step 2: When it was time for tissue collection in theatre, the T5 culture flask (incubated at 2% O_2 for 24 hours) was removed from its incubator and taken to theatre. The first tissue sample was put into RNA*later*® solution i.e. baseline reference (A) (figure 2-2). All other tissue samples were collected into the 2% O_2 T5 culture flask.

Step 3: Tissue was transported back to laboratory in the 2% O_2 T5 culture flask.

Step 4: Once back in the laboratory, the tissue transported in the T5 culture flask was removed and cut up into equally sized pieces. Some tissue was removed at this point giving the second reference point i.e. expression at start of incubation (B).

Step 5: The three 24 well culture plates were removed from their relevant incubators and the rest of the tissue was processed as already described except this was done over three plates instead of two. The plates were incubated for 4 hours in the second experiment instead of 1.5 hours. 4 hours was chosen in order to cause an exaggerated effect on any changes in gene expression due to changes in O_2 concentrations that might not have been apparent during only 1.5 hours. Also, 4 hours was indicated in the literature to be an appropriate length of time to imitate reperfusion (Hua *et al*., 2007; Ha *et al*., 2010). After incubation, the culture plates

were removed and the tissue from each plate was put into RNA*later*® solution (tissue collection points C (2%), D (20%) and E (60%)).

Figure 2-2. Diagram showing the set-up of tissue processing during hyperoxic reperfusion model

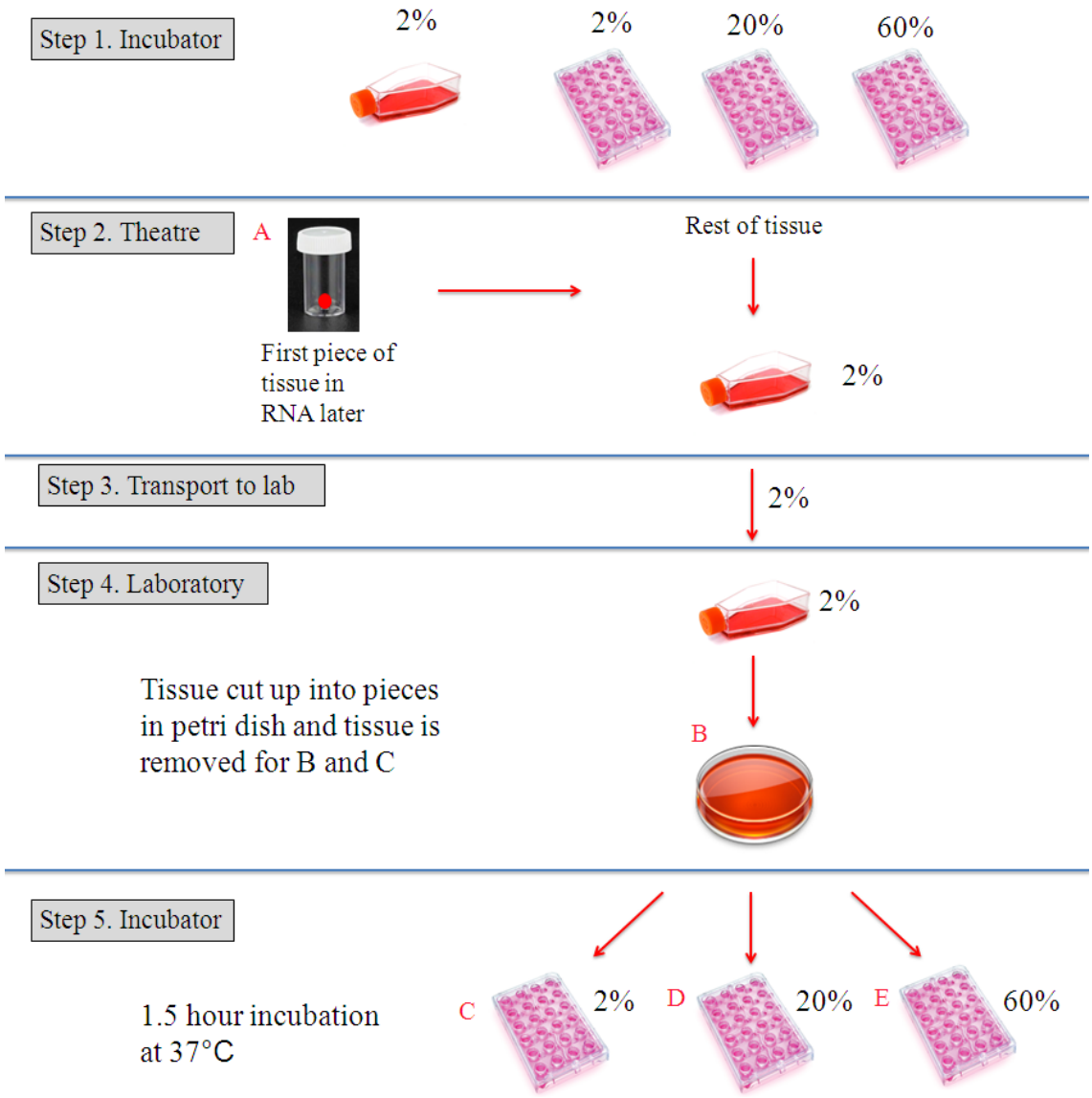


Figure showing the time course of experiment 1. Labelling on the diagrams refers to the following: A – collection of first reference samples, B – collection in flask containing 2% O_2, C – after 2% incubation, D – after 20% incubation and E – after 60% incubation.

2.4 HSP induction experiments: mRNA and protein level analysis of HSP gene expression induced by heavy metal chemical inducers

The aim of these experiments was to determine if heavy metals, known to be able to induce HSPs in other cell types, could induce HSPs in infant myocardium taken from both the cyanotic and acyanotic patient groups. This investigation into HSP expression was performed at both the mRNA and protein level. The expression of several inflammatory mediators was also investigated but only at the mRNA level. The methodology for the experiments described in sections 2.4.1 and 2.4.2 are summarised in figure 2-3.

Figure 2-3. Diagram showing the set-up of tissue processing during HSP chemical induction experiments.

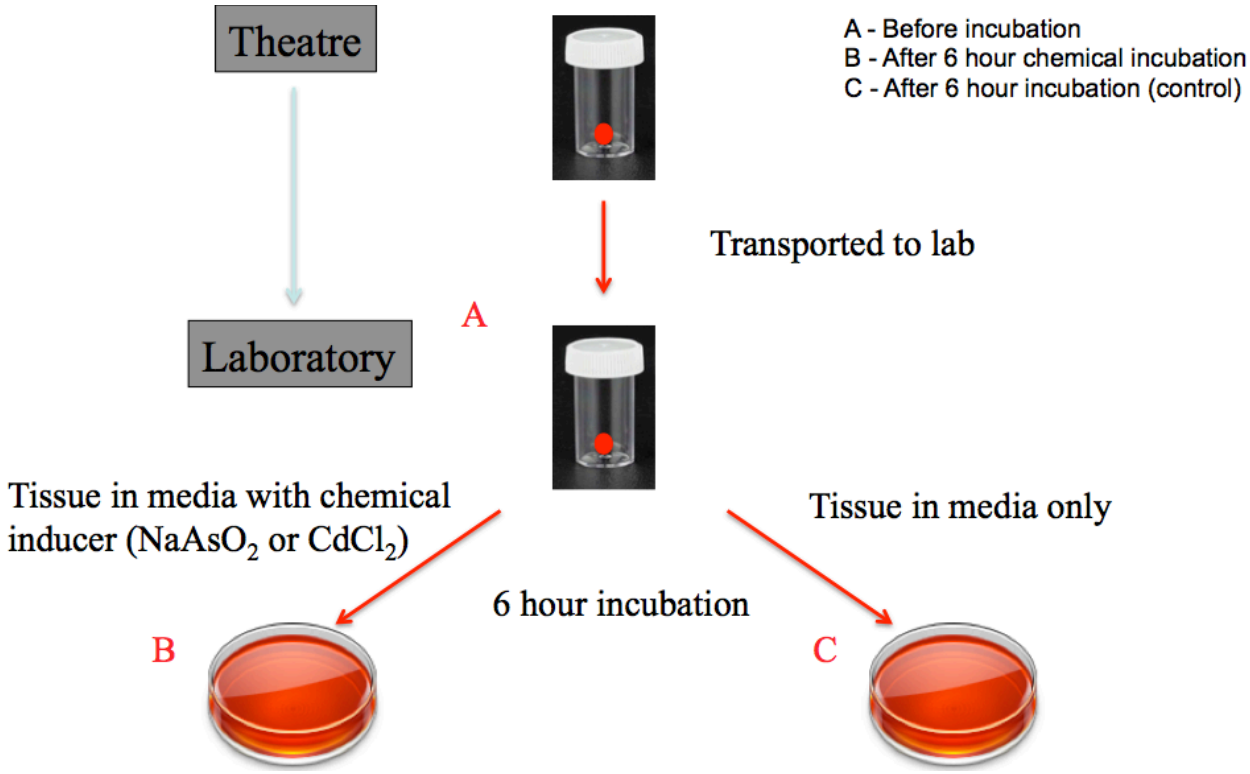


Figure showing the time course of HSP induction experiment. Labelling on the diagrams refers to the following: A – start of incubation, B – after incubation with chemical inducer for 6 hours, C – after incubation with media only for 6 hours (control).

2.4.1 mRNA level analysis of HSP gene expression induced by heavy metal chemical inducers

Myocardium collected in theatre was incubated for 6 hours in media containing a chemical inducer, either sodium arsenite ($NaAsO_2$) or cadmium chloride ($CdCl_2$), or in media only that would act as a comparable experimental control. Based on previous experiments performed within the laboratory using placental tissue, 6 hours was agreed as an appropriate length of time for incubation (Barber *et al*., 1999).

Two medium sized petri dishes were retrieved and labelled appropriately depending on the chemical inducer being used (one with either '6 hour $CdCl_2$' or '6 hour $NaAsO_2$' and one with '6 hour control'). 10ml of media was added to the correct petri dish. For each dish, the medium contained either the relevant concentration of the chemical inducer ($CdCl_2$ or $NaAsO_2$ at 100 μM) (refer to appendix 4) or with supplemented media only (refer to appendix 4) that was the experimental control. A separate amount of supplemented media only was also prepared in a universal container, which was to be used for tissue collection. Once prepared the petri dishes and universal container were put into a NuAire® CO_2 incubator set at 20% O_2 and at 37°C.

When tissue was ready to be collected the universal container was removed from the incubator and taken to theatre and all tissue that was collected was put into it. The samples were brought back to laboratory and then all tissue was removed and cut into equal sized pieces. In order to show expression levels at the start of the 6 hour incubation period a piece of tissue was removed at this point and put into 3ml of RNA*later*® solution. This sample was used as an experimental baseline reference sample (A). Then the 2 petri dishes (with or without the chemical inducer) were removed from the incubator and half of the tissue prepared was put into each. The petri dishes were then put back into the incubator and left for 6 hours.

Once the incubation period was complete, the tissue was removed from the 2 petri dishes and put into 3ml of RNA*later*® solution. Tissue that had been incubated in media with the chemical inducer ($CdCl_2$ or $NaAsO_2$) would act as the second tissue reference sample (B) and tissue incubated in media only would act as the third tissue reference sample (C).

The tissue was left in RNA*later*® solution overnight at 4°C and then mRNA was extracted from the myocardium (see section 2.5) and then reverse transcribed (see section 2.6) into cDNA to be used in qPCR using primers designed for the genes of interest (see sections 2.9 and 2.7.3 respectively). Any differences in gene expression caused by the chemical inducers compared to the appropriate controls were analysed.

2.4.2 Protein level analysis of HSP gene expression induced by heavy metal chemical inducers

The method of protein level analysis of induced HSP gene expression is as described in 2.4.1 except for the following differences. A piece of tissue was not removed at the start of incubation therefore no experimental baseline reference sample (A) was collected. This is because investigating differential expression at the protein level requires larger amounts of tissue and there was insufficient tissue to perform all the steps as for the qPCR experiments, which require less tissue. Instead all tissue collected was split and either incubated for 6 hours in media with the chemical inducer ($CdCl_2$ or $NaAsO_2$ at 100 μM) that gave the sample reference sample B or incubated in media only (experimental control) that gave the sample reference sample C.

After the 6 hour incubation was complete, all samples were snap frozen in liquid N_2 and then stored at -80 °C until needed. This tissue was then homogenised (see section 2.11.1) and the concentration of each sample was calculated using a Bradford assay (see section 2.11.2). The samples were then used to perform Western blotting (see section 2.12) in order to investigate any differences in gene expression caused by the chemical inducers compared to the appropriate controls at the protein level and any differences observed were analysed.

2.4.3 Protein level analysis of HSP gene expression induced by heavy metal chemical inducers with recovery time

An additional experiment was performed after further investigation of the literature. Some studies show that in order to see differential expression of HSP at the protein level, the tissue should be allowed to recover for a certain period of time after exposure to a cellular stress (Kim *et al*., 2001; Rossi *et al*., 2002; Somji *et al*., 2002). After treatment, all aspects of the experimental set up are as in section 2.4.2, except that when the tissue was spilt, half of it

was exposed to $NaAsO_2$ for 4 hours instead of 6 hours then allowed to recover for 20 hours in media only. The other half of the tissue was in media only for the entire 24 hours that acted as the experimental control.

2.5 RNA extraction from heart tissue

Right ventricle myocardium tissue samples were stored overnight at 4°C in RNA*later*® solution to stabilize the RNA (as recommended by manufacturer) in preparation for extraction using the RNeasy® Fibrous Tissue Midi Kit (Qiagen, cat. no. 75742). Recovery of RNA from snap frozen tissue stored at -80°C storage was also attempted on initial samples collected but poor yields of RNA were obtained therefore all subsequent samples were put into RNA*later*® solution.

When RNA extraction was carried out the tissue was removed from the RNAlater® solution and placed in a pre-weighed container, which contained 2ml RLT buffer (buffer provided with kit) supplemented with beta-mercaptoethanol (ß-ME) (10 µl ß-ME per 1 ml buffer RLT). The tissue was then homogenised using a Polytron PT1600E homogeniser (Kinematica) at setting full speed for 3x20 seconds, then RNA was extracted from the homogenate as follows.

After homogenising, each sample (2ml) was mixed with 4ml RNase free water (provided in kit) and 65µl proteinase K solution (provided in kit) then incubated at 52°C for 20 minutes. The mixture was then spun in the centrifuge (4000xg, 25°C for 5 minutes). The supernatant was removed and put into new sterile 15ml Falcon tubes (Sigma-Aldrich, cat. no. Z617849) (typically 6ml of supernatant is retrieved) and a half volume of 100% ethanol (typically 3ml ethanol per 6ml of supernatant) was then added to the supernatant and mixed well by pipetting. An appropriate number of RNasey® Midi kit spin columns already contained within the 15ml collection tubes (provided in kit) were retrieved and 3 ml of the mixed supernatant (ethanol and supernatant) was added to the column which was then spun in the centrifuge (4000xg, 25°C for 5 minutes). This was repeated twice until all the mixed supernatant had gone through the column. 2ml of RW1 buffer (provided with kit) was then added to each spin column then spun down in the centrifuge (4000xg, 25°C for 5 minutes). DNase I solution (140µl of RDD buffer and 20µl of DNase I stock solution both provided in kit) was made and was then added to the membrane of all spin columns for 15 minutes to

ensure there was no genomic DNA (gDNA) contamination. Once completed, 2ml of RW1 buffer was added to each spin column and left to stand for 5 minutes then spun down in the centrifuge (4000xg, 25°C for 5 minutes). 2.5ml of RPE buffer (provided in kit) was added to each spin column then spun down in the centrifuge (4000xg, 25°C for 2 minutes). Another 2.5ml of RPE buffer was added to each spin column then again spun down in the centrifuge (4000xg, 25°C for 5 minutes). The spin columns were removed from their original collection tubes and put in new ones to ensure no ethanol in the RPE buffer in the flow through from the last step of centrifugation was present on the spin column.

For RNA elution, 150µl of RNase free water was added directly to the membrane of the spin columns and then left on the bench for 1 minute. After this they were spun down in the centrifuge (4000xg, 25°C for 3 minutes) and then this step was repeated. Once RNA was successfully eluted in RNase free water, the RNA concentration (ng/µl) was then determined using a NanoDrop® (Thermo Scientific) to find the concentration of the RNA (either in a final volume of 150µl or 300µl), which once determined was entered into the appropriate database. The RNA was then separated into 50µl aliquots then put into -80°C storage.

The concentration (ng/ ul) of RNA was determined on a NanoDrop® (see figure 2-4). The ratio of absorbance at 260 nm and 280 nm gives an indication of RNA purity. A value between 1.8 and 2.1 indicates optimal purity. Using high purity RNA during reverse transcription (RT) ensures optimal quality cDNA is used in the subsequent PCR/ qPCR.

The issue of tissue viability is complex. Even if end stage markers of death are unaltered it is very likely for any tissue removed from the body that the events leading to death will begin. This could cause subtle changes in signalling molecules such as caspases and other apoptotic events. It would be useful in future work to examine tissue viability using the lactate dehydrogenase assay. However the RNA stability was examined in tissues and was shown to be non-degraded. Since cell death would result in RNA degradation, this as least suggests the tissue was viable.

Figure 2-4. NanoDrop® measurement of mRNA concentration for patient sample

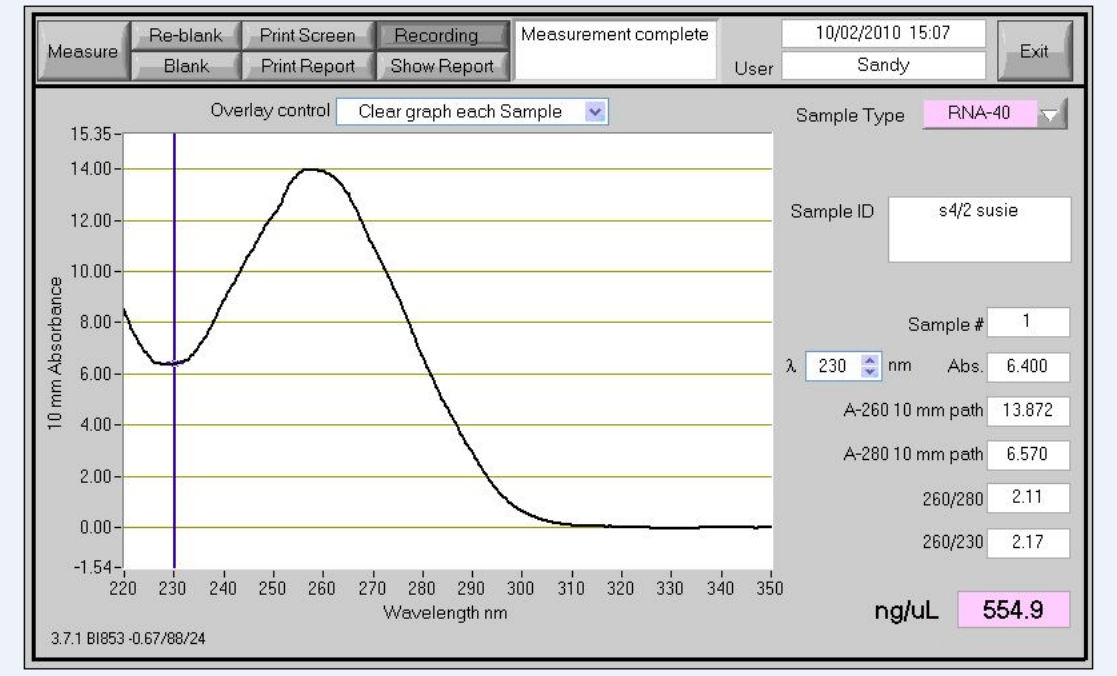


Figure shows the RNA concentration of a sample 4.1 in ng/ul (554.9). The ratio of absorbance at 260 nm and 280 nm (260/ 280) gives an indication of RNA purity.

2.6 Reverse transcription (RT) of mRNA

mRNA was removed from -80°C storage and kept on wet ice at all times (4°C). Using the QuantiTect® Reverse transcription Kit (Qiagen, cat. no. 205310) and GoScript™ reverse transcriptase (Promega, cat. no. A5003) the RNA was reverse transcribed into cDNA. The amount of RNA required for 100ng total RNA is calculated for each RNA sample. Experimental variation was minimized by using a standardized amount of RNA (100ng). This assumes almost 100% efficiency of the RT reaction.

The first step of this reaction using the QuantiTect® RT kit was the gDNA elimination reaction that was carried out on wet ice in PCR tubes (Thistle Scientific, cat. no. AX-PCR-02-C). For each DNA elimination reaction in each PCR tube:

- Wipeout buffer – 2µl
- Template RNA (100ng) – Xµl and RNase free water – Yµl (X+Y=12µl)
- Total reaction volume – 14µl

Once the reaction was set up, using a thermal cycler (DNA Engine®, Bio-Rad) the tubes were put through the 'GOUT' program:

- 3 minutes at 42°C

Once the program was complete, the PCR tubes were removed and a master mix was made up for the RT reaction (GoScript™ reverse transcriptase (not from QuantiTect® kit), RT buffer and RT primer mix) and 6µl of master mix was then added to each tube containing the RNA that had gone through the gDNA wipeout reaction. In each tube there was now the following:

- DNA wipeout reaction - 14µl (already in tube)
- GoScript™ reverse transcriptase – 1µl (from Promega, cat. no. A5003)
- RT Buffer - 4µl
- RT Primer Mix - 1µl
- Total reaction volume - 20µl

Once the RT reactions were step up they were again put into the thermal cycler using the 'RT4' program:

- 30 minutes at 42°C
- 5 minutes at 95°C (enzyme deactivation)

Once the reaction was completed, the newly made cDNA was put into -20°C storage.

The GoScript™ reverse transcriptase was used instead of the one in the QuantiTect® kit because during the optimisation of the RT reaction, it was shown, having compared both, that the GoScript™ reverse transcriptase produced better quality cDNA. The quality of cDNA was checked using the Human cDNAOK! ® kit (Microzone, cat. no. 2HCDOK-150). This involved a PCR reaction containing 5 different primers pairs. Better quality cDNA will be able to amplify all 5 amplicons of up to 600 base pairs (bp). If there is no cDNA, only the positive PCR control should be visible (500 bp). This method was also used to periodically check the integrity of cDNA using the following reaction in each tube:

- Human cDNAOK! ® kit mix – 7.5µl
- MasterMix Gold – 12.5µl
- H_20 - 4µl
- cDNA - 1µl
- Total reaction vol. – 25µl

A mastermix of Human cDNAOK! ® kit mix, MasterMix gold and H_20 was made and 24µl of this was added to suitably labelled PCR tubes, to which 1µl of cDNA was added. Once completed, the tubes were put into a thermal cycler using the program 'cDNA-OK':

- 95°C for 5 minutes.
- For 35 cycles:
  - 95°C for 30 sec.
  - 59°C for 20 sec.
  - 72°C for 45 sec.

Once completed, the products of this PCR reaction were separated on a 2% agarose gel at 50 volts constant voltage (see section 2.10). An example of this is shown below (figure 2-5).

Figure 2-5. Human cDNAOK! ® kit PCR products

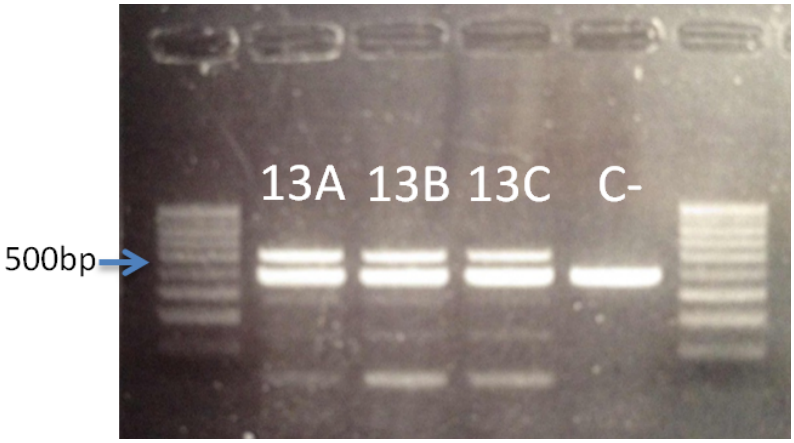


Figure showing a PCR experiment using the Human cDNAOK! ® kit (Microzone, cat. no. 2HCDOK-150. The cDNA used was from samples 13A, 13B AND 13C. The negative control (C-) only contained distilled water and only the positive PCR control is visible at 500 bp. cDNA was made using the Quantitect® kit and GoScript™ reverse transcriptase. Products are run on a 2% agarose gel using a 100 bp ladder (Sigma-Aldrich, cat. no. P1473). 6µl of DNA ladder and 10µl of PCR product were loaded into the gel.

As already stated, in order to check the quality of the cDNA produced the Human cDNAOK!® kit was used. This demonstrated that the cDNA made was of optimal quality as it allows amplification of amplicons of up to 600bp. It was not possible to calculate the concentration of cDNA after the RT reaction was complete on the NanoDrop®. This is because there are Deoxynucleotide Triphosphates (dNTPs) left over within the cDNA from the RT reaction that would affect any reading on the NanoDrop®. Therefore any concentration obtained would not be exclusively for the cDNA produced.

2.7 Primers

2.7.1 Design

In order to design primers the website ensembl (http://www.ensembl.org/index.html) was used to look up the gene of interest (name or symbol) in order for the sequence of the gene, including the position of exons and introns to be determined. Once a suitable DNA sequence was selected, primer 3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used to try and design suitable primers. Specific requirements were as followed:

- A product size of between 100-200bp.
- Primer size minimum 18bp, optimum 20bp, maximum 22bp.
- T_m of primers to – optimum 61°C (–/+ 1°C).
- Primer GC% - minimum 40%, optimum 50%, maximum 60%.
- Select 'advanced setting' tab:
  - Set the no. of GC clamps to 2 (preferable one at either end of primer).
- Press the 'pick primers' button.
- Hopefully a selection of possible primer pairs will be generated.

Once potential primers were selected, they were checked for single-nucleotide polymorphisms (SNP) (https://ngrl.manchester.ac.uk/SNPCheckV2/snpcheck.htm). If no SNP were detected, using the website reverse ePCR (http://www.ncbi.nlm.nih.gov/projects/e-pcr/reverse.cgi) the primers were checked to ensure they would bind specifically within the gene of interest only and not in multiple areas of the transcriptome. The website DNA calculator (http://www.sigma-genosys.com/calc/DNACalc.asp) was then used to check the secondary structure of the primers to ensure they would not likely create primer dimers. If the chosen primers pass all the checks were then ready to be ordered.

2.7.2 Primer preparation (Sigma-Aldrich)

Before preparation, the workspace and all relevant equipment were cleaned with 100% ethanol and appropriate protective clothing was worn. Primers ordered from Sigma-Aldrich arrived as lyophilised primers labelled with the name given to the primers when they were designed and ordered.

A primer stock solution was made as stated on the data sheet that arrived with the primers to give a 100µM primer stock solution. From this, a 5µM working solution of each primer was made, by performing a 1/20 dilution using sterile RNase free water (380µl of RNase free H_2O to 20µl of each primer). This concentration of working primer stock was based on optimisation experiments for the endpoint and qPCR machine used in the laboratory. Both the stock and primer stock solution were stored at -20°C.

2.7.3 Primer sequences used for genes of interest

Beta Actin (β-Actin*)* (designed by A. Fletcher)

- F – GCGGGAAATCGTGCGTGACATT
- R – GATGGAGTTGAAGGTAGTTTCGTG
- Amplicon size – 232bp

HSP20 (self-designed)

- F – ACGAGGAGCGCCCGGATGAG
- R – CTGGTGCGGCCTGGATGGAC
- Amplicon size – 138bp

HSP27 (Oba *et al.,* 2008)

- F – CAGTCTCATCGGATTTTGCAGC
- R – TGTCCCTGGATGTCAACCACTT
- Amplicon size – 318bp

HSP32 (Shokawa *et al*., 2006)

- F – GAGGCCAAGACTGCGTTCC
- R – GGTGTCATGGGTCAGCAGC
- Amplicon size - 72bp

HSP60 (self-designed)

- F – AAGGCATGAAGTTTGATCGAG

- R – CATTGGCAATTTCAAGAGCA
- Amplicon - 153bp

HSP72 (self-designed)

- F – GGTGCAGGTGAGCTACAAGG
- R – TGCGAGTCGTTGAAGTAGGC
- Amplicon – 156bp

HSP90 (self-designed)

- F – TTTGTGGAGAAGGAACGTGA
- R – CAGGTTTGTCTTCCGACTCTTT
- Amplicon – 109bp

4th TLR4 (self-designed primer 3 plus)

- F – AGTTGAACGAATGGAATGTGC
- R – ACACTGAGGACCGACACACC
- Amplicon size – 114bp

MYD88 (Isnardi *et al.,* 2008)

- F – GACGACGTGCTGCTGGAGCTG
- R – GATGAAGGCATCGAAACGCTCAG
- Amplicon size – 201bp

TLR2 (self-designed with primer 3 plus)

- F – GCCTCTCCAAGGAAGAATCC
- R – TGTTGTTGGACAGGTCAAGG
- Amplicon size – 141bp

Apelin receptor (APJ) (Melgar-Lesmes *et al*., 2011)

- F – CTATCCTGTTTTCTGAGTGTGAGG
- R – CTAAGGGCTGGAGCACTAATTATC
- Amplicon size – 89bp

GRP78 (HSPA5)

- Solaris® qPCR Gene Expression Assays for (Thermo Scientific, cat. no. AX-008198-00-0100)

β-Actin

- Solaris® qPCR Gene Expression Assays for (Thermo scientific, cat. no. AX-003451-00-0100)

There was a long optimisation period of several months involved with TLR4 primers ordered from Sigma-Aldrich used in the work herein. The first 3 TLR4 primer pairs used were tried and discarded until the fourth set used was finally optimised under the standard qPCR assay conditions. After this, no more problems were encountered.

2.8 Polymerase Chain reaction (PCR)

Before PCR was carried out all equipment was cleaned with 70% ethanol. Blue MegaMix-Double® (Microzone, cat. no. 2MMBD-5) was used for all endpoint PCR reactions. Primers suitable to analyze the genes of interest were ordered from Sigma-Aldrich (usually working stock of 5μM). A mastermix of relevant reagents was made up and 1μl of cDNA was used. A typical reaction in a single tube contained:

- 12.5 μl Blue MegaMix Double®.
- 2.5μl Forward primer (5μM).
- 2.5μl Reverse primer (5μM).
- 6.5μl RNAse water.
- 1μl sample cDNA/ positive control cDNA/ distilled water (dH_2O) negative control
- 25μl total reaction volume.

Once all reactions were set up they were placed into a thermal cycler (DNA Engine®, Bio-Rad) and then run with the standard program 'SAN1':

- 95°C for 5 minutes.
- For 35 cycles:
  - 94°C for 30seconds.
  - 61°C for 30seconds.
  - 72°C for 30seconds.
- 72°C for 10 minutes.

Once the reaction was complete the PCR products were placed in the fridge until gel electrophoresis was performed (see section 2.10).

2.8.1 Toll like receptor (TLR) screening with endpoint PCR

The aim of these experiments was to screen ischemic myocardial tissue from cyanotic and control patients, collected in theatre, that had not been exposed to any type of experimental conditions in order to establish their expression profile for all known human 10 TLRs and demonstrate if there were any identifiable differences between cyanotic and acyanotic patient groups. This was done using the TLR-RT Primer Set® (Invivogen, cat. no. rts-htlrs) that contained primers for all 10 human TLRs (figure 2-6). The sizes of the primers used were unknown as they were part of the kit.

Figure 2-6. Primers for 1-10 TLR from the TLR-RT Primer Set®

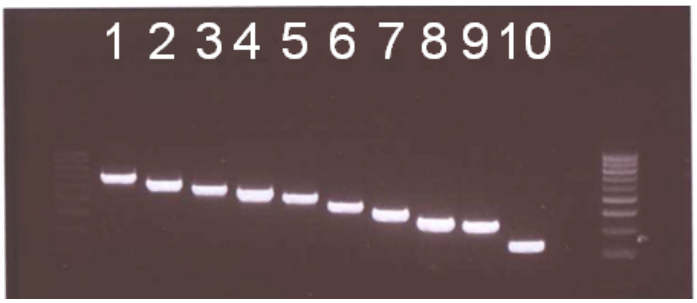


Figure shows an endpoint PCR reaction using the Invivogen kit control cDNA with all 10 TLR primers. 100bp DNA ladder (Sigma-Aldrich, cat. no. P1473-1VL) was used despite the primer sizes being unknown. 6µl of the DNA ladder and 10µl of PCR product were loaded.

RNA was extracted from the heart tissue (see section 2.5) and then the RNA was used for RT to make cDNA. This cDNA was used to run a series of PCR experiments using primer pairs for all 10 human TLRs. The method of PCR mentioned in 2.8 was used but the reagents and the PCR program used were slightly different. The primers used were provided in the kit but their sequence and precise size was not given. A reaction in a single tube contained:

- 12.5 µl Blue MegaMix Double®.
- 1µl TLR primer pair (from kit).
- 10µl RNase water.
- 1.5µl sample cDNA/ positive control cDNA/ dH_2O negative control or
- 25µl total reaction volume.

The following conditions (program 'TLR') were used for the PCR reaction on the thermal cycler:

- 95°C for 2 minutes.
- For 35 cycles:
  - 95°C for 30 seconds.
  - 60°C for 30 seconds.
  - 72°C for 2 minutes.
- 72°C for 5 minutes.

A period of optimization was carried out when this kit was initially used. It was found it would only work with the positive control provided with the kit. It was discovered that the original PCR protocol provided by Invivogen was not correct and upon correction the problems encountered were solved.

2.9 Quantitative real time polymerase chain reaction (qPCR)

Before this method was undertaken all work space and equipment to be used was sterilized with 70% ethanol. Disposable sterile plastic consumables were used as bought or were sterilized under ultra-violet (UV) light. cDNA was removed from -20°C storage and allowed to thaw on wet ice. The GoTaq® qPCR Master Mix (Promega, cat. no. A6001) was used to undertake mRNA level investigation of differential gene expression using the StepOnePlus® qPCR machine (Applied Biosystems). The GoTaq® qPCR Master Mix contains

an intercalating dye that binds to double stranded DNA between adjacent base pairs. Once bound it emits a fluorescent signal. As the PCR amplification proceeds and more double stranded product is made, the fluorescent signal will increase.

Primers used were ordered from Sigma-Aldrich and the endogenous control used was β-Actin. The choice of endogenous control is important. If its expression is not uniform in all samples then it does not account for intra sample variation. For this work the endogenous control was β-Actin, which has previously been demonstrated as a suitable endogenous control in this laboratory.

Sample and reference sample (for correction of expression data and positive control) cDNA were diluted at 1/5 (2µl cDNA + 8µl dH_2O). A mastermix was made for every primer used within a single experiment. A typical master mix plus cDNA per number of wells would be as follows:

- 12 µl GoTaq® master mix (provided in kit).
- 1.*25*µl Forward primer (5µM).
- 1.*25*µl Reverse primer (5µM).
- 5.25µl RNase water.
- 0.25 CRX reference dye (provided in kit)
- 5µl cDNA (1/5 dilution)
- 25µl total volume.

The experiment would be set up in Microamp® fast optical 96 well plates (Applied Biosystems, cat. no. 4346906). Once the plate was set up, a plastic cover was placed and sealed on top of the plate. The plate was then spun in a centrifuge (1500xg for 1 minute) to remove any bubbles and then it was put into the qPCR machine and the run was started. The following qPCR programme was used:

- Holding stage - 95°C for 20seconds
- Cycling stage - 95°C for 3 seconds, 60°C for 30 seconds - 50 cycles
- Melt curve – 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15seconds.

Once the reaction was complete, the plate was removed from machine and kept in the fridge. The PCR products were run on a standard agarose gel (see section 2.10) to check size and confirm a single discreet product.

The disadvantage with GoTaq® qPCR Master Mix is that the dye it contains binds indiscriminately to any double stranded product. Therefore, to ensure a single discreet qPCR product is amplified during the reaction, melting curve analysis is used. This is where the qPCR products are exposed to a temperature gradient. The point at which 50% of the product denatures is measured i.e. when the double stranded PCR product separates to become single stranded DNA. If only one single discreet peak is present then there should only be one product. Great care was taken when the primers were designed. It was ensured they did not contain any self-complementary sequence and that they did not form primer dimers. This ensured that the primers did not form secondary structures or result in non-specific primer binding that would result in undesired application. If any amplification occurs after 35 cycles during a qPCR reaction it is caused by non-specific primer binding and any results were discarded.

The correct size (bp) of amplicon is known for each primer pair when they are designed (see section 2.7.1). Therefore the size of a PCR product can be established by gel electrophoresis (see section 2.10).

It should be noted that the sensitivity of the GoTaq® assay used in all the qPCR reactions in this project was optimised by the use of copy number serial dilutions; a sample with a known copy number was serially diluted by 1/10 down to one copy number (demonstrated by Mr Alexander Fletcher).

Biological replicates instead of technical replicates were used for these experiments. Specifically, tissue samples from the same patient group were grouped together and used as replicates of each other. Such variability between biological replicates is normal.

Differential gene expression was then calculated using the Comparative C_t method to give relative quantification (RQ) of gene expression using the StepOne Software® v2.1 (Applied Biosystems).

2.9.1 qPCR with Solaris® qPCR gene expression assays (GRP78)

These assays are an alternative to TaqMan® Gene Expression Assays (Applied Biosystems). They contain fluorescently labelled probes as well as primers for a gene of

interest that bind within the amplicon. These probes have fluorescent labels and a quencher molecule. The presence of the quencher prevents the fluorescent label from emitting a signal. During a successful qPCR reaction, the fluorescent label on the probe is successfully cleaved from the quencher by the activity of the Taq polymerase during the reaction, allowing the signal to be detected. This means a fluorescent signal will only be detected when the primers and probes bind together and amplify the target i.e. the gene of interest.

Before this method was undertaken all work space and equipment to be used was sterilized with 70% ethanol. Disposable sterile plastic consumables were used as bought or were sterilized under UV light. cDNA was removed from -20°C storage and allowed to thaw on wet ice. Solaris© qPCR Gene Expression Assays for HSPA5 (GRP78) (Thermo Scientific, cat. no. AX-008198-00-0100) and β-Actin (Thermo Scientific, cat. no. AX-003451-00-0100) were used to undertake mRNA level investigation of differential gene expression using the StepOnePlus® qPCR machine (Applied Biosystems). The endogenous control used was β-Actin.

Sample and reference sample (for correction of expression data and positive) cDNA were not diluted. A mastermix was made for every Solaris® qPCR Gene Expression Assay used within a single experiment. A typical master mix plus cDNA per number of wells would be as follows:

- 12.5 µl Solaris® qPCR master mix (provided in kit).
- 1.25µl probe/ primer (provided in kit)
- 10.25µl RNAse water (provided in kit)
- 1µl cDNA (provided in kit)
- 25µl total volume.

The experiment would be set up in a Thermo-fast® 96 PCR plate (Thermo Scientific, cat. no. AB-1900/W). Once the plate was setup, a plastic cover was placed and sealed on top of the plate. The plate was then spun in a centrifuge (1500xg for 1 minute) to remove any bubbles and then it was put into the qPCR machine and the run was started. Once the reaction was complete, the plate was removed from the machine and kept in the fridge if products were to be separated on an agarose gel (see section 2.10) to ensure the produced qPCR product was of the expected size. Differential gene expression was then calculated using the Comparative C_t

method to give RQ of gene expression using the StepOne Software® v2.1 (Applied Biosystems).

2.9.2 Calculation of gene expression by comparative C_t method

Differential gene expression of all the genes of interest was determined using the comparative C_t method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). This method is a relative quantification (RQ) method used to assess changes in mRNA levels of a gene of interest in one sample relative to the mRNA levels in other samples. It is not necessary for the exact amount of mRNA of the gene of interest in each sample to be known. This RQ of gene expression in all samples is determined against the level of expression of the gene of interest found in an arbitrary reference sample (described below). The resulting RQ or fold change of gene expression is expressed as arbitrary values with no units.

The resulting RQ values were calculated using the StepOne® Software v2.1 (Applied Biosystems). The RQ value was calculated using the following equations:

- $\Delta C_t = C_t$ [Gene of interest] - Ct [Endogenous control]
- $\Delta\Delta C_t = \Delta C_t$ [sample]- ΔC_t [reference sample]
- $RQ = 2^{-\Delta\Delta Ct}$ (normalised against reference sample)

The C_t is the cycle number at the threshold chosen by the StepOne® Software v2.1 at which to measure and compare the level of transcript found in each sample. This is the point at which PCR product amplification is optimal. As amplification proceeds, the dye in the GoTaq® qPCR Master Mix intercalates into the double stranded PCR product and a fluorescent signal is emitted. This signal therefore indicates the degree of product amplification in each sample.

The endogenous control is a housekeeping gene, whose mRNA expressions levels are fairly constant in all cell types. In these experiments the endogenous control used was β-Actin. In any sample you measure the known expression of a gene (β-Actin) against the unknown expression of a gene of interest. This is performed in order to account for intra sample variation. In this project, such variation is also accounted for by the fact that the concentration of RNA used in all RT reactions (see section 2.6) is standardised (100ng). Therefore, the level

of expression i.e. C_t of the endogenous control in each sample is substracted from the Ct of the gene of interest in each sample. This provides the ΔC_t value. This is done for an arbitrary reference sample as well. The level of expression in this reference sample is what is used to calculate the RQ of gene expression of the gene of interest in all other samples. The ΔC_t of each sample has the ΔC_t of the reference sample subtracted from it, which gives the $\Delta\Delta C_t$ for each sample. The RQ value is then calculated as shown above.

The reference samples used were myocardial tissue samples obtained from 1) a TOF patient exposed to a chemical inducer that was used for the HSP primer set (13B) and 2) a control sample exposed to 2% O_2 (18B). These references samples were tested to ensure they were appropriate for all the primers they were for (shown in figure 2.7 and figure 2.8). The fact this reference sample is known to work with all primers used and has been through the same procedure as all other samples means it also acts as a positive control for this assay.

The fact the same references samples were used for all qPCR experiments allows the RQ results from one qPCR experiment to be compared to the results from another therefore it helped minimize inter experimental variation. Any inter experimental variation experienced in different reactions will affect the RQ value produced for the reference sample the same as it would affect the RQ values produced for all other samples. Therefore any variability in RQ values is minimized by its use in every reaction.

Figure 2-7. Reference sample 13B with HSP primer set

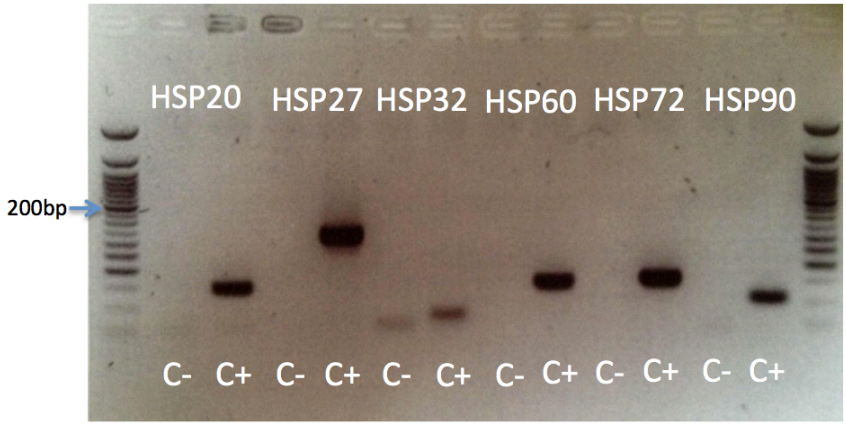


Figure shows a PCR experiment using patient 13B cDNA with primers for HSP20, HSP27, HSP32, HSP60, HSP72 and HSP90. Products are separated on a 2% agarose gel. 50 base pair ladder was used (Bio-Rad, cat. no. N32365). 6µl of DNA ladder and 10µl of PCR product were loaded.

Figure 2.8. Reference sample 18B with inflammatory mediator primer set

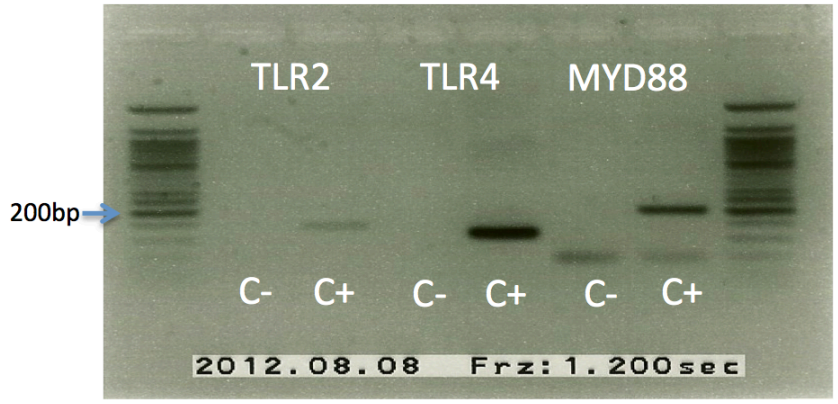


Figure shows a PCR experiment using patient 18B cDNA with primers for TLR2 (141bp), TLR4 (114bp) and MYD88 (201bp). Products are separated on a 2% agarose gel. 50 base pair ladder was used (New England Biolabs, cat. no. N04735). 6µl of DNA ladder and 10µl of PCR product were loaded. MYD88 has a known secondary product, which is apparent in the figure.

2.10 Gel electrophoresis for cDNA after endpoint PCR/ qPCR

PCR/ qPCR products were separated by size in a 2%, 50ml agarose gel. 1.0g of agarose (Sigma-Aldrich, cat. no. 05066-50G) was mixed with 50ml of Tris-Acetate EDTA (TAE) buffer (Flowgen Bioscience, cat. no. H19109) (1x buffer diluted from a 50x stock) and thoroughly mixed. This was heated in the microwave at full power for 1minute and then 7.5μl of SYBR safe (Invitrogen, cat. no. S33102) was added before the gel was poured in order to make the cDNA in the gel visible under UV light. The gel was allowed to polymerise for up to 1 hour, then once ready samples and a suitable molecular ladder (100bp DNA ladder from Sigma-Aldrich, cat. no. P1473-1VL) were prepared for loading. When reagents used did not have a dye already present within them, 2μl of gel loading dye (Sigma-Aldrich, cat. no. G2526-5ML) was added. Once loaded, the gel was run at 50 volts at constant voltage. Usually after running for about 20 minutes the gel was observed under a UV trans-illuminator in order to visualise the bands.

2.11 Heart tissue preparation for protein expression analysis using Western blotting

2.11.1 Homogenisation

Tissue samples were removed from -80°C storage and ground to a fine powder in liquid N_2 with a mortar and pestle and were added to an appropriate volume of homogenising buffer (see appendix 4) supplemented with protease inhibitor cocktail. Using a Polytron PT1600E homogeniser (Kinematica) set at setting 10, the sample containers were surrounded by ice and homogenized for 3x10s intervals with a 1 minute cool down at 4°C in between. The homogenate was spun in a centrifuge (5000xg for 10 minute at 4°C) to remove debris and the resultant supernatant containing the total cell protein (both particulate and cytosolic fractions) was extracted.

2.11.2 Bradford assay for protein estimation

The Bradford protein assay (Bradford, 1976) was used to quantify total protein concentration. A stock standard (STD) solution of Bovine Serum Albumin (BSA) (1mg per ml) (see appendix 4) was used to create the standard curve using the following standards:

Table 5. Standards (µg/ml) used for Bradford Assay

STD concentration (µg/ml)	Vol. stock std (µl)	Vol. of dH_2O (µl)
0	n/a	50
200	10	40
400	20	30
600	30	20
800	40	10
1000	50	n/a

Samples were diluted by 1/40 (39µl of dH_2O and 1µl of homogenate) or by an applicable dilution factor (e.g. 1/20). Measurement of concentrations was prepared using a BioPhotometer® (Eppendorf).

2.12 Western Blotting

2.12.1 Sample and gel preparation

Samples were mixed 1:1 with loading buffer (see appendix 4) and boiled for 5 minutes at 95°C before loading. Samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide resolving gel with a 4% stacking gel (see appendix 4 for buffers used and for the composition of resolving and stacking gels) using the Protean II® xi Cell System (Bio-Rad, Hemelhempstead, U.K.) at 200 volts for a maximum of 4 hours (see appendix 4 for composition of running buffer). Each well was loaded with 50µg of protein unless otherwise stated. Pre-stained SDS-PAGE Standards (Low range, Bio-Rad, cat. no. 161-0305) were loaded beside the samples to demonstrate the size of proteins identified. Homogenised term placental villous tissue (50µg), known to express HSP27 and HSP72, were loaded and used as a reference sample and positive control. Although loading controls are not shown, they were performed to ensure the amount of protein being loading was equal for all samples. This was done using a β-actin antibody (see section 2.12.4).

2.12.2 Transfer of proteins to Nitrocellulose

Transfer of proteins to Whatman Protran® nitrocellulose transfer membranes (Sigma-Aldrich, cat. no. Z61360) in transfer buffer (see appendix 4) was carried out using the Trans-

Blot SD Semi-Dry® Electrophoretic Transfer Cell (Bio-Rad, cat. no. 170-3940) at 22 volts and a variable constant current (2.5 x membrane area) for 30 minutes.

2.12.3 Immuno-detection of proteins (Immuno-blotting)

Filters were blocked in 5% donkey serum (Serotec, cat. no. CO6SBZ) in TBSTB (see appendix 4) for 1 hour at room temperature unless otherwise stated. Primary antibodies were pre-absorbed (minimum of 30 minutes) in 5% human serum (Sera Laboratories International, cat. no. S-123-H) in TBSTB at room temperature during the blocking process. Filters were rinsed in TBSTB and incubated for 1 hour at room temperature with appropriate primary antibody solution (e.g. HSP27, New England Biolabs, cat. no 2402, mouse monoclonal, 1:1000 concentration). Filters were rinsed and washed in TBSTB (3x5 minutes) and then incubated for 1 hour at room temperature with an appropriate horseradish peroxidase conjugated secondary antibody (e.g. Donkey anti mouse, Abcam,, cat. no. ab6820, 1:1000) diluted in TBSTB. Filters were rinsed again and washed with TBSTB (3x5 minutes) and then washed once for 5 minutes with dH_2O. Immunologically reactive proteins were visualised using the Amersham ECL® Western Blot detection system (Amersham Pharmacia Biotech, GE healthcare, cat. no. RPN2106). Bands on the exposed films were scanned using a Bio-Rad GS-700® imaging densitometer connected to a Macintosh computer using Bio-Rad MultiAnalyst® software. Band densities were expressed relative to the density of the internal placental control sample included on every gel i.e. as relative optical density (ROD).

2.12.4 Antibodies

Primary antibodies:

- HSP27
    - Cell signaling technology, cat. no. #2402
    - Mouse monoclonal
    - 1/1000 concentration
- HSP32 (HO-1)
    - Enzo Life Sciences, cat. no. ADI-SPA-896
    - Rabbit polyclonal
    - 1/1000 concentration
- HSP72
    - Enzo Life Sciences, cat. no. ADI-SPA-812

    - Rabbit polyclonal
    - 1/1000 concentration
- APJ
    - Novus Biologicals, cat. no NBP1-30869
    - Rabbit polyclonal
    - 1/1000 concentration
- β-actin
    - Sigma Aldrich, cat. no. WH0000060M1
    - Mouse monoclonal
    - 1/3000 concentration

Secondary antibodies:

- Donkey polyclonal Secondary Antibody to Mouse IgG - H&L (HRP)
    - Abcam, cat. no. ab6820
    - 1/1000 concentration
- Donkey anti-Rabbit IgG H&L (HRP) secondary antibody
    - Abcam, cat. no. ab7083
    - 1/3000 concentration

2.13 Decay curve experiments

The aim of this experiment (figure 2-9) was to determine if the expression pattern of the genes of interest seen during the qPCR experiments within the excised myocardium from both the acyanotic group and the cyanotic group was influenced by the conditions of the experiments performed on the myocardial tissue or if it was due to degradation of RNA during the time period of incubation. The steps 1 to 5 in the diagrams correspond with steps 1 to 5 described within the text.

Step 1: In preparation for tissue collection 1ml of supplemented media (see appendix 4) was added to 4 wells of a sterile 24 well culture plate (4ml in total). A separate amount of supplemented media was also prepared in a universal container, which was to be used for tissue collection. Once prepared, the 24 well culture plate and universal container were put into a NuAire® CO_2 incubator set at 20% O_2 and at 37°C. Also 6 bijous were prepared, each containing 3ml of RNA*later*® solution.

Step 2: When tissue was ready to be collected, the universal container was removed from the incubator and taken to theatre and all tissue that was collected was put into it. A bijou containing 3ml of RNA*later*® solution was also taken over to theatre and a piece of tissue was removed at this point and put into it. This sample was used as an experimental baseline reference sample (A).

Step 3: The samples within the universal container and the reference sample within the bijou were brought back to the laboratory.

Step 4: When back in the laboratory, all tissue was removed from the universal and cut into equal sized pieces. Another piece of tissue was removed at this point and was put into another bijou containing 3ml of RNA*later*® solution that would act as a second baseline reference (B).

Step 5: When tissue was ready for further processing, the 24 well culture plate was removed from the incubator. The rest of the tissue was then distributed into the four wells of the 24 well plate that contained supplemented media. Once done, the 24 well culture plate was then put back into the incubator. Tissue was removed after 1.5 hours (C), 3 hours (D), 4 hours (E) and 6 hours (F) of incubation. These times were chosen as they represented the incubation times used in both the HSP induction (see section 2.4) and reperfusion experiments (see section 2.3). At each stage that tissue was removed, the culture plate was removed from the incubator and tissue in the relevant well of the 24 well culture plate was removed and put into a bijou containing 3 ml of RNA*later*® solution.

The tissue was left in RNA*later*® solution overnight at 4°C and then RNA was extracted from the tissue (see section 2.5) and then reversed transcribed (see section 2.6) into cDNA to be used in qPCR reactions using primers designed for the genes of interest (see sections 2.7.3). Any differences in gene expression seen were analysed using qPCR (see section 2.9).

Figure 2-9 Decay curve experiment

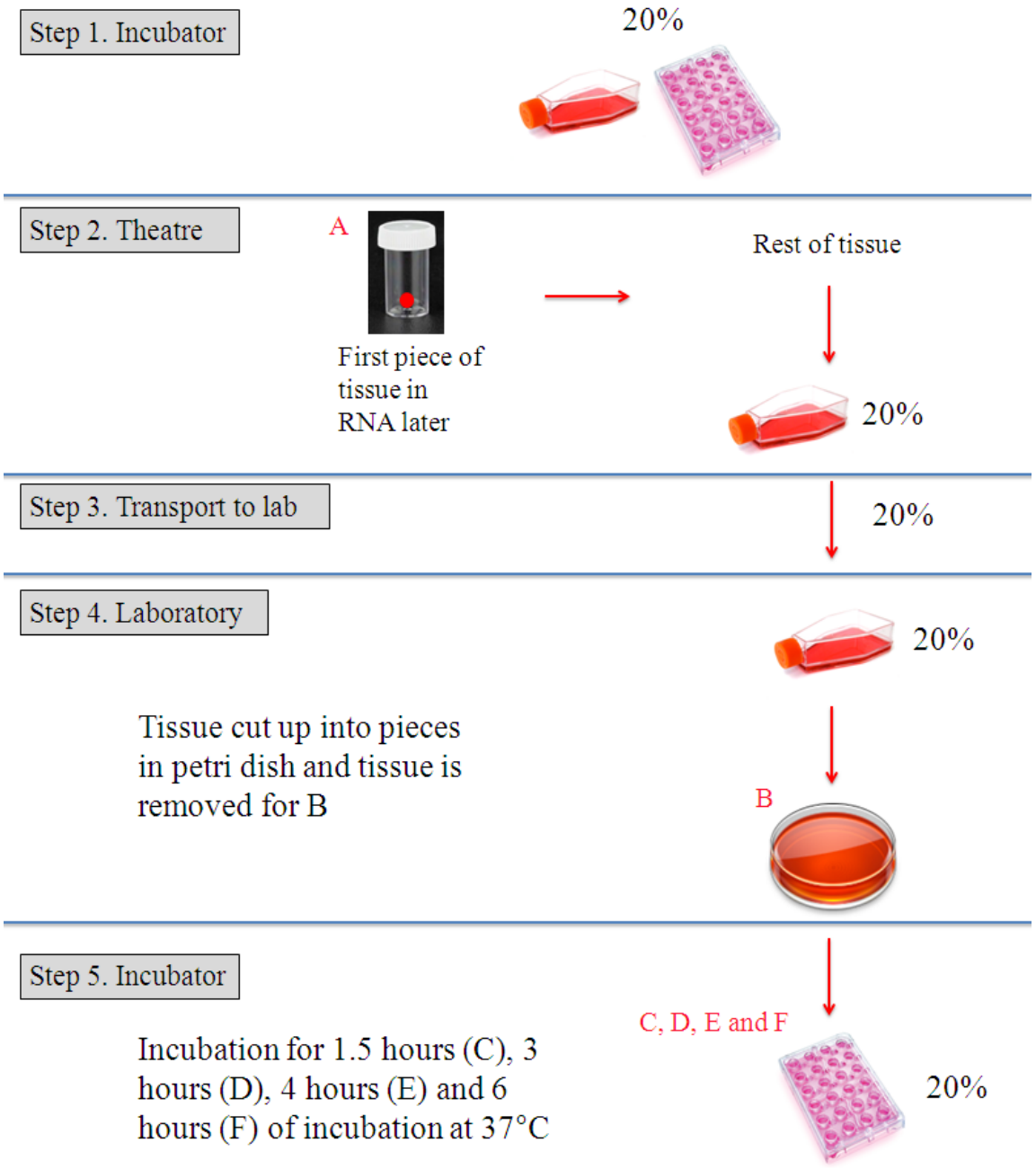


Figure 2.9 showing the time course of decay curve experiment. Labelling on the diagrams refers to the following: A – collection of first reference samples, B – collection in flask containing 20% O_2, C – after 1.5 hours incubation, D – after 3 hours incubation, E – after 4 hours incubation, F – after 6 hours incubation.

CHAPTER 3 – RESULTS

3.1 Screening of differential gene expression in ventricular myocardium excised from cyanotic and control (acyanotic) patient groups

3.1.1 Introduction

Children with congenital heart defects such as TOF (see section 1.1.3) and SAS (see section 1.1.4) require surgical correction that involves cardiac surgery with CPB (Bailliard and Anderson, 2009). The major difference between these patient groups is that pre-operatively, TOF patient myocardium is cyanotic due to chronic exposure to insufficiently oxygenated blood shunted from the right to the left side of the heart through the VSD while SAS patient myocardium is acyanotic. It is thought that patients that are cyanotic pre-operatively are more vulnerable to damage sustained during cardiac surgery, for example, to IR injury (Merante *et al*., 1998; Najm *et al*., 2000; Corno *et al*., 2002).

3.1.2 Methods and Results

The aim was to investigate the differential expression of several genes of interest in myocardium excised from children undergoing corrective heart surgery in both cyanotic and acyanotic patients. 12 patients were recruited (table 6). Seven patients were cyanotic and were comprised of TOF and fallot variant (FV) patients. Five were control (acyanotic) patients, comprising of SAS and truncus arteriosus (TA) patients. There are 2 experiments described in this section and they both used the same sample set shown below in table 6.

Table 6. Patients recruited for cyanotic and acyanotic patients groups

Patient	Type	Group	Gender	Age
3(1)	TOF	Cyanotic	Female	1 year 1 month
4(1)	TOF	Cyanotic	Male	1 year 2 months
9(1)	TOF	Cyanotic	Male	1 year 2 months
10(1)	TOF	Cyanotic	Male	0 years 11 months
11A	TOF	Cyanotic	Female	1 year 0 months
16A	TOF	Cyanotic	Male	0 year 11 months
23A	FV	Cyanotic	Female	1 year 1 month
5(1)	SAS	Acyanotic	Male	0 years 7 months
6(1)	SAS	Acyanotic	Female	12 years 4 months
7(1)	SAS	Acyanotic	Male	2 years 8 months
8(1)	TA	Acyanotic	Male	0 years 1 month
18A	SAS	Acyanotic	Female	0 years 11 months

3.1.2.1 Experiment 1: Toll Like Receptor (TLR) screening by endpoint PCR

Myocardial tissue (ischemic) samples collected (LV and RV) from acyanotic and cyanotic patients respectively that have not been exposed to any type of experimental conditions were screened in order to establish their expression profile for human TLRs 1-10 using a specialised kit (Invivogen, cat. no. rts-htlrs). This was done in order to see if there were any identifiable differences of TLR expression within the myocardium from the cyanotic and acyanotic patient groups using endpoint PCR (see section 2.8.1). To date it is not known which TLRs are expressed in infant myocardium. The PCR products were separated on an agarose gel for size analysis (see section 2.10). It should be noted that the size of primers was unknown, as the company did not provide this information.

In the cyanotic patient group (figure 3-1) TLR2-8 were expressed to some degree. TLR1 was not expressed in any patients. TLR9 and TLR10 were expressed at very low levels. TLR4 is expressed highly in all cyanotic patients. For the acyanotic group (figure 3-2), the observations were the same except for sample 6(1) where TLR8 was highly expressed. Also for sample 18A, TLR9 and TLR10 were highly expressed. There was no obvious noticeable

difference in the TLR gene expression profile of cyanotic patients compared to control patients. These observations are not quantitative and statistical analysis in this case is not appropriate. Overall TLRs, including TLR4 and TLR2 that became the main TLRs of interest in this thesis, are expressed in infant myocardial tissue. Such expression was found in myocardium from both the cyanotic and acyanotic patient groups.

Figure 3-1. TLR1-10 expression of 7 cyanotic patients

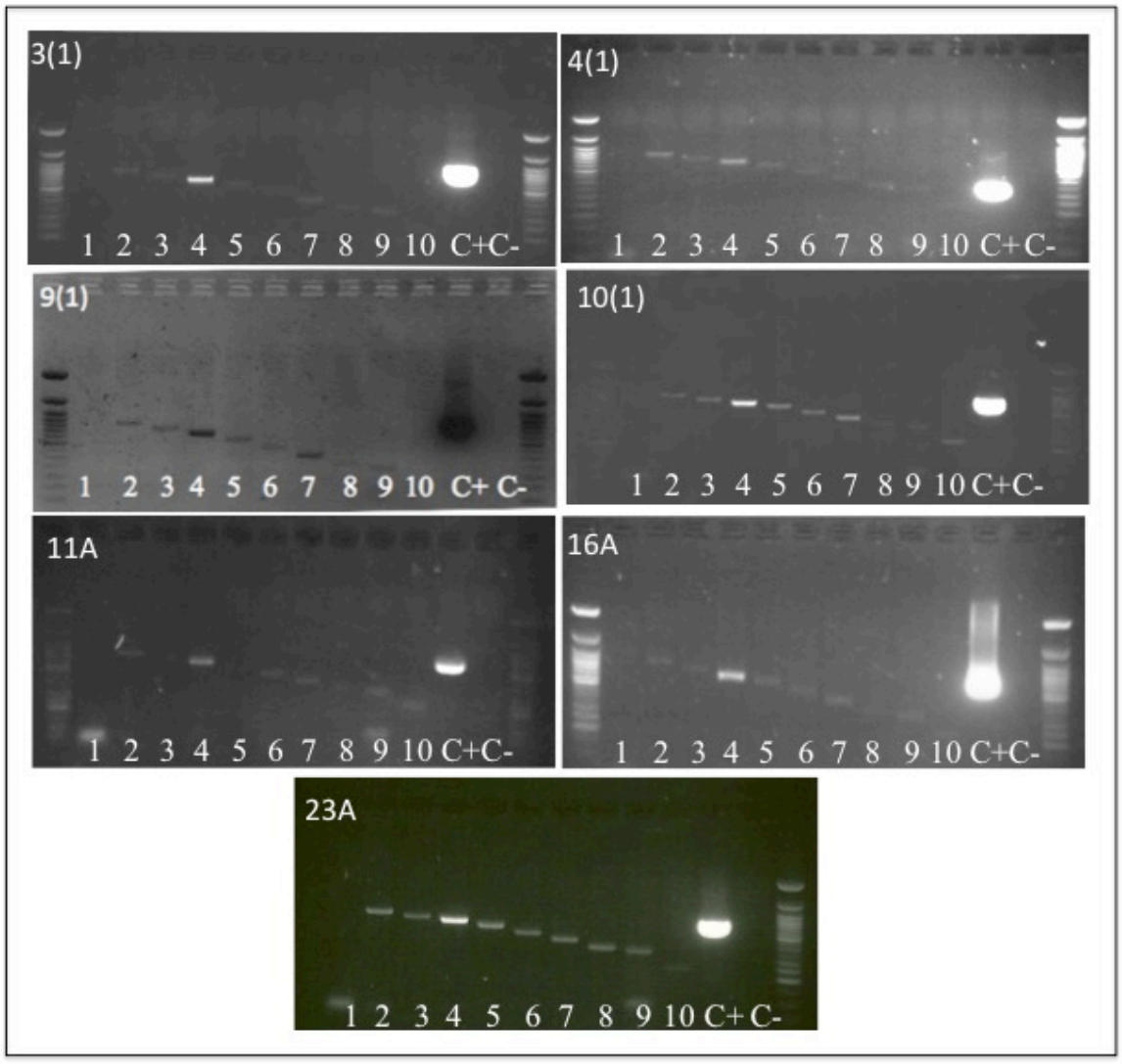


Figure showing TLR1-10 expression profile of 7 cyanotic patients. Products are separated a 2% agarose gel using a 100bp DNA ladder (Sigma-Aldrich, cat. no. P1473-1VL) despite the primer sizes being unknown. The numbers on each gel correspond to each of the 10 human TLRs. Positive control (C+) is provided for in kit and negative control (C-) is dH_2O.

Figure 3-2. TLR1-10 expression of 5 acyanotic patients

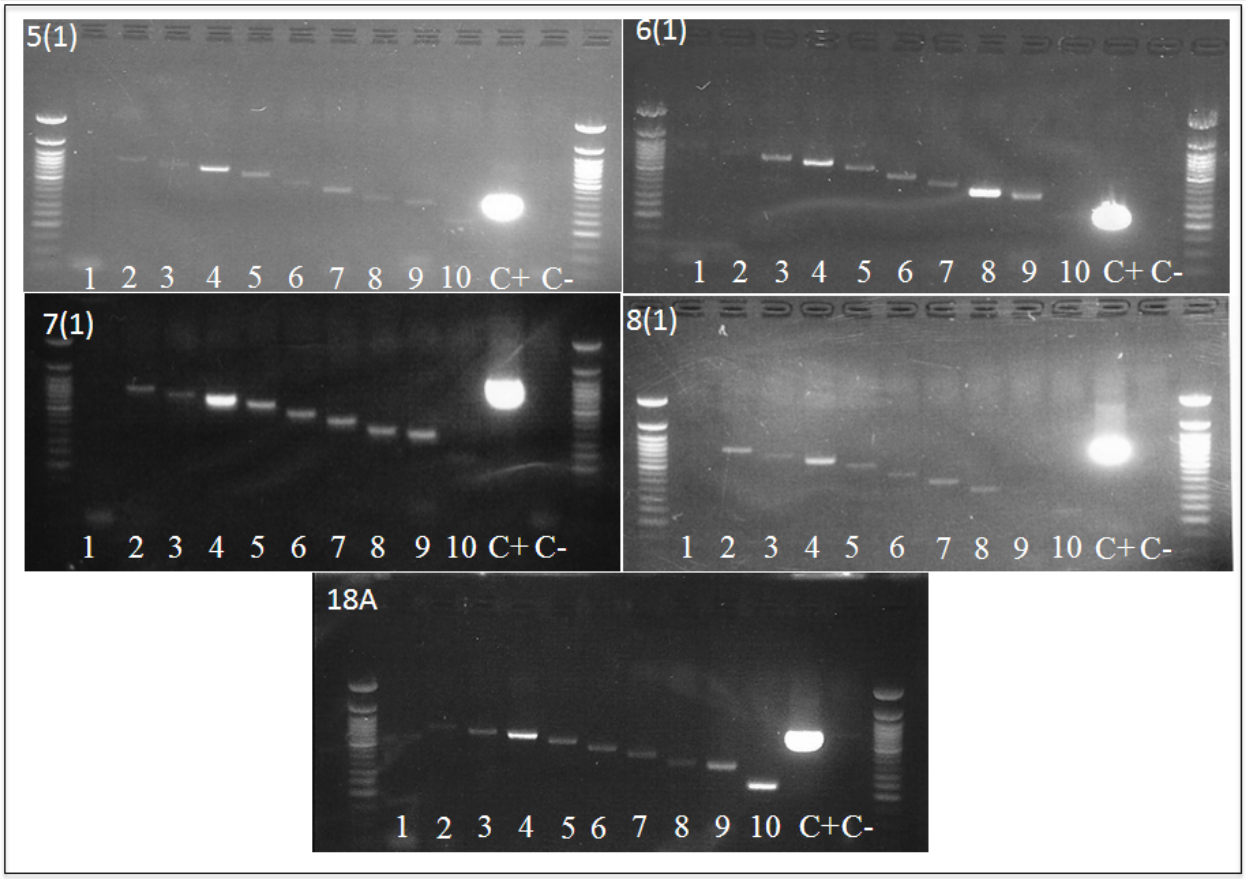


Figure showing TLR1-10 expression profile of 5 acyanotic patients. Products are separated on a 2% agarose gel using a 100bp DNA ladder (Sigma-Aldrich, cat. no. P1473-1VL) despite the primer sizes being unknown. The numbers on each gel correspond to each of the 10 human TLRs. Positive control (C+) is provided for in kit and negative control (C-) is dH_2O.

3.1.2.2 Experiment 2: Screening the differential expression of several genes of interest by qPCR

Ischemic myocardial tissue samples (LV and RV) excised from cyanotic and control patients that had not been exposed to any type of experimental conditions were collected. The samples were screened in order to establish if there were any differences in expression seen for the genes of interest within the myocardium from both the cyanotic and control patient groups. This was done using qPCR (see section 2.9). The genes of interest include the inflammatory markers TLR2, TLR4, MYD88 and the stress genes HSP20, HSP27, HSP32 (HO-1), HSP60, HSP70 and HSP90 (for primer sequences see section 2.7.3).

qPCR (see section 2.9) was used to investigate changes in expression and any differences seen in gene expression were calculated using the Comparative C_t method (see section 2.9.2) to give relative quantification (RQ) of gene expression using the StepOne Software® v2.1 (Applied Biosystems).

Statistical analysis was performed using GraphPad Prism 6 Software©. Due to its biological variation the data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. Therefore the test used was the Mann-Whitney U test where data was expressed by the median and the interquartile range. A p-value of <0.05 (two-tailed) is considered as statistically significant.

Inflammatory mediators:

The mRNA expression of TLR4, TLR2, and MYD88 was investigated (figure 3-3). TLR2 expression was significantly higher in the cyanotic group compared to the acyanotic group (1.23 vs. 0.68; p=0.0177). TLR4 expression was significantly higher in the cyanotic group compared to the acyanotic group (0.51 vs. 0.35; p=0.0303). MYD88 did not show a significant difference between the two groups.

Stress proteins:

The mRNA expression of HSP20, HSP27, HSP32, HSP60, HSP72 and HSP90 were investigated (see figure 3-4). Only HSP27 showed significantly higher expression in the cyanotic group compared to the acyanotic group (0.12 vs. 0.05; p=0.0303). HSP20. HSP32, HSP60, HSP70 and HSP90 showed no significant differences in expression between the two groups.

Figure 3-3. Figure shows the expression of inflammatory mediators in both the cyanotic and acyanotic patient groups.

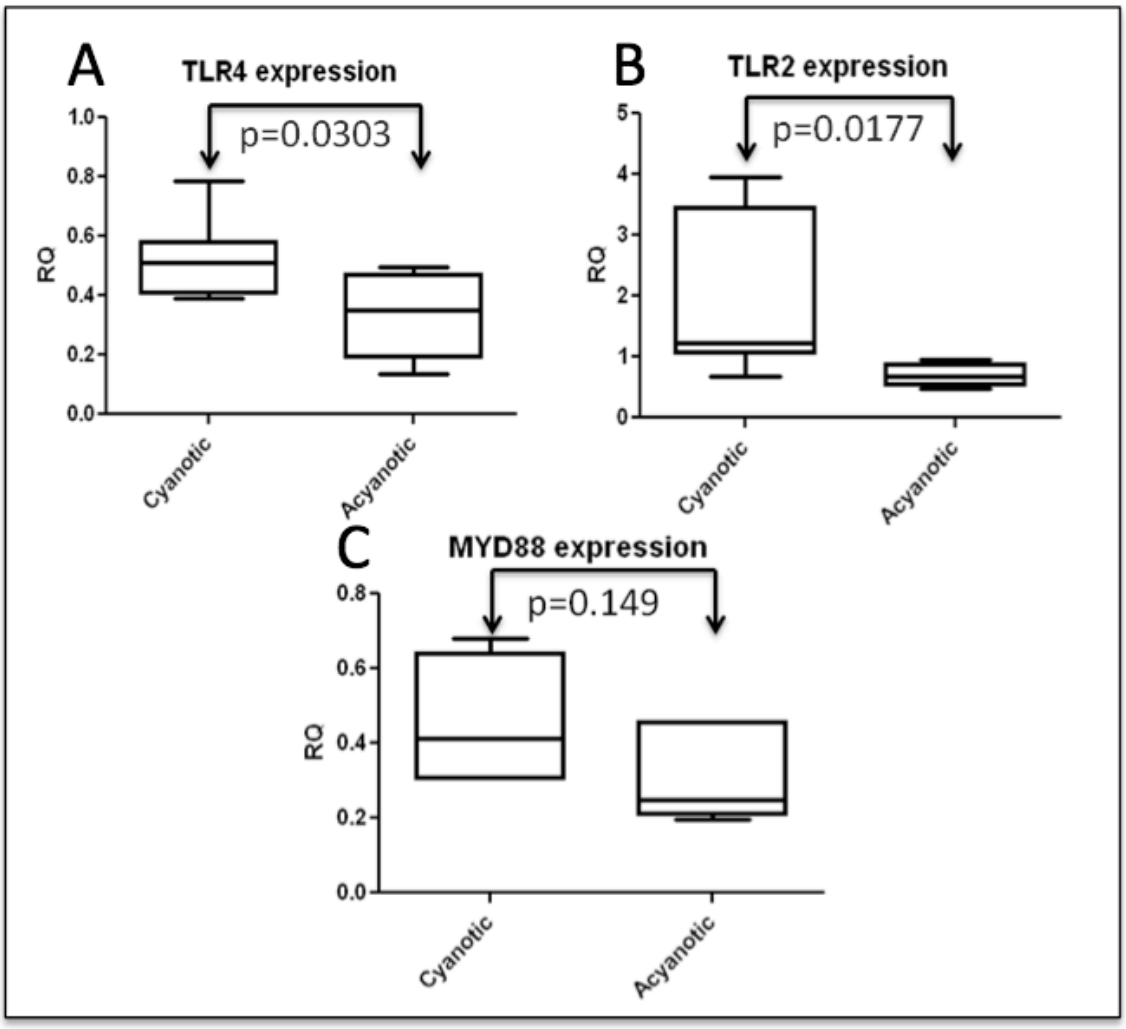
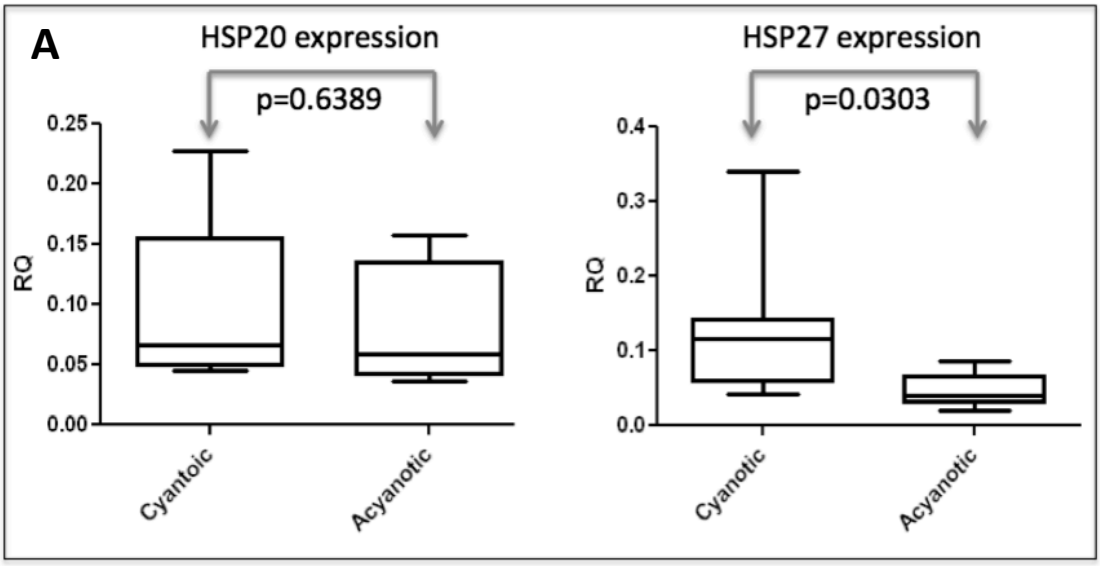
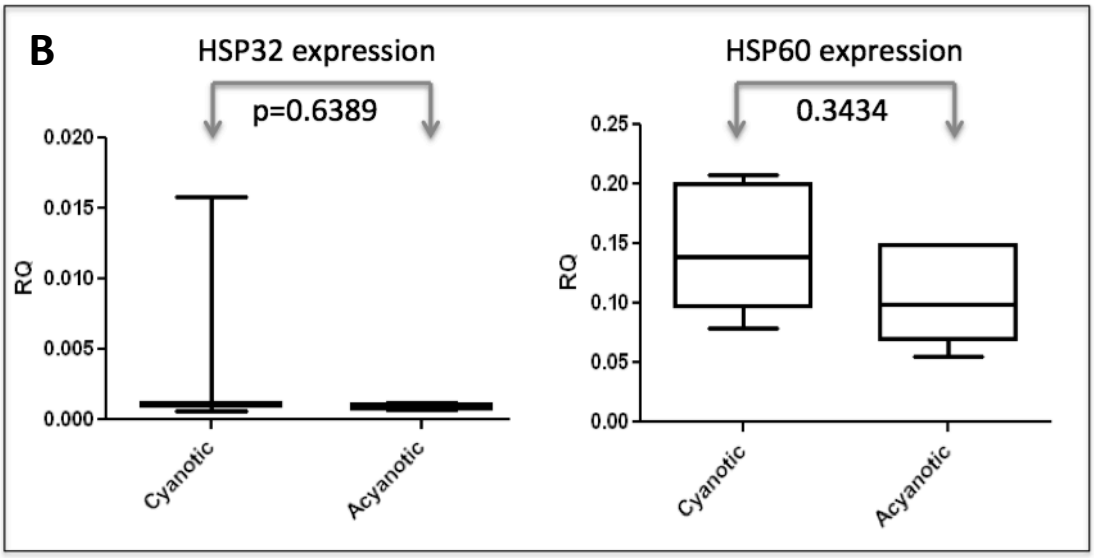


Figure shows comparison in the expression of TLR4 (A), TLR2 (B) and MYD88 (C) in both the cyanotic (n=7) and acyanotic (n=5) patient groups. Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give relative quantification (RQ) of gene expression using the StepOne Software® v2.1 (Applied Biosystems). Statistical analysis was done using the Mann-Whitney U test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-4. Figure shows the expression of stress proteins in both the cyanotic and acyanotic patient groups. A show HSP20 and HSP27, B shows HSP32 and HSP60 and C shows HSP72 and HSP90.





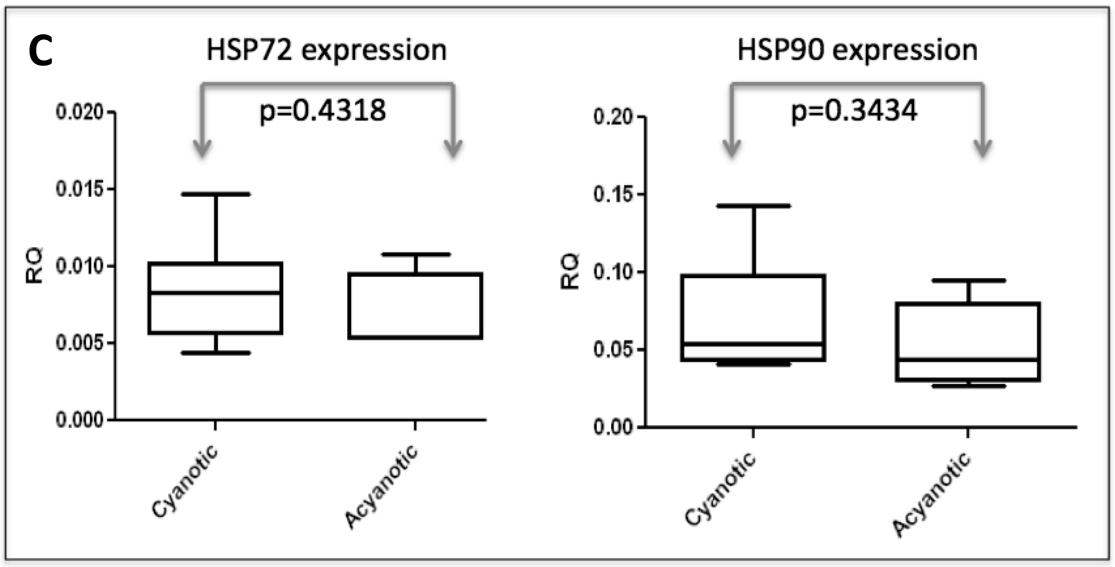


Figure shows comparison in the expression of HSP20 and HSP27 (A), HSP32 and HSP60 (B), HSP72 and HSP90 (C) in both the cyanotic (n=7) and acyanotic (n=5) patient groups. Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression using the StepOne® Software v2.1 (Applied Biosystems). Statistical analysis was done using the Mann-Whitney U test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Summary of main findings:

- Experiment 1:
  - TLR4 mRNA expression was the highest in both patient groups.
  - No difference in expression between cyanotic and acyanotic patient groups.
- Experiment 2:
  - TLR4 (p=0.0303), TLR2 (p=0.0177) and HSP27 (0.0303) mRNA expression is significantly higher in the cyanotic patient group compared to the acyanotic group.

3.2 Induction of HSP gene expression in excised ventricular myocardium: mRNA and protein level analysis of HSP gene expression induced by heavy metal chemical inducers

3.2.1 Introduction

HSPs are a highly conserved and ubiquitously expressed protein family. Expression of many HSPs is increased by a variety of different cellular insults. Consequently, HSPs are also known as "stress proteins" as they are expressed in response to a range of such stresses in order to repair or prevent any intracellular damage caused (Madden *et al*., 2008).

The types of cellular insult that increase HSP expression have been mentioned before but some examples include hypoxia (Patel *et al*., 1995), inflammation (reviewed in Horvath *et al*., 2008), ischemia (Richard *et al.,* 1996), bacterial infection (Zheng *et al.,* 2004), ROS (oxidative stress) (Kukreja *et al*., 1994) and sodium arsenite ($NaAsO_2$) (Li *et al*., 1983). The one of note here would be $NaAsO_2$, which is one of the classical inducers of HSP expression used in this investigation.

There are several examples of studies that have shown that heavy metal exposure can lead to increased expression of HSPs. For example, in a study involving the exposure of post-implantation rat embryos *in vitro* to different levels of several different chemical teratogens, including cadmium chloride ($CdCl_2$) and $NaAsO_2$, HSP72 expression levels were measured using western blotting and immunohistochemistry. $NaAsO_2$ was shown to cause a measurable increase of HSP72 while $CdCl_2$ was not (Mirkes *et al*., 1994). Another study involved the exposure of various types of human endothelial cell types to $CdCl_2$ and other chemical stressors. The results showed that cellular $CdCl_2$ exposure for 3 hours caused the induction of HSP70, HSP60, HSP32 and HSP27 mRNA and protein expression. The most noticeable differences were seen for HSP32 and HSP72 in cadmium treated cells compared to untreated controls (Wagner *et al*., 1999). A more recent study used a transgenic mouse model, in which the firefly luciferase reporter gene was under the control of the murine *hsp70-1* promoter. When the mice were exposed to $CdCl_2$, HSP-70 gene expression was increased, the level of which was at maximum after 10 hours. This was indicated by the elevated activity of the reporter gene controlled by the *hsp70-1* promoter and the elevated level of endogenous HSP70 protein found in tissue samples taken from the transgenic mice after exposure (Wirth *et al*.,

2002). Another similar study involving HSP32 (HO-1) was performed. This involved transgenic mice where the *ho-1* promoter controlled the luciferase gene. The mice were exposed to acute levels of various chemical inducers, including $CdCl_2$. The level of HSP32 activation and expression, which was indicated by the level of the reporter gene, was used as an induction of the amount of induced HSP expression by the chemical inducers. In the case of $CdCl_2$, HSP32 induction was seen in various tissues but the liver had the highest level of activity of the reporter gene and therefore the highest level of HSP32 induced expression (Malstrom *et al*., 2004).

Other studies more specific for HSP induction due to $NaAsO_2$ exposure include one involving the use of the cultured human urothelial cell line UROtsa, which were exposed to $NaAsO_2$. The affect this had on the expression of HSP27, HSP60, HSC70, and HSP70 was investigated by qPCR and Western blotting in order to determine any changes in both mRNA and protein expressions levels. Only HSP70 showed significantly induced expression due to $NaAsO_2$ exposure at both the mRNA and protein level (Rossi *et al*., 2002). Another study was performed that looked at the expression of HSP 32, HSP 72, HSP 60, and HSP 90 in precision-cut rat lung slices when exposed to various concentrations $NaAsO_2$. Only HSP32 and HSP72 were induced upon $NaAsO_2$ exposure (Wijeweera *et al*., 2001).

Despite this wide literature on HSP induction by heavy metals, there is a distinct lack of evidence in the literature of this action in the heart. As HSP are potential mediators of protection in the heart from insults like IR injury that occurs during cardiac surgery, the ability to induce HSPs in cardiac tissue is of obvious benefit. This is especially important in children undergoing cardiac surgery for the correction of congenital cardiac defects, as such children often are associated with pre-operative cyantoic damage that results in apoptosis, meaning such children are much more vulnerable to additional damage.

There have been studies that show that the induction of HSPs by the use of heavy metals has provided protection against IR injury in other tissues. One such study involved hepatic IR injury in mice, which investigated the role of HSP70 in such injury. HSP70 expression was induced by the direct administration of $NaAsO_2$ into the liver tissue before the liver was exposed to IR injury and it was found that there was a 50% reduction in liver injury sustained determined by western blotting analysis (Kuboki *et al*., 2007). Also, HSP70 null

mice also pre-exposed to $NaAsO_2$ in the same manner did not acquire the same protection. This highlighted it was the expression of HSP70 that provided the protection against the IR injury (Kuboki *et al*., 2007).

Thus in this project it was of interest to determine whether it is actually possible to induce HSPs in human infant myocardium using heavy metals. If so, this might open the pathway to seek 'non-toxic' ways of inducing HSPs in the heart. This set of experiments also acted as a control for the reperfusion experiments (see section 3.3) by investigating if it was actually possible to induce HSPs in the neonatal heart.

3.2.2 Methods and Results

The aim of these experiments was to determine the effect of heavy metal chemical inducers on the gene expression within infant myocardial tissue taken from the ventricle of children undergoing corrective surgery from both the cyanotic and acyanotic patient groups (see section 2.4). This investigation was done at both the mRNA and protein level. These experiments were undertaken while the hyperoxic reperfusion set up was being established.

3.2.2.1 mRNA level gene expression in response to heavy metal exposure

Differential gene expression was investigated in infant myocardial tissue taken from the ventricle of children undergoing corrective surgery from both the cyanotic and acyanotic patient groups. This tissue was exposed to heavy metal chemical inducers for 6 hours (see section 2.4.1). The two heavy metal chemical inducers used were $NaAsO_2$ and $CdCl_2$. The genes of interest were the stress genes HSP20, HSP27, HSP32 (HO-1), HSP60, HSP70 and HSP90. The inflammatory markers TLR2, TLR4, MYD88 were also investigated.

In order to investigate the differential gene expression of the genes of interest, the method of qPCR (see section 2.9) was used and RQ of gene expression was calculated using the comparative C_t method (see section 2.9.2). Differential expression statistical analysis was investigated by looking at both chemical inducers separately and also with both inducers together.

Statistical analysis was performed using GraphPad Prism 6 Software©. The data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. The Wilcoxon matched-pairs signed rank test was used to look for any statistical differences in expression for all genes of interest seen due to the exposure to heavy metal chemical inducers. A p-value of <0.05 (two-tailed) is considered as statistically significant for these experiments. Seven patients (table 7) were recruited, comprising of TOF and TA patients. These patients were used for the mRNA gene expression analysis only.

Table 7. Patients recruited for mRNA level gene expression in response to heavy metals

Patient	Type	Group	Inducer	Gender	Age
13	TOF	Cyanotic	Na	Male	1 year 3 months
15	TOF	Cyanotic	Cd	Female	1 year 2 months
19	TOF	Cyanotic	Na	Female	0 years 9 months
20	TOF	Cyanotic	Cd	Female	1 year 2 months
21	TOF	Cyanotic	Na	Female	0 years 8 months
22	TOF	Cyanotic	Cd	Male	1 year 8 months
28	TA	Acyanotic	Na	Male	0 years 2 months

(Na = Sodium aresenite, Cd = Cadmium Chloride)

Analysis of data from induction by the heavy metal inducers separately:

When looking at the differential mRNA expression of all HSPs and inflammatory mediators when exposed to either $NaAsO_2$ or $CdCl_2$ separately, there were no statistically significant differences observed in the expression (see table 8) of any of the HSPs or the inflammatory mediators. One possibility is that due to the low number of patient samples exposed to each chemical inducer (3 patients exposed to $CdCl_2$ and 4 exposed to $NaAsO_2$) may have meant that any significant difference in expression that may have been present was not identified.

Table 8. Statistical analysis of chemical inducers when analysed separately using the Wilcoxon matched-pairs signed rank test (p-value of <0.05 is considered as statistically significant). The p values are shown.

Condition	HSP20	HSP27	HSP32	HSP60	HSP72	HSP90	TLR4	TLR2	MYD88
$NaAsO_2$ baseline vs. $NaAsO_2$ induction	0.125	0.125	0.125	0.125	0.125	0.125	0.88	0.13	0.875
$NaAsO_2$ baseline vs. $NaAsO_2$ control	0.125	0.875	0.125	0.625	0.125	0.125	0.13	0.63	0.125
$NaAsO_2$ induction vs. $NaAsO_2$ Control	0.25	0.25	0.125	0.125	0.125	0.125	0.38	0.88	0.125
$CdCl_2$ Baseline vs. $CdCl_2$ induction	0.25	0.25	0.25	0.75	0.25	0.25	1	0.75	0.25
$CdCl_2$ Baseline vs. $CdCl_2$ control	1	0.75	0.25	0.25	0.25	0.25	0.75	1	0.25
$CdCl_2$ induction vs. $CdCl_2$Control	0.25	0.5	0.25	0.25	0.5	0.25	0.75	1	0.25

Analysis of data from induction by the heavy metal inducers combined:

Inflammatory mediators:

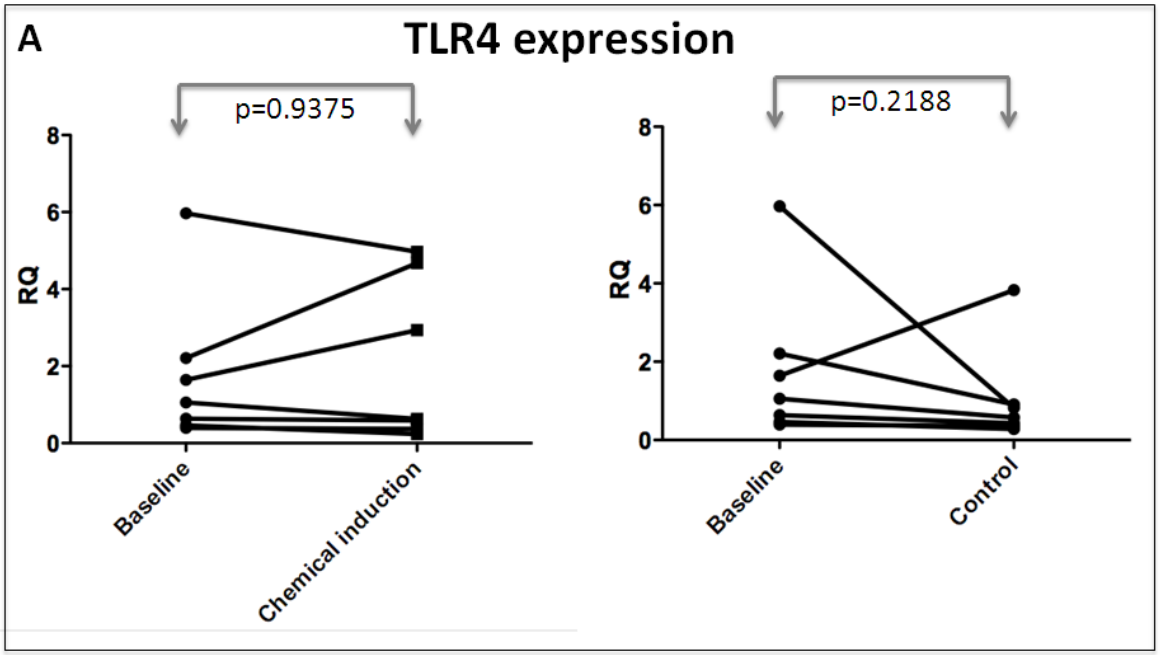
The mRNA expression of TLR4, TLR2 and MYD88 were investigated (figure 3-5). There was no significant difference in the expression of TLR2, TLR4 and MYD88 upon exposure to heavy metal chemical induction after 6 hours. This was also true for TLR2 and TLR4 expression in the tissue incubated in media only for 6 hours. MYD88 expression was significantly decreased (p=0.0156) in the tissue incubated in media only for 6 hours.

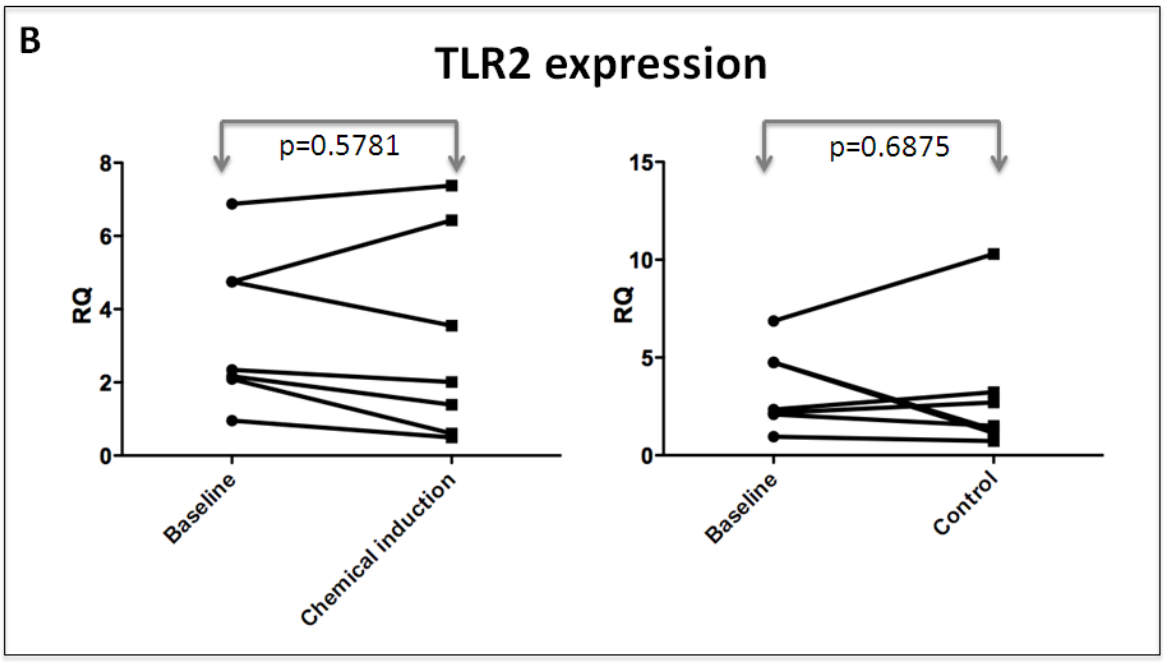
Stress proteins:

The mRNA expression of HSP20, HSP27, HSP32 (figure 3-6) and the expression of HSP60, HSP72 and HSP90 (figure 3-7) were investigated. HSP27 and HSP60 had no significant differences when exposed to chemical inducers for 6 hours. This was the same for the tissue exposed to media only for 6 hours (control). HSP20 showed a significant decrease (p=0.0156) in expression when exposed to heavy metal chemical inducers for 6 hours but this was not shown in tissue exposed to media only for 6 hours (control). HSP32 (p=0.0156), HSP72 (p=0.0156) and HSP90 (p=0.0156) all showed a significant increase in expression when exposed to heavy metal inducers for 6 hours. This was also true for tissue exposed to media only for 6 hours (control).

The increase in expression due to exposure to the chemical inducer for 6 hours for HSP32, HSP72 and HSP90 was significantly higher (figure 3-8) when compared to the increase in the expression due to exposure to media only for 6 hours. Therefore the increase due to the chemical inducer was greater.

Figure 3-5. Figure showing the differential mRNA expression of inflammatory mediators upon exposure to heavy metal chemical inducers or media only (control).





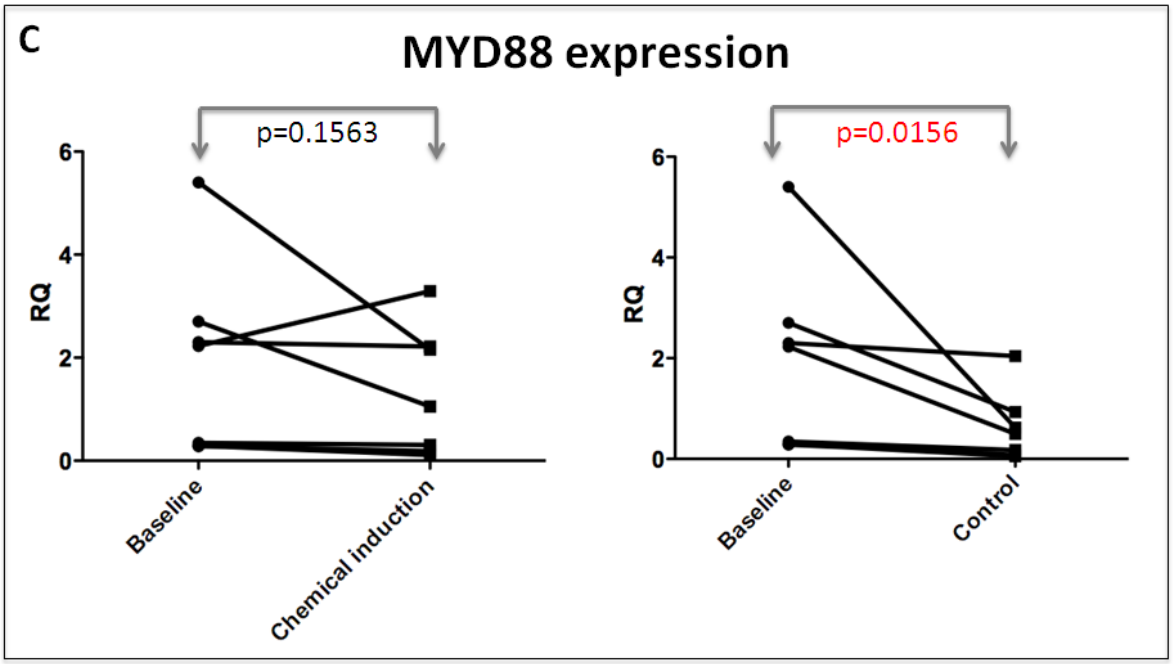
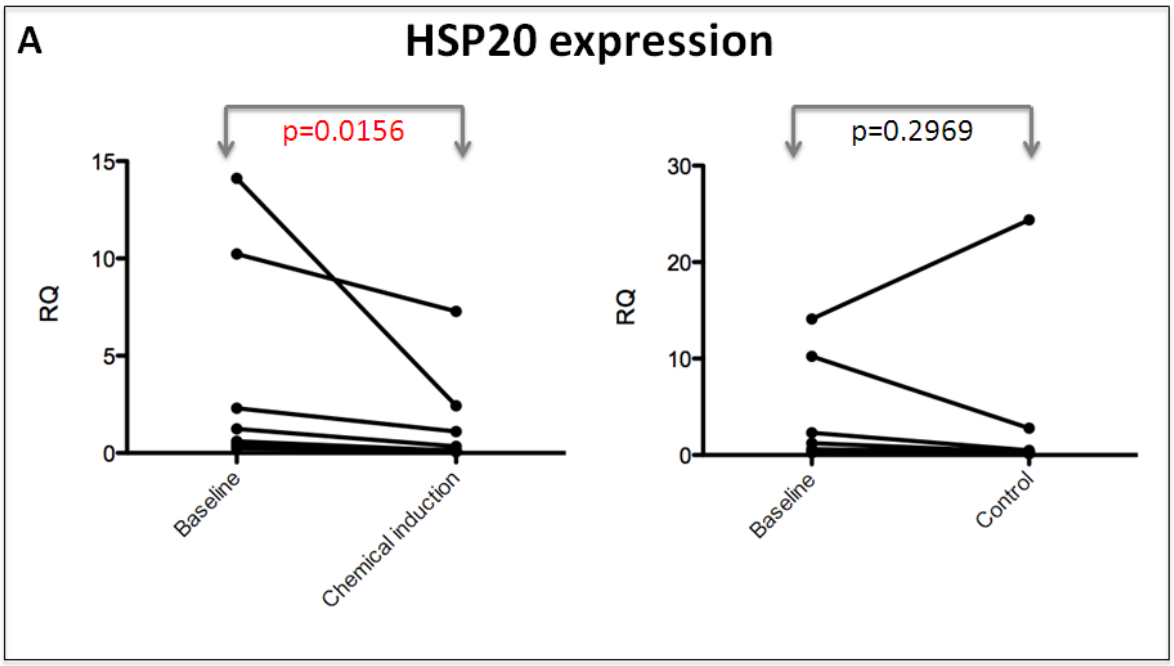
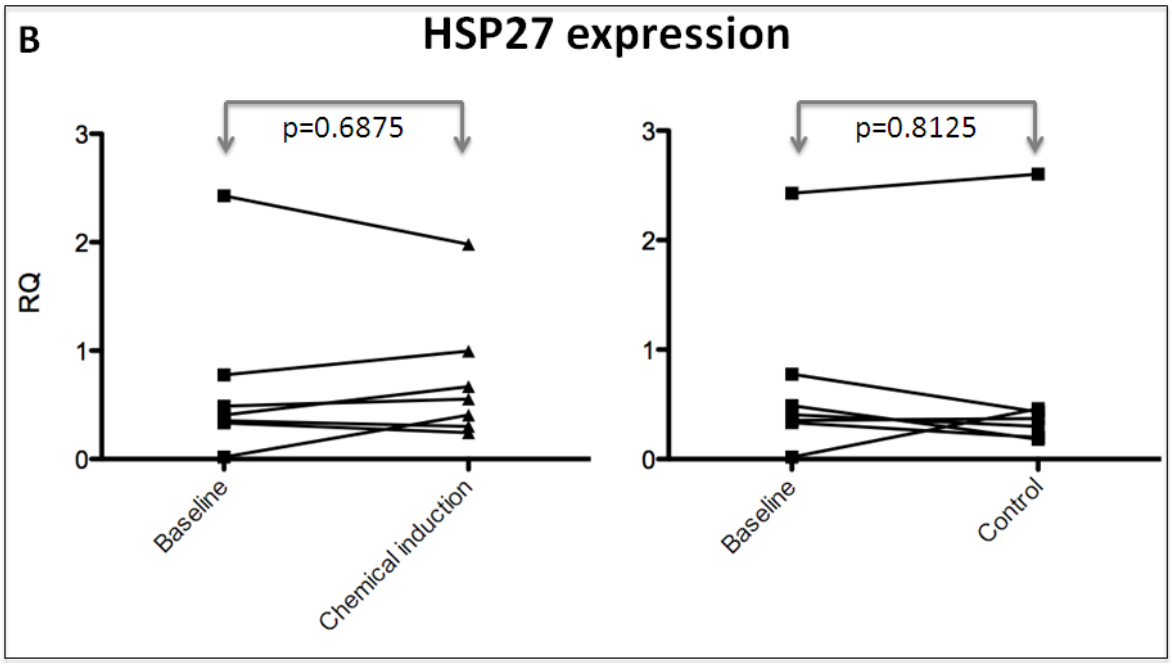


Figure showing the differential mRNA expression of TLR4 (A), TLR2 (B) and MYD88 (C) in tissue exposed to heavy metals for 6 hours or tissue exposed to media only for 6 hours (control). Samples taken from each patient (n=7) were used in both groups. Data is shown as line and symbol graphs (each sample's data is plotted separately). The graphs (from left to right) show the expression from the start of incubation (baseline) and expression after 6 hours of incubation (with chemicals or control). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-6. Figure showing the differential mRNA expression of the stress genes HSP20, HSP27 and HSP32 upon exposure to heavy metal chemical inducers or media only (control).





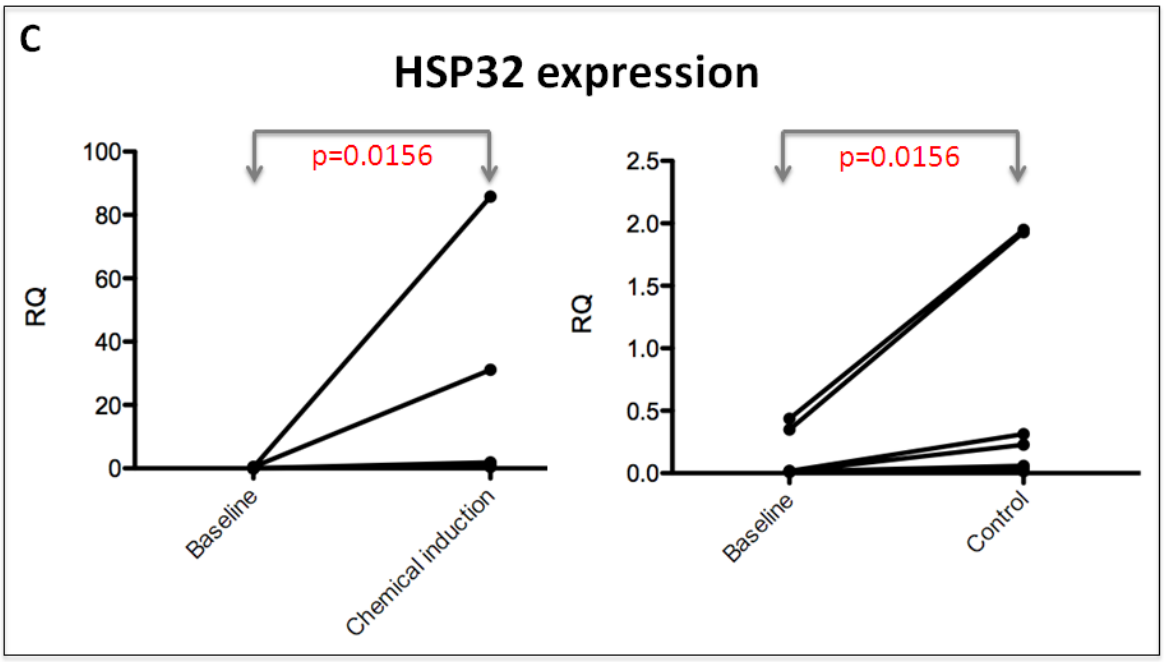
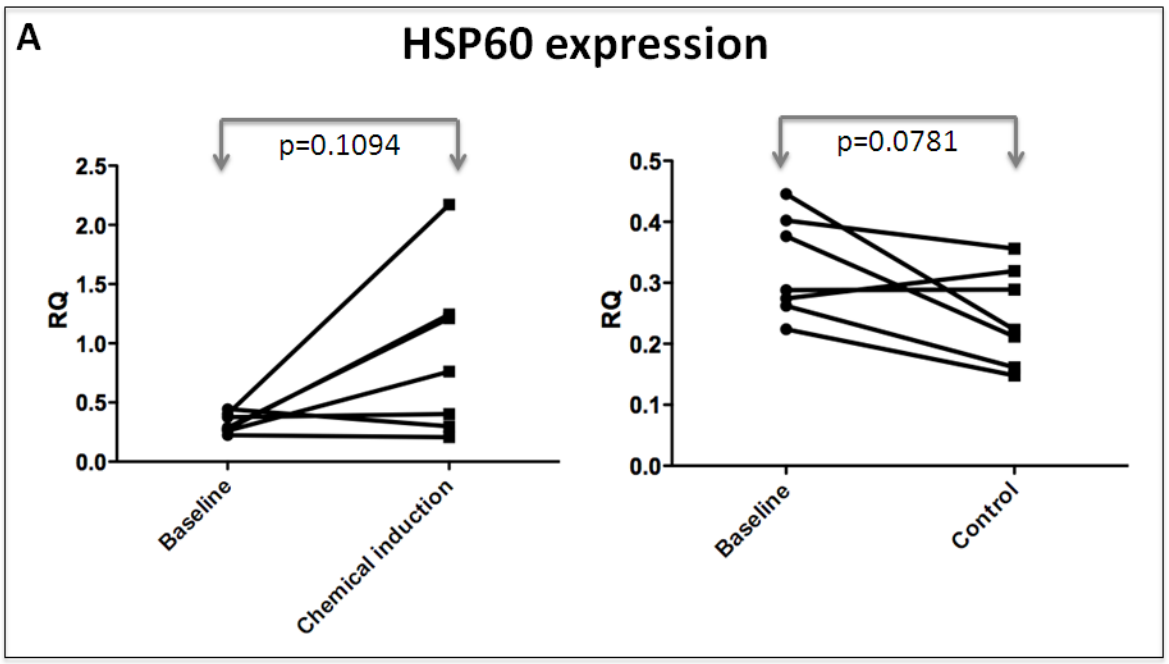
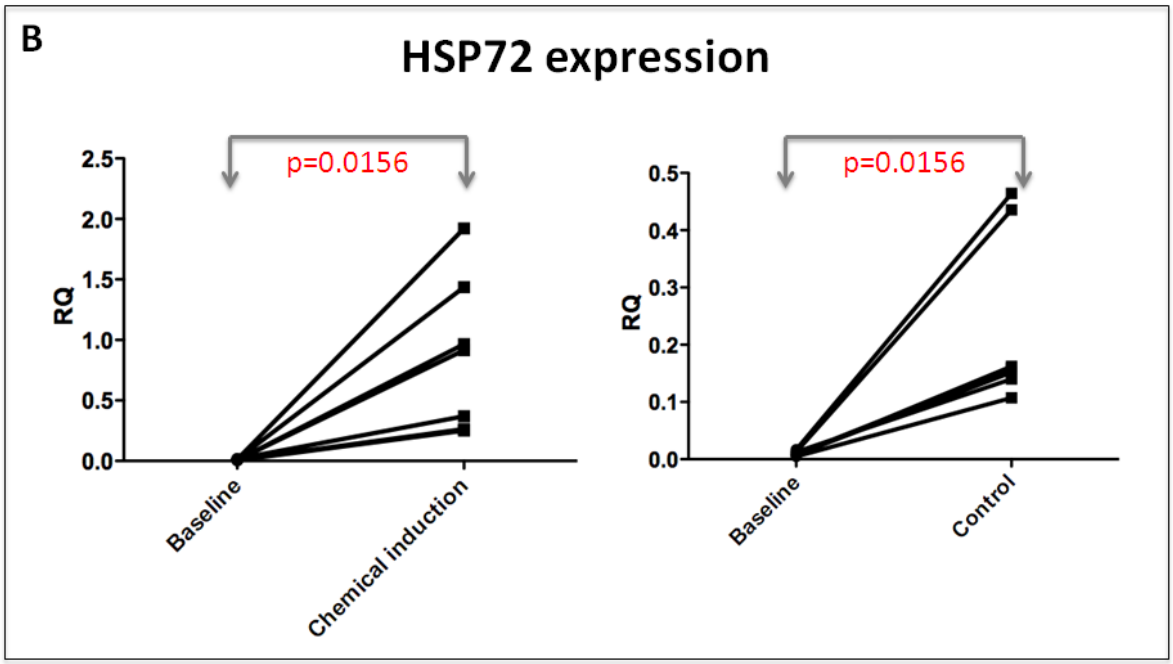


Figure showing the differential mRNA expression of HSP20 (A), HSP27 (B) and HSP32 (C) in tissue exposed to heavy metals for 6 hours or tissue exposed to media only for 6 hours (control). Samples taken from each patient (n=7) were used in both groups. Data is shown as line and symbol graphs (each sample's data is plotted separately). The graphs (from left to right) show the expression from the start of incubation (baseline) and expression after 6 hours of incubation (with chemicals or control). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-7. Figure showing the differential mRNA expression of the stress genes HSP60, HSP72 and HSP90 upon exposure to heavy metal chemical inducers or media only (control).





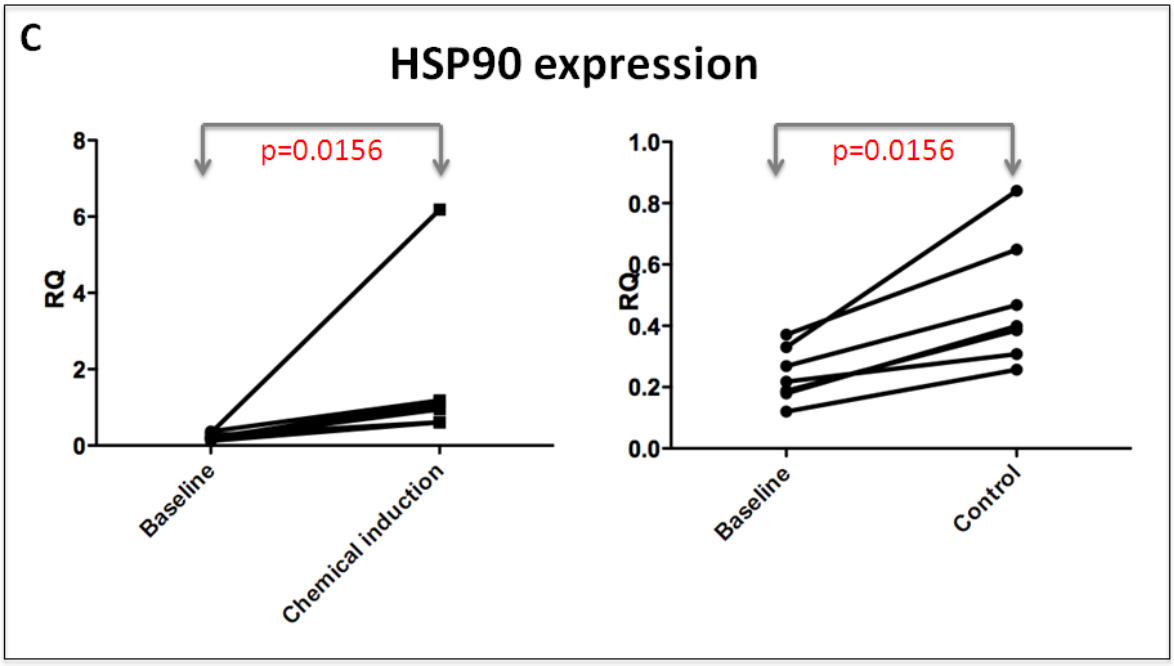


Figure showing the differential mRNA expression of HSP60 (A), HSP72 (B) and HSP90 (C) in tissue exposed to heavy metals for 6 hours or tissue exposed to media only for 6 hours (control). Samples taken from each patient (n=7) were used in both groups. Data is shown as line and symbol graphs (each sample's data is plotted separately). The graphs (from left to right) show the expression from the start of incubation (baseline) and expression after 6 hours of incubation (with chemicals or control). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-8. Figure shows the differential mRNA expression of the stress genes HSP32, HSP72 and HSP90 in tissue exposed to chemical inducers compared to media only (controls).

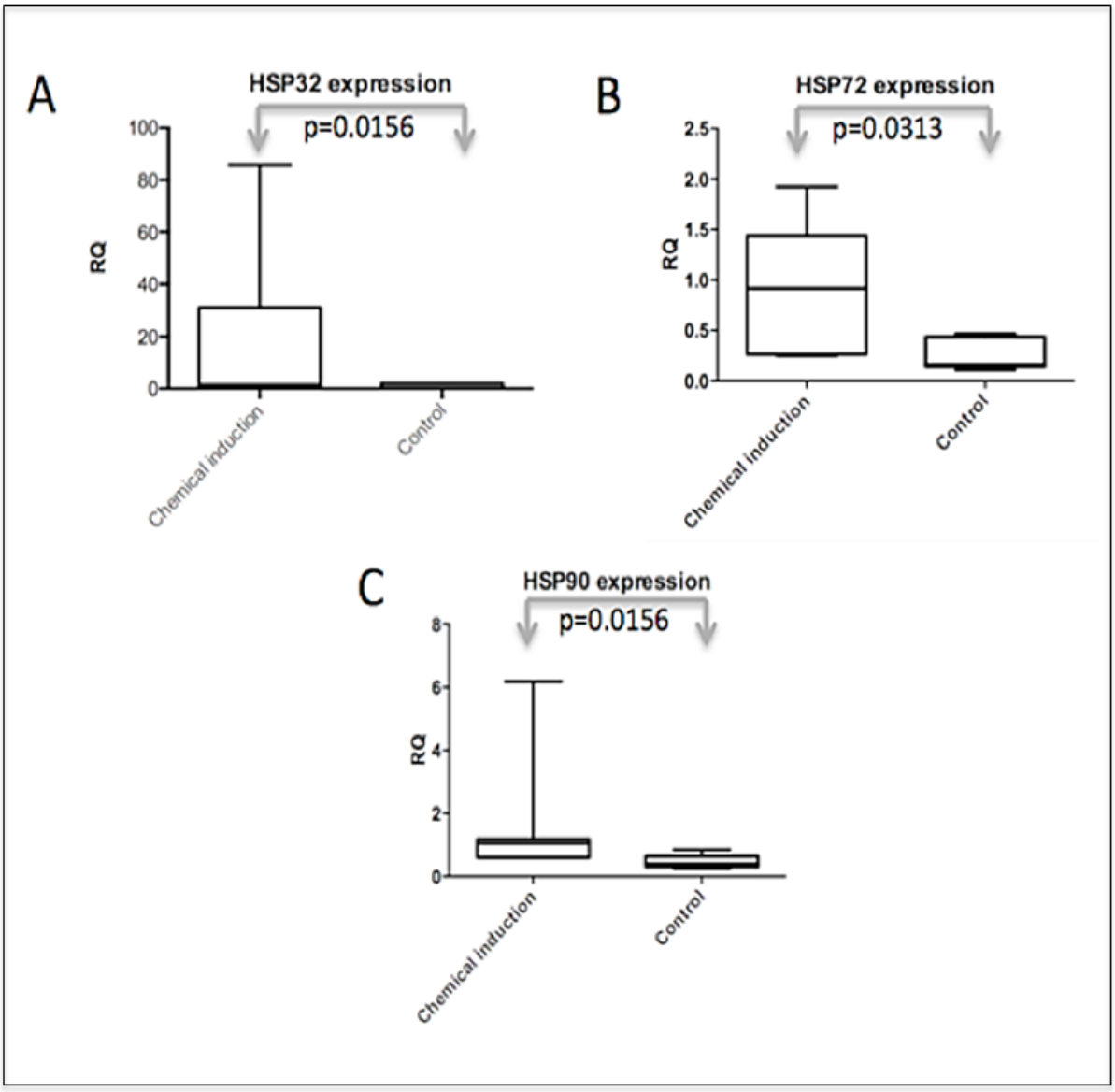


Figure showing the differential mRNA expression of the stress proteins HSP32 (A), HSP72 (C) and HSP90 (C) of tissue exposed to chemical inducers for 6 hours compared to tissue exposed to media only for 6 hours (control). Samples taken from each patient (n=7) were used in both groups. Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

3.2.2.2 Protein level gene expression in response to heavy metal exposure

The expression of HSPs in response to heavy metal exposure was also investigated at the protein level (see section 2.4.2) in infant myocardial tissue taken from the ventricle of children undergoing corrective surgery from the cyanotic patient group. Tissue was exposed to either 100μM $NaAsO_2$ or 100μM $CdCL_2$ for 6 hours at 37°C and then compared to appropriate controls. Differential expression at the protein level was performed using Western blotting (see section 2.12). The expression of HSP27, HSP72 and HSP32 were investigated. To see the relevant antibodies used see section 2.12.4. Five cyanotic patients (table 9) were recruited for this experiment, consisting of TOF and double outlet right ventricle (DORV) patients. These patients were used for protein gene expression analysis only.

Table 9. Patients recruited for protein level gene expression in response to heavy metals

Patient	Type	A or C	Inducer	Gender	Age
26	TOF	C	Na	M	2 years 11 months
27	TOF	C	Cd	F	0 years 9 months
30	DORV	C	Na	F	4 years 1 months
31	TOF	C	Cd	M	0 years 10 months
44	TOF	C	Na	M	0 years 8 months

(Na = Sodium arsenite, Cd = Cadmium Chloride)

Differential expression of HSP32:

Investigation of the differential expression of HSP32 was attempted but there did not seem to be any HSP32 expression present in the heart tissue samples being used for the experiments. To ensure this was not because of the antibody, we used an appropriate positive control (Microsome extract, Sigma-Aldrich, cat. no. MO317 (20mg/ul)). An example of this is shown below in figure 3-9. There was no expression observed in the heart tissue but there was expression seen for the positive control.

Figure 3-9. Results of Western blotting that shows the differential expression of HSP32 when induced by chemical inducers.

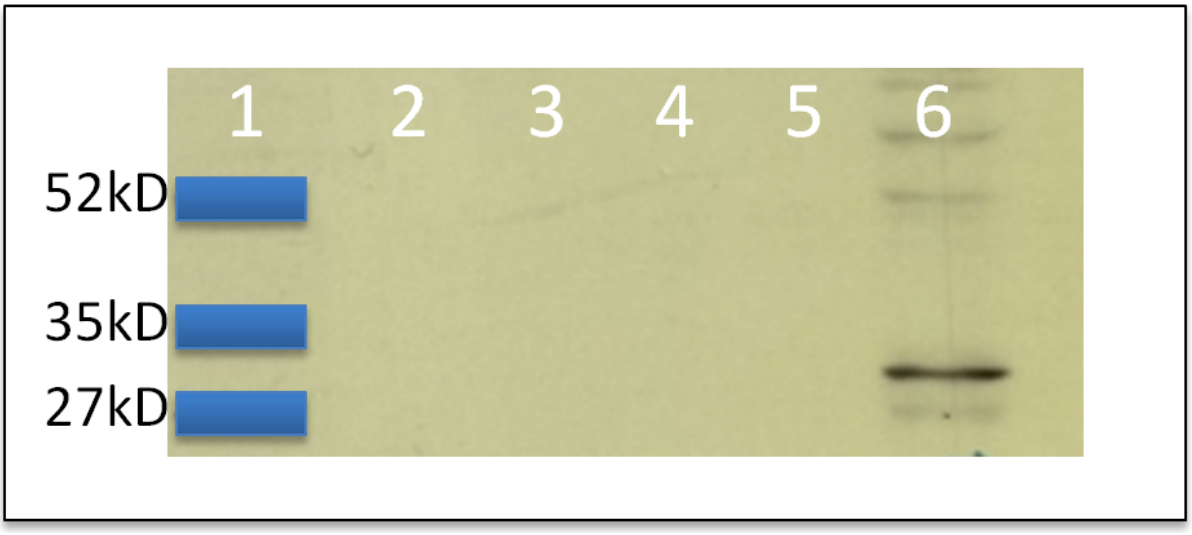


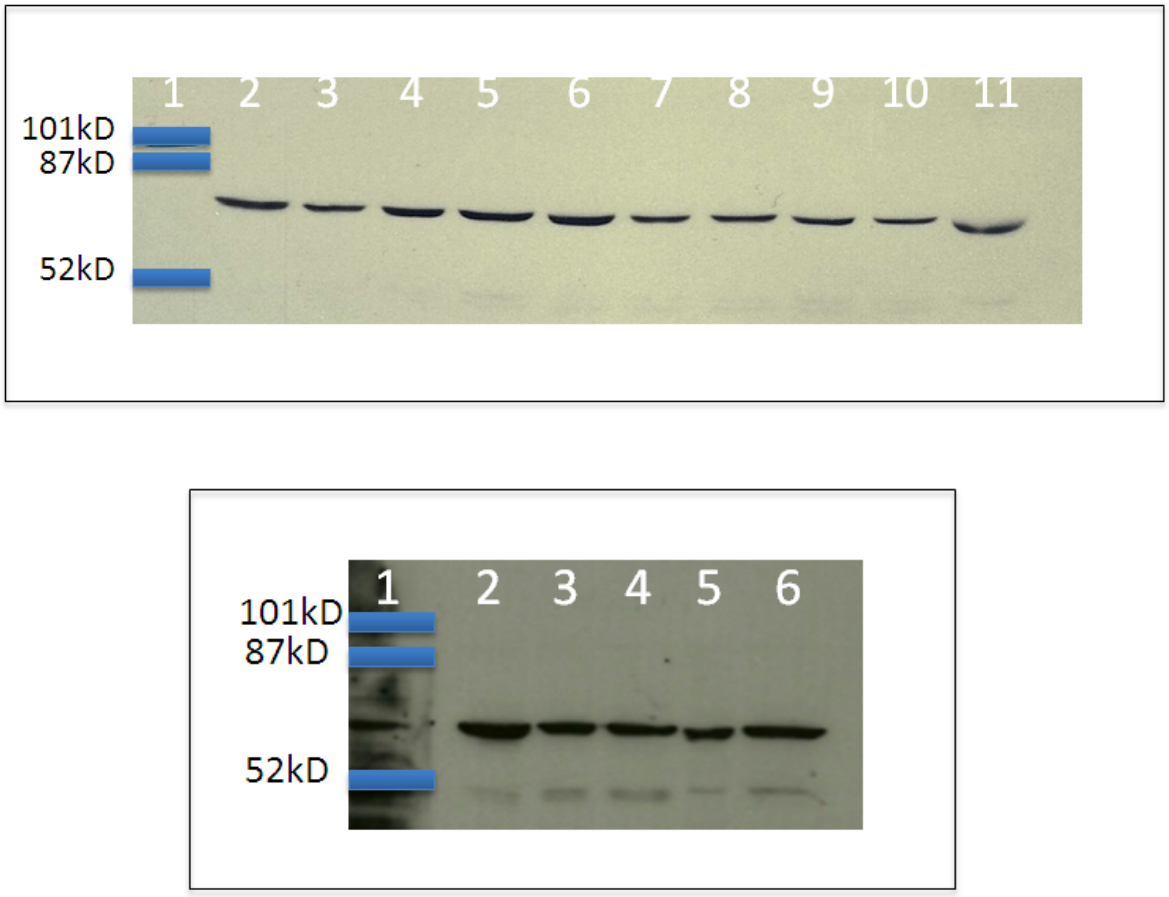
Figure shows an autoradiograph representing the differential expression of HSP32 with placental tissue, heart tissue and a positive control. Lane 1 – SDS pre-stained standards (Bio-rad, cat. no. 161-0305), lane 2 - heart control, lane 3 –, placental control, lane 4 – $NaAsO_2$ 4 hour induction with 20 hour recovery, lane 5 – $NaAsO_2$ control 24 hour induction, lane 6 – Microsome extract (C+).

Differential expression of HSP72 and HSP27:

The expression of HSP72 (figure 3-10) and HSP27 (figure 3-11) were investigated by Western blotting. Upon completion of HSP gene expression analysis by Western blotting, densitometry was then applied to the resulting autoradiographs shown below using Quality One® software and then used to calculate the ROD. The Wilcoxon matched-pairs signed rank test was used to look for any statistical differences in the expression HSP72 and HSP27 due to the exposure to heavy metals compared to the appropriate controls (media only). All data was analysed by combining the tissue exposed to both $NaAsO_2$ and $CdCl_2$.

There was no significant difference in the expression of HSP27 and HSP72 (figure 3-12) seen between tissue that had been incubated with a chemical inducer for 6 hours and tissue that had been incubated in media only for 6 hours.

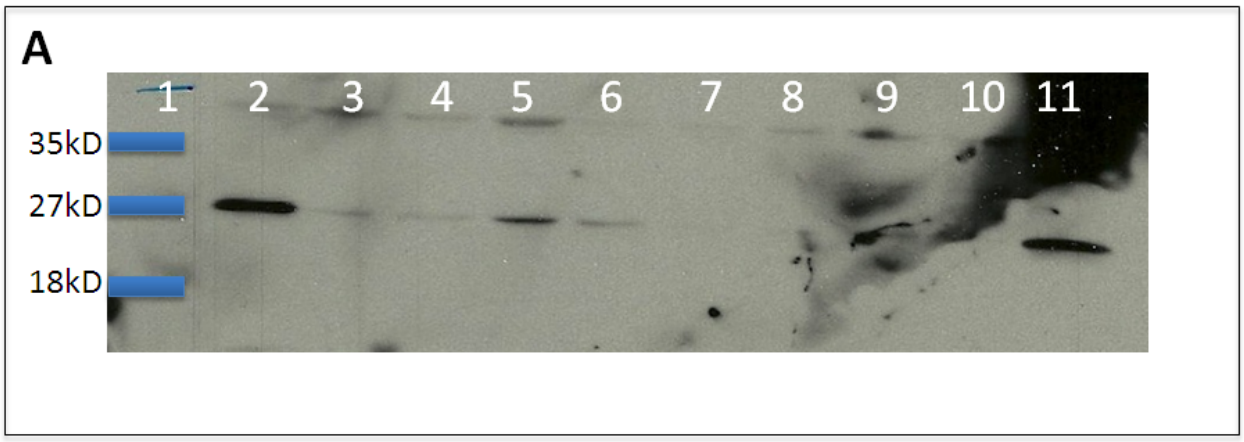
Figure 3-10. Results of Western blotting that shows the differential expression of HSP72 in tissue exposed to chemical inducers.

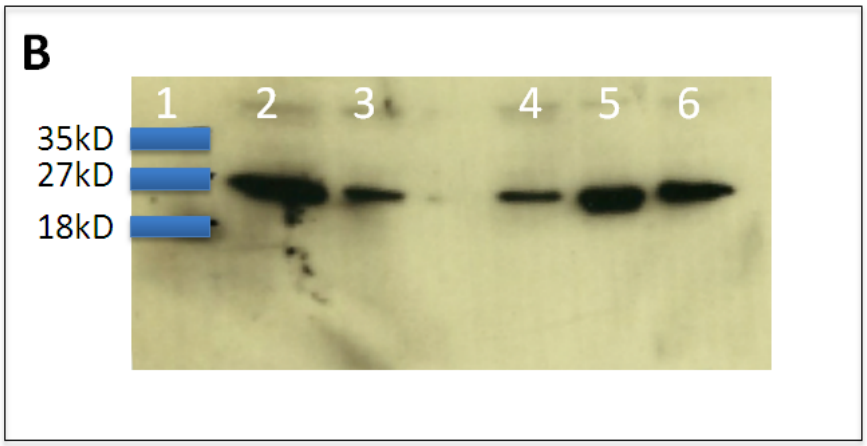


A: Autoradiograph representing the differential protein expression of HSP72 when induced by $NaAsO_2$ and $CdCl_2$. Lane 1 – SDS pre-stained standards (Bio-Rad), lane 2 – heart control sample, lanes 3 and 7 - $NaAsO_2$ 6 hour induction, lanes 4 and 8 - $NaAsO_2$ control 6 hour induction, lanes 5 and 9 - $CdCl_2$ 6 hour induction, lanes 6 and 10 - $CdCl_2$ control 6 hour induction, lane 2 – heart control, lane 11 – placental control.

B: Autoradiograph representing the differential protein expression of HSP72 when induced by $NaAsO_2$. Lane 1 – SDS pre-stained standards (Bio-Rad), lane 2 – start of incubation, lane 3 - $NaAsO_2$ 6 hour induction, lane 4 - $NaAsO_2$ control 6 hour induction, lane 5 - placental control, lane 6 - heart control.

Figure 3-11. Results of Western blotting that shows the differential expression of HSP27 in tissue exposed to chemical inducers.





A: Autoradiograph representing the differential protein expression of HSP27 when induced by $NaAsO_2$ and $CdCl_2$. Lane 1 – SDS pre-stained standards (Bio-Rad), lane 2 – heart control sample, lanes 3 and 7 - $NaAsO_2$ 6 hour induction, lanes 4 and 8 - $NaAsO_2$ control 6 hour induction, lanes 5 and 9 - $CdCl_2$ 6 hour induction, lanes 6 and 10 - $CdCl_2$ control 6 hour induction, lane 2 – heart control, lane 11 – placental control.

B: Autoradiograph representing the differential protein expression of HSP27 when induced by $NaAsO_2$. Lane 1 – SDS pre-stained standards (Bio-Rad), lane 2 – start of incubation, lane 3 - $NaAsO_2$ 6 hour induction, lane 4 - $NaAsO_2$ control 6 hour induction, lane 5 - placental control, lane 6 - heart control.

Figure 3-12 Differential expression of HSP27 (A) and HSP72 (B) seen in tissue that had been incubated in tissue with a chemical inducer compared to tissue incubated in media only for 6 hours (control).

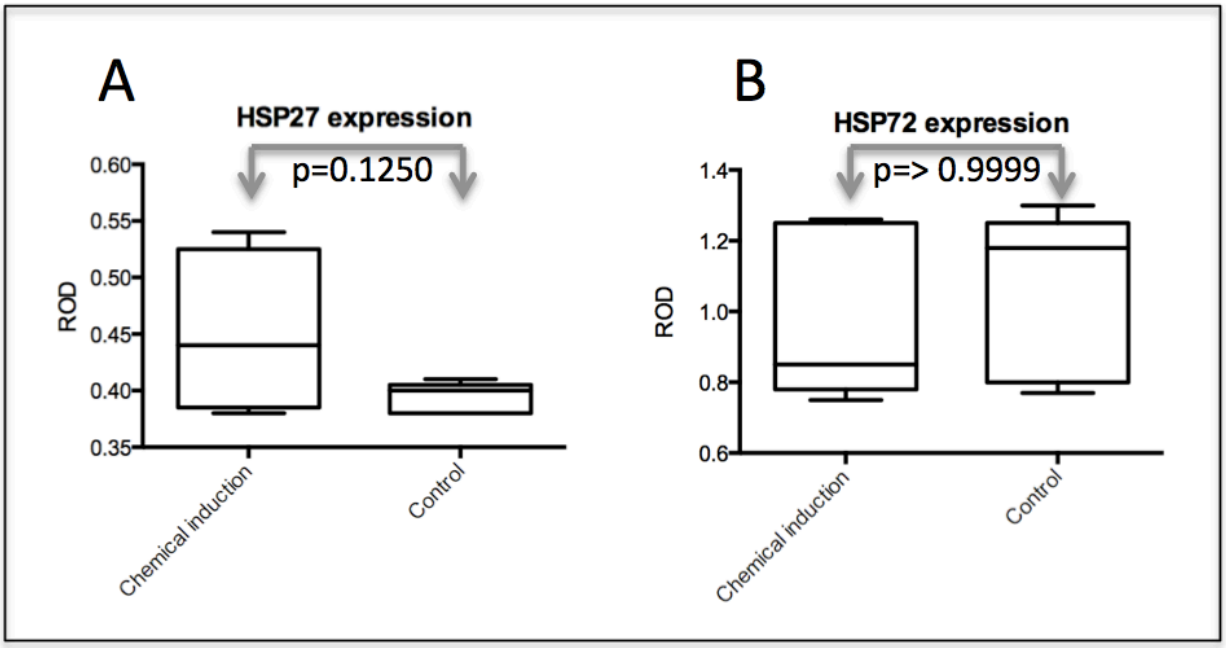
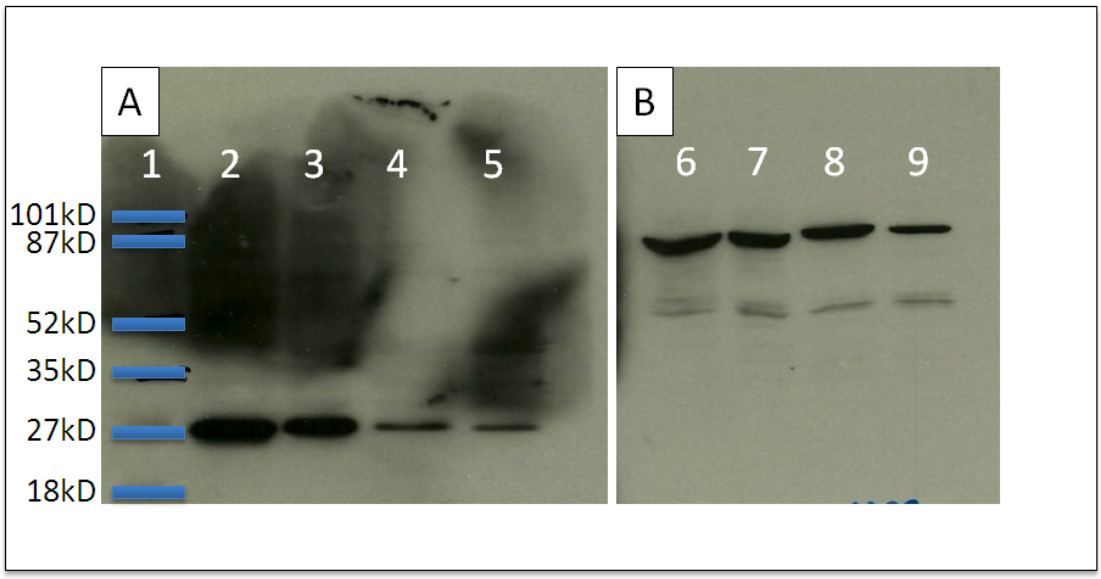


Figure showing the differential protein expression of HSP27 (A) and HSP72 (B) seen in tissue that had been incubated in tissue with a chemical inducer for 6 hours compared to tissue incubated in media only for 6 hours (control). Samples taken from each patient (n=5) were used in both groups. Data is shown as box and whiskers plots (median, interquartile ranges). Expression is shown as ROD. The Wilcoxon matched-pairs signed rank test was used to look for any statistical differences in expression.

Heavy metal exposure with recovery period:

An additional experiment was performed where there was a period of recovery allowed after the initial exposure to the chemical inducer (see section 2.4.3). Specifically, tissue from a TOF patient (patient 40: TOF, cyanotic, male) was exposed to $NaAsO_2$ for 4 hours then allowed to recover in media only for 20 hours. Part of the tissue was exposed to media only for 24 hours in order to act as an appropriate experimental control. There was no obvious difference in the expression of HSP27 (figure 3-13A) and HSP72 (figure 3-13B) in the tissue exposed to heavy metals with a recovery period when compared to tissue exposed to media only (control).

Figure 3-13. Figure showing the results of Western blotting that shows the differential expression of HSP27 (A) and HSP72 (B) when induced by $NaAsO_2$ for 4 hours with 20 hours recovery.



A: Autoradiograph representing the differential protein expression of HSP27 when exposed to $NaAsO_2$ for 4 hours with 20 hours recovery and appropriate controls. Lane 1 – SDS pre-stained standards (Bio-rad, cat. no. 161-0305), lane 2 - placental control, lane 3 – heart control, lane 4 – $NaAsO_2$ 4 hour induction with 20 hour recovery, lane 5 – $NaAsO_2$ control 24 hour induction.

B: Autoradiograph representing the differential expression of HSP72 when exposed to $NaAsO_2$ for 4 hours with 20 hours recovery and appropriate controls. Lane 6 - placental control, lane 7 – heart control, lane 8 – $NaAsO_2$ 4 hour induction with 20 hour recovery, lane 9 – $NaAsO_2$ control 24 hour induction.

Summary of main findings:

- Experiment 1:
  - No difference in mRNA expression seen for any genes when heavy metal inducers analysed separately.
  - mRNA expression of HSP32 (p=0.0156), HSP72 (p=0.0156) and HSP90 (p=0.0156) were significantly increased by heavy metals (analysis of both inducers combined.
- Experiment 2:
  - HSP27 and HSP72 protein expression was not induced by heavy metal exposure (separate or combined analysis).

3.3 Differential gene expression in RV myocardium excised from TOF and control patient groups exposed to differential O_2 levels

3.3.1 Introduction

Surgical correction of congenital heart defects on bypass can have adverse effects on post-operative cardiac output. During such procedures, the myocardium is exposed to high O_2 tensions (Ihnken *et al*., 1998; reviewed in Allen *et al*., 2001; reviewed in Morita, 2012). There are some concerns that this re-oxygenation of ischemic tissue (IR injury) could cause adverse post-operative complications. Patients with TOF have chronic ischemic damage results in cyanosis in the RV. Such children are thought to be more vulnerable to re-oxygenation injury (Modi *et al*., 2002; Bulutcu *et al*., 2002).

The molecular mechanisms involved in the myocardial response to IR injury have been investigated extensively. These investigations have shown that myocardium exposed to IR injury is prone to an inflammatory response that is regulated by varying factors, for example, by TLR4 (Chong *et al*., 2004; Cha *et al*., 2008). High level O_2 exposure can also result in TLR downstream signalling that will result in an inflammatory response, primarily in the lungs (Zhang *et al*., 2005; Ogawa *et al*., 2007; Qureshi *et al*., 2006; Huang *et al*., 2011).

HSPs, which are well known stress proteins, have been shown to provide protection against IR injury in rat myocardial tissue. In particular, HSP72 and HSP27 were shown to provide enhanced protection for rat myocardium against IR injury (Pantos *et al*., 2007). HSPs have also been shown be provide protection against damage done by hyperoxic O_2 exposure (Wong *et al*., 1998; Shao *et al*., 2009).

HSP70 has been shown to interact with TLR4, specifically when HSP72 was unregulated it was shown to modulate the inflammatory response and subsequent myocardial dysfunction caused by IR injury using TLR4 (Zou *et al*., 2008). This shows an interaction between HSPs and TLRs, both of which are of major interest in this project.

Investigating the expression of the genes discussed herein, within the myocardium of patients undergoing cardiac surgery with CPB, could help improve the current knowledge of the molecular mechanisms active within the myocardium during heart surgery. This could allow us to discover new and novel methods of preventing such damage by reducing

inflammation caused by IR injury and other forms of insult or providing enhanced myocardial protection against such cellular insult. This would be of greater benefit for paediatric heart surgery for cyanotic patients, who are more vulnerable to re-oxygenation injury (Merante *et al*., 1998; Najm *et al*., 2000; Corno *et al*., 2002).

3.3.2 Methods and Results

The aim of these experiments was to investigate the effect of exposing ischemic heart tissue, taken from patients undergoing corrective cardiac surgery with CPB for congenital heart defects, to 2% O_2 (hypoxia) representing the surgical state of myocardium, 20% O_2 (normoxia) representing what would happen if the child's myocardium were exposed to normal O_2 during surgery and 60% (hyperoxia) O_2 representing what actually happens to myocardium during surgery i.e. exposed to hyperoxia. The aim was to investigate the effect this had on the differential gene expression of genes of interest within such tissue. A level of 60% O_2 was chosen as the highest level of O_2 exposure because this was the highest that could be achieved *in vitro*. The genes of interest include both the inflammatory markers TLR2, TLR4, MYD88 and the stress genes HSP20, HSP27, HSP32 (HO-1), HSP60, HSP70 and HSP90.

3.3.2.1 Experiment 1: exposing myocardial tissue to 2% (hypoxia) and 20% (normoxia) O_2

The first experimental model undertaken involved exposing myocardial tissue to 2% (hypoxia) and 20% (normoxia) O_2 (outlined in section 2.3.1) for 1.5 hours. This first experiment was undertaken due to initial problems encountered establishing a model for hyperoxic reperfusion (60%) (see section 2.3.2). The tissues were exposed to either O_2 concentration for a period of 1.5 hours as this is this length of time the myocardium is exposed to re-oxygenation in the clinical setting of such surgery i.e. upon removal of the aortic clamp. Five patients (table 10) were recruited for this experiment, consisting of TOF, SAS and FV patients. These patients were used for the mRNA gene expression analysis upon exposure to 2% and 20% O_2 for 1.5 hours only.

Table 10. Patients recruited for exposure to 2% (hypoxia) and 20% (normoxia) O_2

Patient	Type	Group	Gender	Age
11	TOF	Cyanotic	Female	1 year 0 months
16	TOF	Cyanotic	Male	0 years 11 months
17	TOF	Cyanotic	Female	1 year 9 months
18	SAS	Acyanotic	Female	0 years 11 months
23	FV	Cyanotic	Female	1 year 1 month

In order to investigate the gene expression of the inflammatory mediators and stress proteins mentioned above, the method of qPCR (see section 2.9) was used and RQ of gene expression was calculated using the comparative C_t method (see section 2.9.2).

Statistical analysis was performed using GraphPad Prism 6 Software©. Due to biological variation the data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. Therefore the Wilcoxon matched-pairs signed rank test was used to look for any statistical differences in expression for all genes of interest seen due to the exposure to differential O_2 levels. A p-value of <0.05 (two-tailed) is considered as statistically significant.

It should be noted that the RQ values for the 20% baseline (C) sample for patient 18 was disregarded due to poor quality of RNA and the resulting anomalous results. This study is an explorative study and is therefore observational in it nature. Due to limited patient numbers, all patients recruited for this study were taken as one group therefore a comparison of cyanotic and acyanotic patients was not possible.

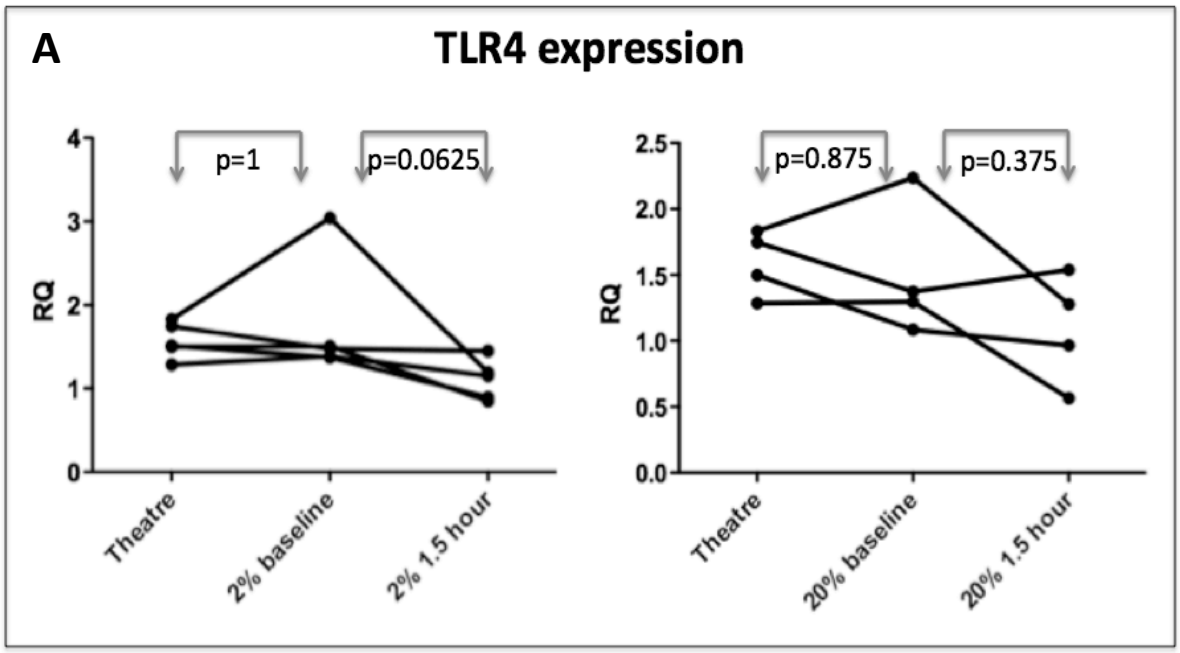
Inflammatory markers:

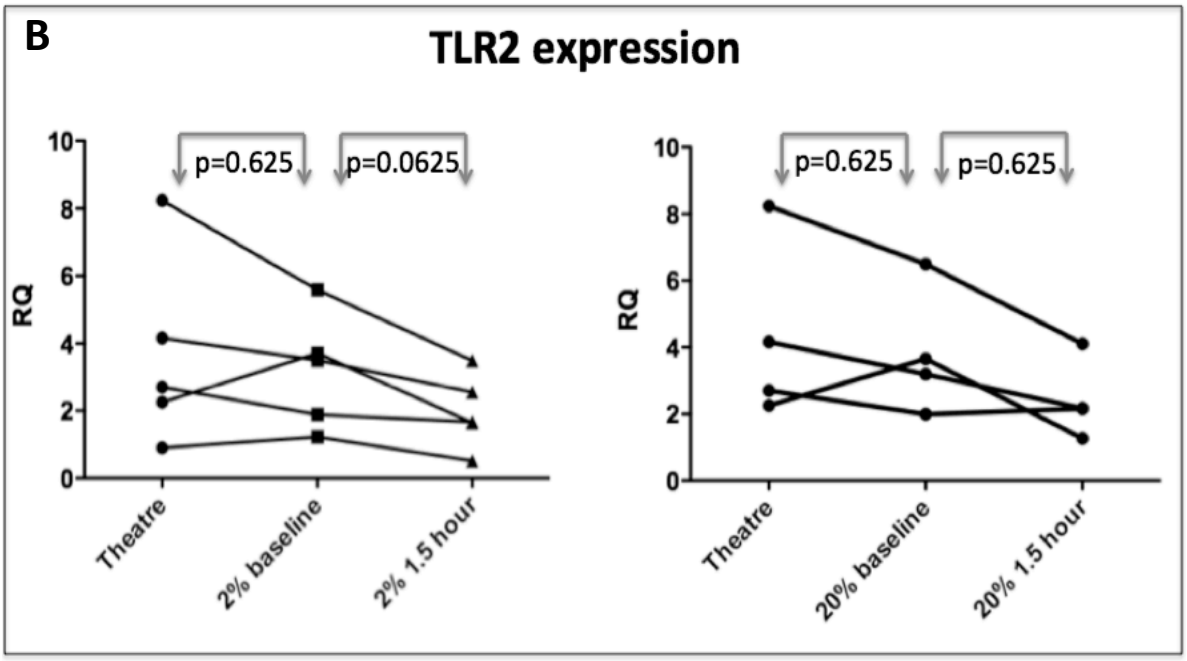
The differential mRNA expression of TLR2, TLR4 and MYD88 (figure 3-14) were investigated. None of these genes showed any significant differences in gene expression upon exposure to either hypoxia or normoxia for 1.5 hours. Due to the low patient number, any potential trends may not be apparent therefore this would require further investigation.

Stress proteins:

The differential mRNA expression of HSP20, HSP27, HSP32 (figure 3-15), HSP60, HSP72 and HSP90 (figure 3-16) were investigated. None of these genes showed any significant differences in gene expression upon exposure to either hypoxia or normoxia for 1.5 hours. Due to the low patient number, any potential trends may not be apparent therefore this would require further investigation.

Figure 3-14. Figure showing the differential expression of inflammatory mediators upon exposure to 2% O_2 and 20% O_2 for 1.5 hours. A shows TLR4, B shows TLR2 and C shows MYD88.





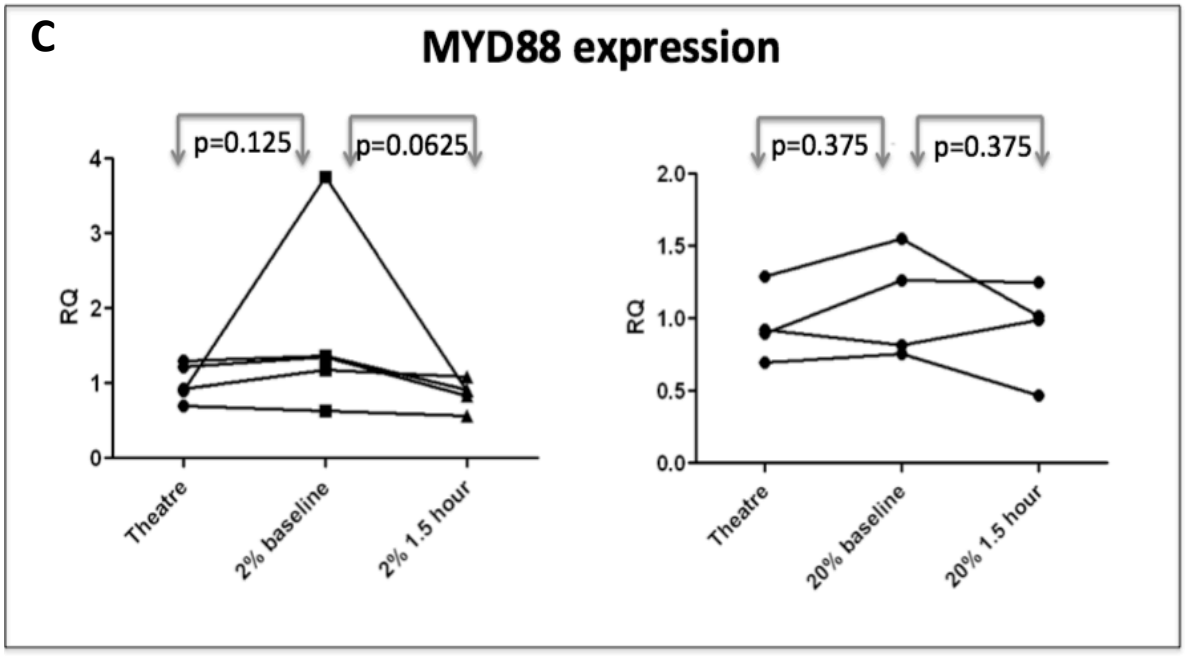
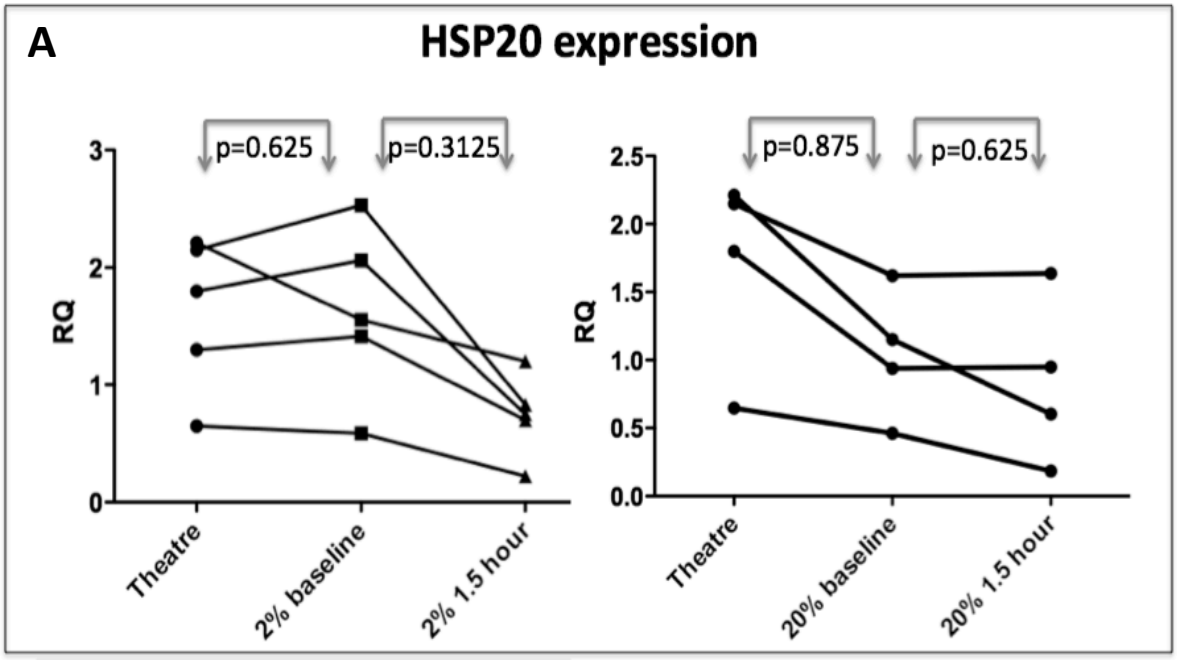
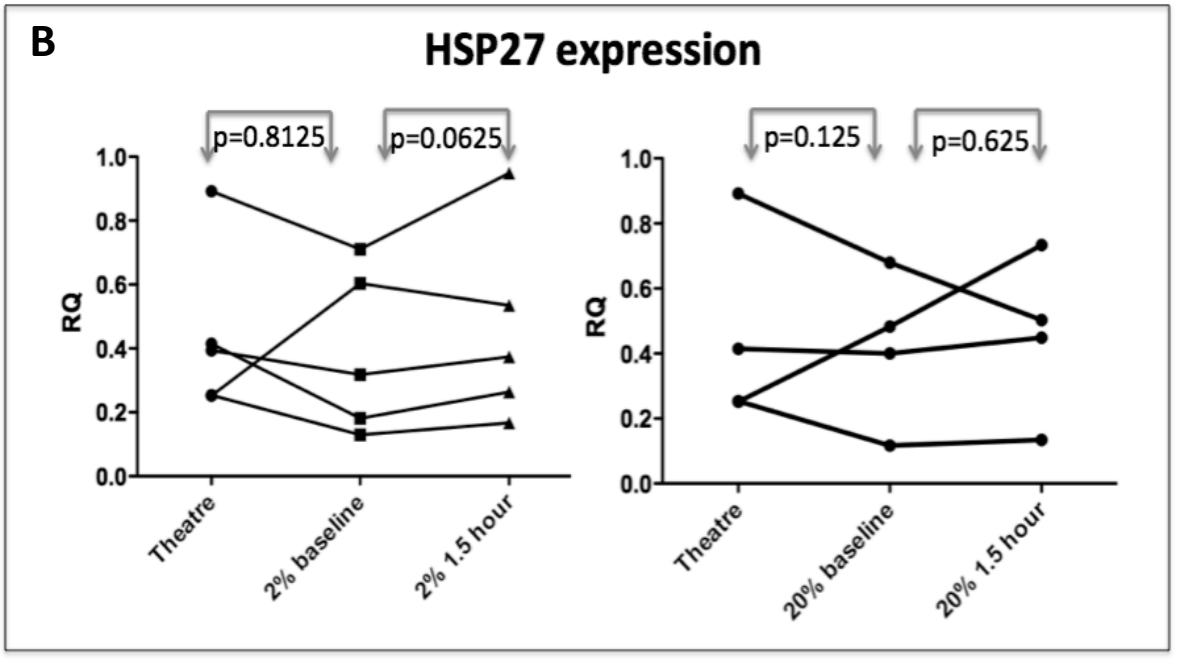


Figure showing the differential expression of TLR4 (A), TLR2 (B) and MYD88 (C) when myocardium exposed to 2% O_2 and 20% O_2 for 1.5 hours. Samples taken from each patient (n=5) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). The graphs show the expression seen at the time of collection in theatre (theatre), at the time of the start of the incubation back in the laboratory (2/ 20% baseline) and after the 1.5 hour incubation (2/ 20% 1.5 hour). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-15. Figure showing the differential expression the stress proteins HSP20, HSP27 and HSP32 upon exposure to 2% O_2 and 20% O_2 for 1.5 hours. A shows HSP20, B shows HSP27 and C shows HSP32.





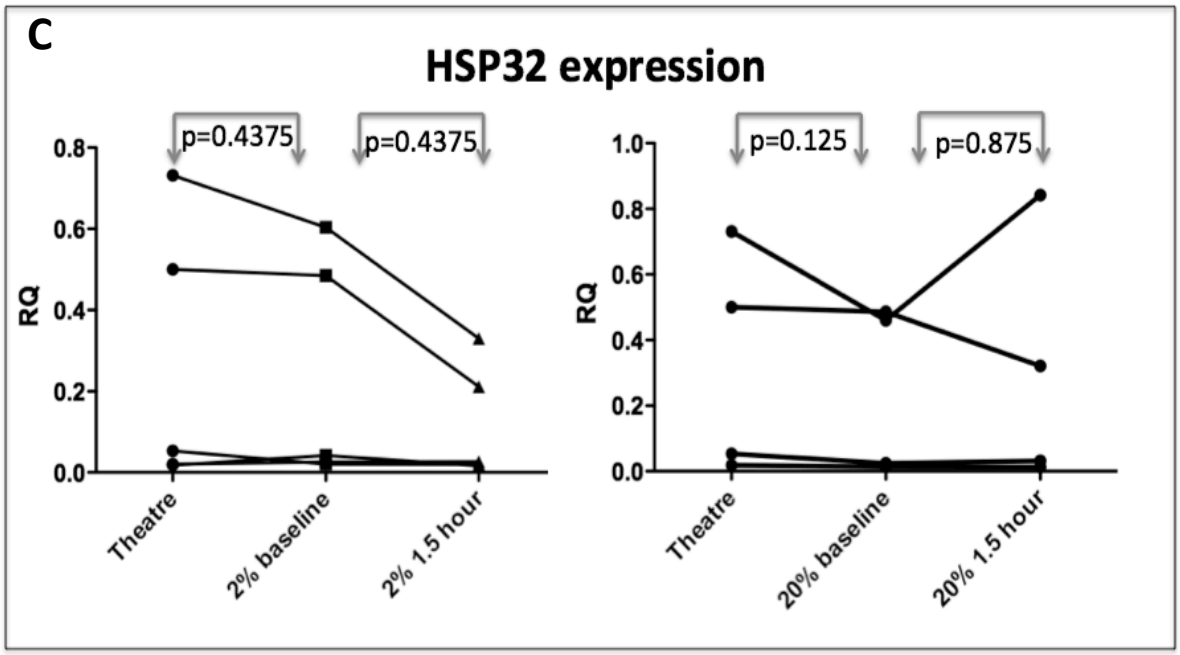
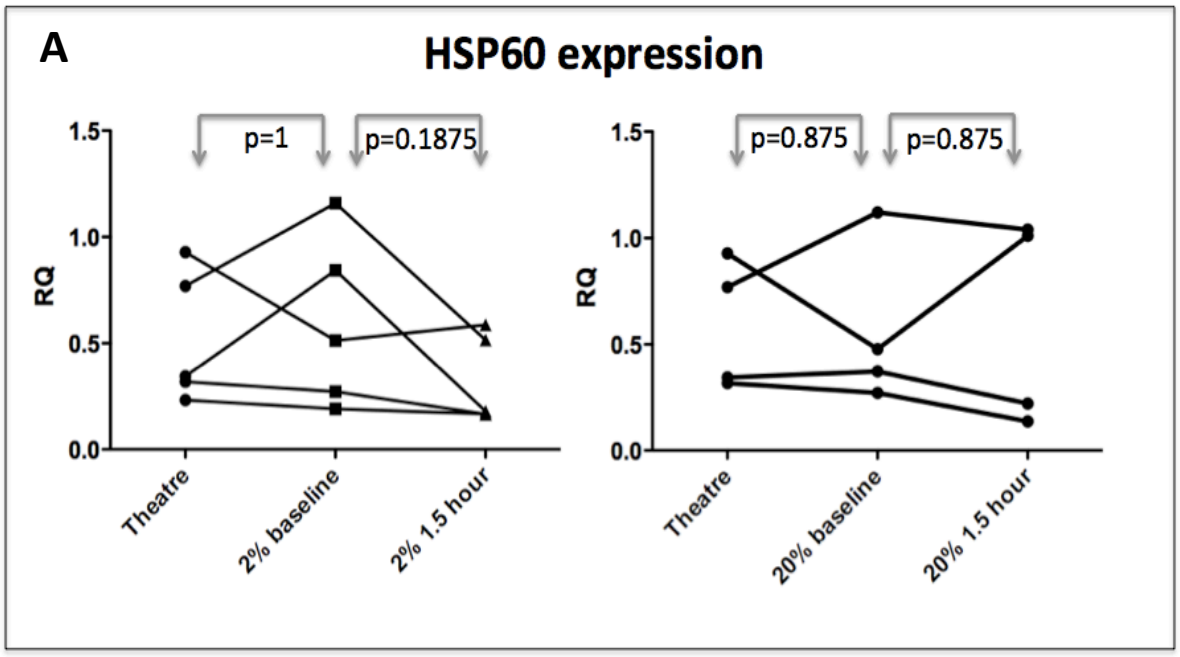
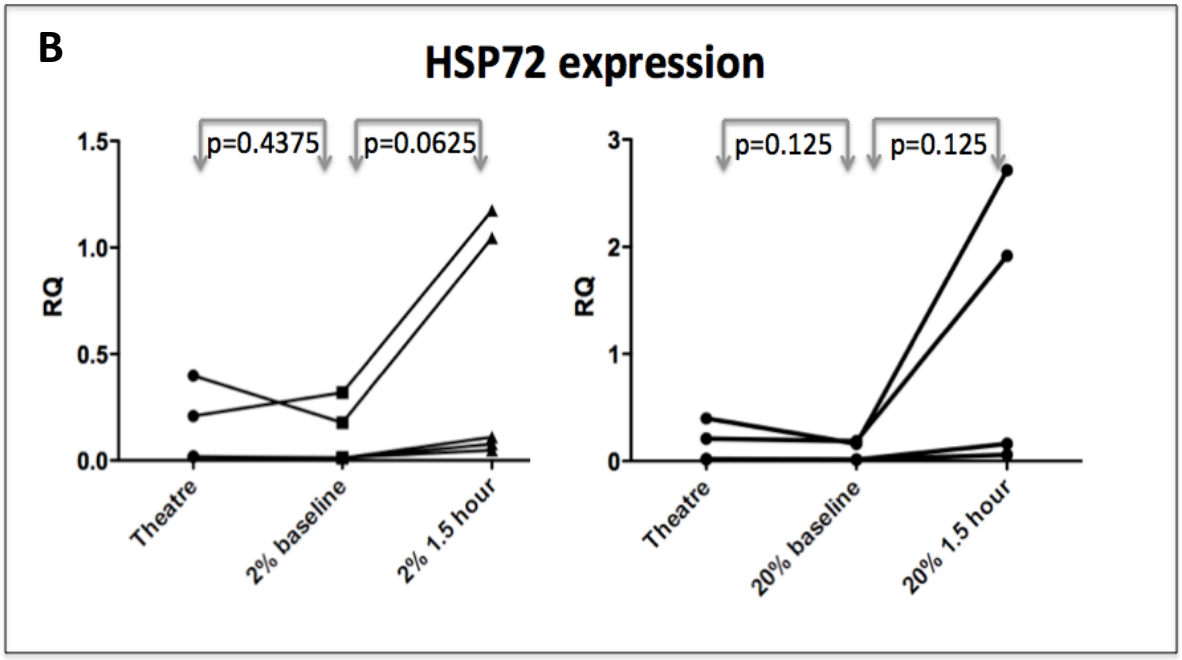


Figure showing the differential expression of HSP20 (A), HSP27 (B) and HSP32 (C) when exposed to 2% O_2 and 20% O_2 for 1.5 hours. Samples taken from each patient (n=5) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). The graphs show the expression seen at the time of collection in theatre (theatre), at the time of the start of the incubation back in the laboratory (2/ 20% baseline) and after the 1.5 hour incubation (2/ 20% 1.5 hour). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-16. Figure showing the differential expression the stress proteins HSP60, HSP72 and HSP90 upon exposure to 2% O_2 and 20% O_2 for 1.5 hours. A shows HSP60, B shows HSP72 and C shows HSP90.





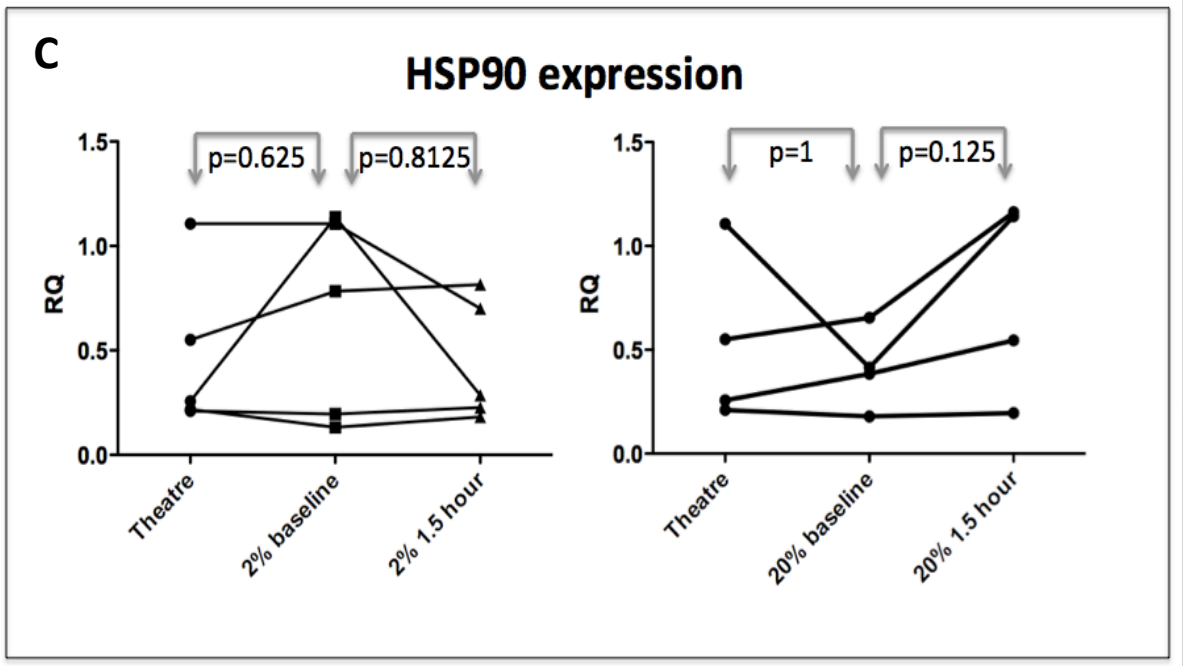


Figure showing the differential expression of HSP60 (A), HSP72 (B) and HSP90 (C) when exposed to 2% O_2 and 20% O_2 for 1.5 hours. Samples taken from each patient (n=5) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). The graphs show the expression seen at the time of collection in theatre (theatre), at the time of the start of the incubation back in the laboratory (2/ 20% baseline) and after the 1.5 hour incubation (2/ 20% 1.5 hour). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

3.3.2.2 Experiment 2: exposing myocardial tissue to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2

The second experimental model involved exposing ischemic infant myocardial tissue to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2 (outlined in section 2.3.2) for 4 hours. It was then determined whether or not such exposure caused any differences to occur in the expression of both inflammatory mediators and stress proteins. Hyperoxic exposure of the myocardium is the most clinically relevant due to its use in the clinical setting. The tissue was exposed for 4 hours rather 1.5 hours in order to determine if a longer exposure would result in enhanced gene expression. Ten patients (table 11) were recruited for this study, consisting of TOF, aortic obstruction and VSD patients. These patients were used for the mRNA gene expression analysis upon exposure to 2%, 20% and 60% O_2 for 4 hours only.

Table 11. Patients recruited for exposure to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2

Patient	Type	Group	Gender	Age
32	PREV TOF	Cyanotic	Male	6 years 0 months
33	TOF	Cyanotic	Male	0 years 10 months
34	TOF	Cyanotic	Female	1 year 4 months
35	TOF	Cyanotic	Female	0 years 6 months
36	REDO TOF (FULLY SATURATED)	Acyanotic	Female	6 years 5 months
37	TOF	Cyanotic	Male	0 years 11 months
38	AORTIC OBSTRUCTION (CTL)	Acyanotic	Male	1 year 3 months
39	TA (CTL)	Acyanotic	Male	0 years 1 month
41	COMPLEX TOF	Cyanotic	Male	5 years 0 months
45	VSD (FULLY SATURATED)	Acyanotic	Female	1 year 2 months

It should be noted that patients 38 and 39 do not have samples collected in theatre (A) or baseline (B) (sample time point on return to laboratory) due to limited tissue availability, therefore because paired statistical analysis was carried out the results for these samples were not included. Due to limited patient numbers, all patients recruited for this study were taken as one group.

In order to investigate the gene expression of the inflammatory mediators and stress proteins mentioned above, the method of qPCR (see section 2.9) was used and RQ of gene expression was calculated using the comparative C_t method (see section 2.9.2). Statistical analysis was performed as described in experiment 1.

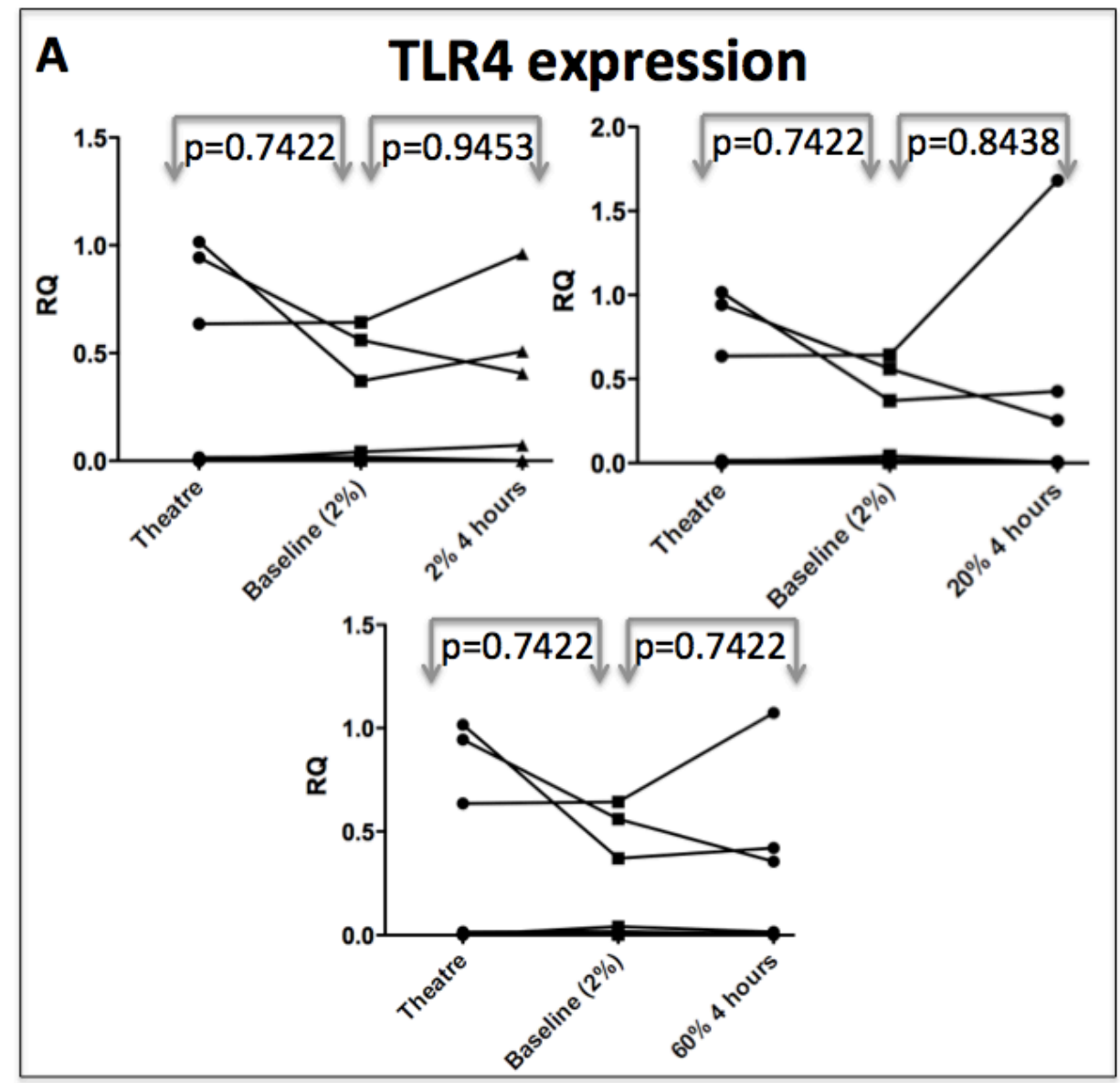
Inflammatory markers:

The differential mRNA expression of TLR4 (figure 3-17A), TLR2 (figure 3-17B) and MYD88 (figure 3-17B) were investigated. None showed any significant differences in expression when exposed to 2% (hypoxia), 20% (normoxia) or 60% (hyperoxia) O_2 for 4 hours. Due to the low patient number, any potential trends may not be apparent therefore this would require further investigation.

Stress proteins:

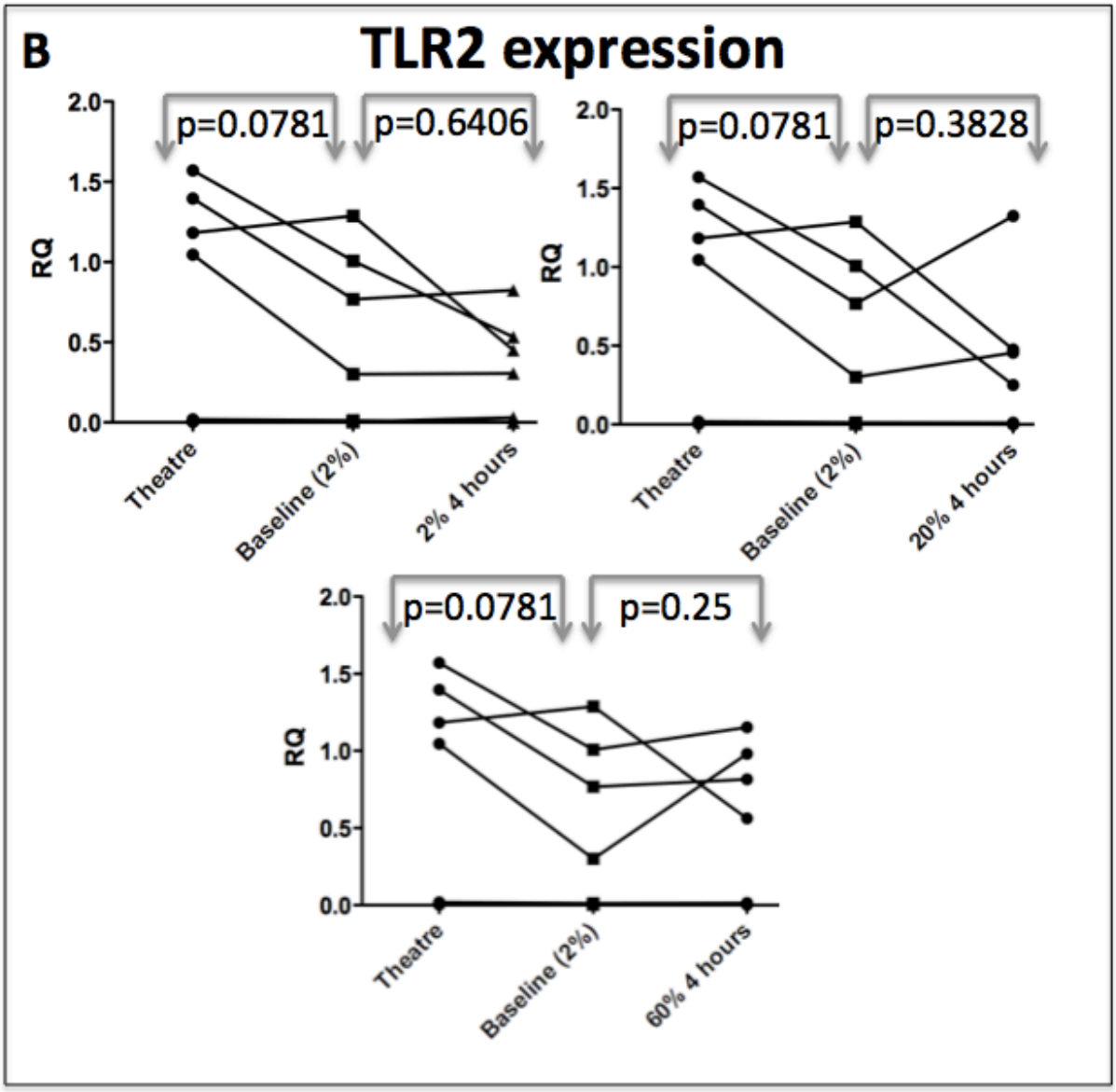
The differential mRNA expression of HSP20, HSP27, HSP32 (figure 3-18), HSP60, HSP72 and HSP90 (figure 3-19) were investigated. HSP20 and HSP27 expression showed no significant differences in expression when exposed to any differential O_2 level. HSP32 showed a significant increase in expression when exposed to normoxia ($p=0.0391$) and hyperoxia ($p=0.0078$) for 4 hours. HSP60 showed no significant differences in expression when exposed to any differential O_2 level. HSP72 showed a significant increase in expression when exposed to normoxia ($p=0.0078$) and hyperoxia ($p=0.0078$) for 4 hours. HSP90 showed a trend of an increase in expression when exposed to normoxia ($p=0.0547$) and hyperoxia ($p=0.0547$) but this did not make statistical significance. There was no change in HSP32, HSP72 and HSP90 expression after exposure to hypoxia for 4 hours.

Figure 3-17A. Figure showing the differential expression of TLR4 upon exposure to hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (60% O_2) for 4 hours



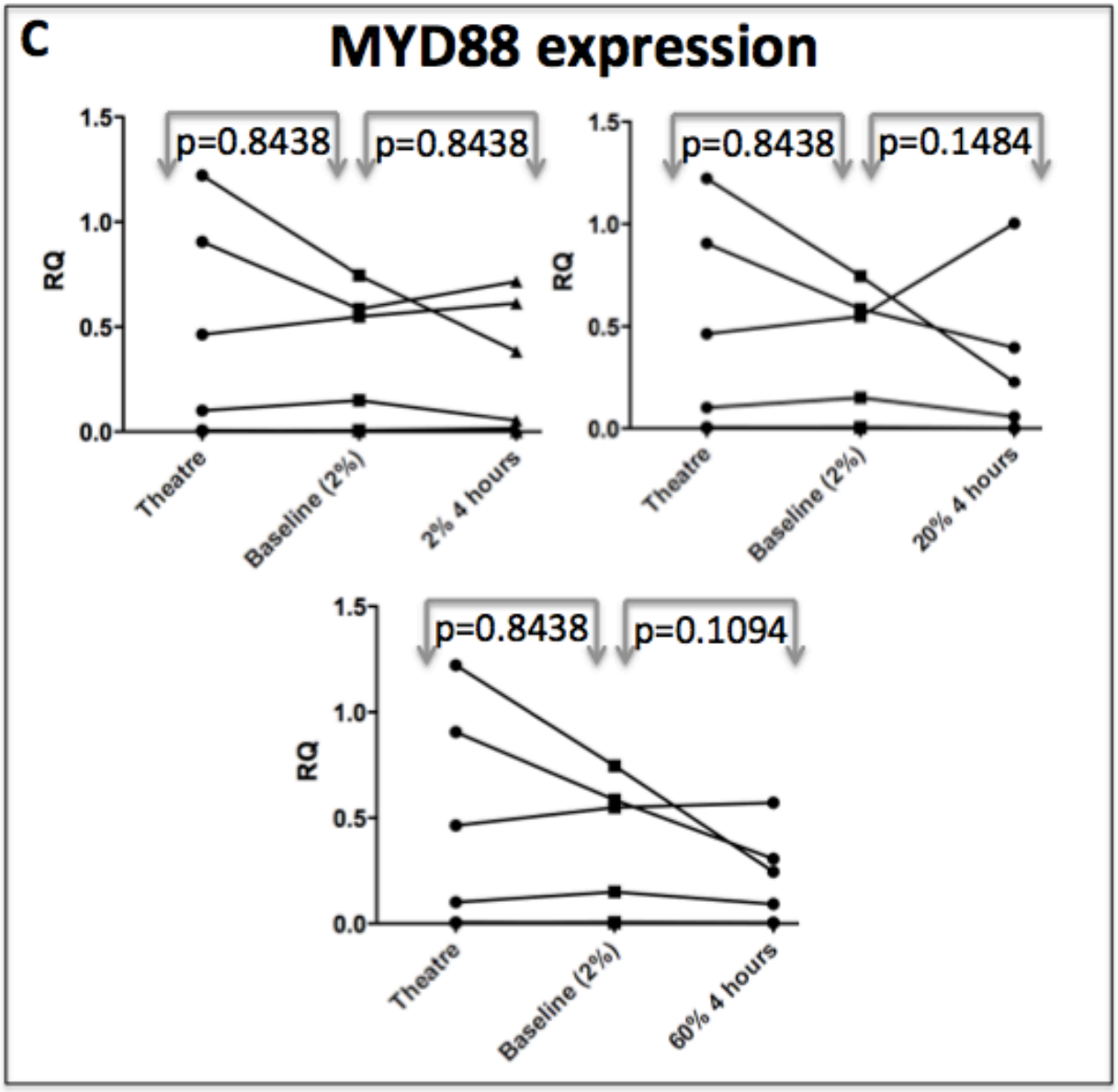
The three points on the x-axis show TLR4 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-17B. Figure showing the differential expression of TLR2 upon exposure to hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (60% O_2) for 4 hours.



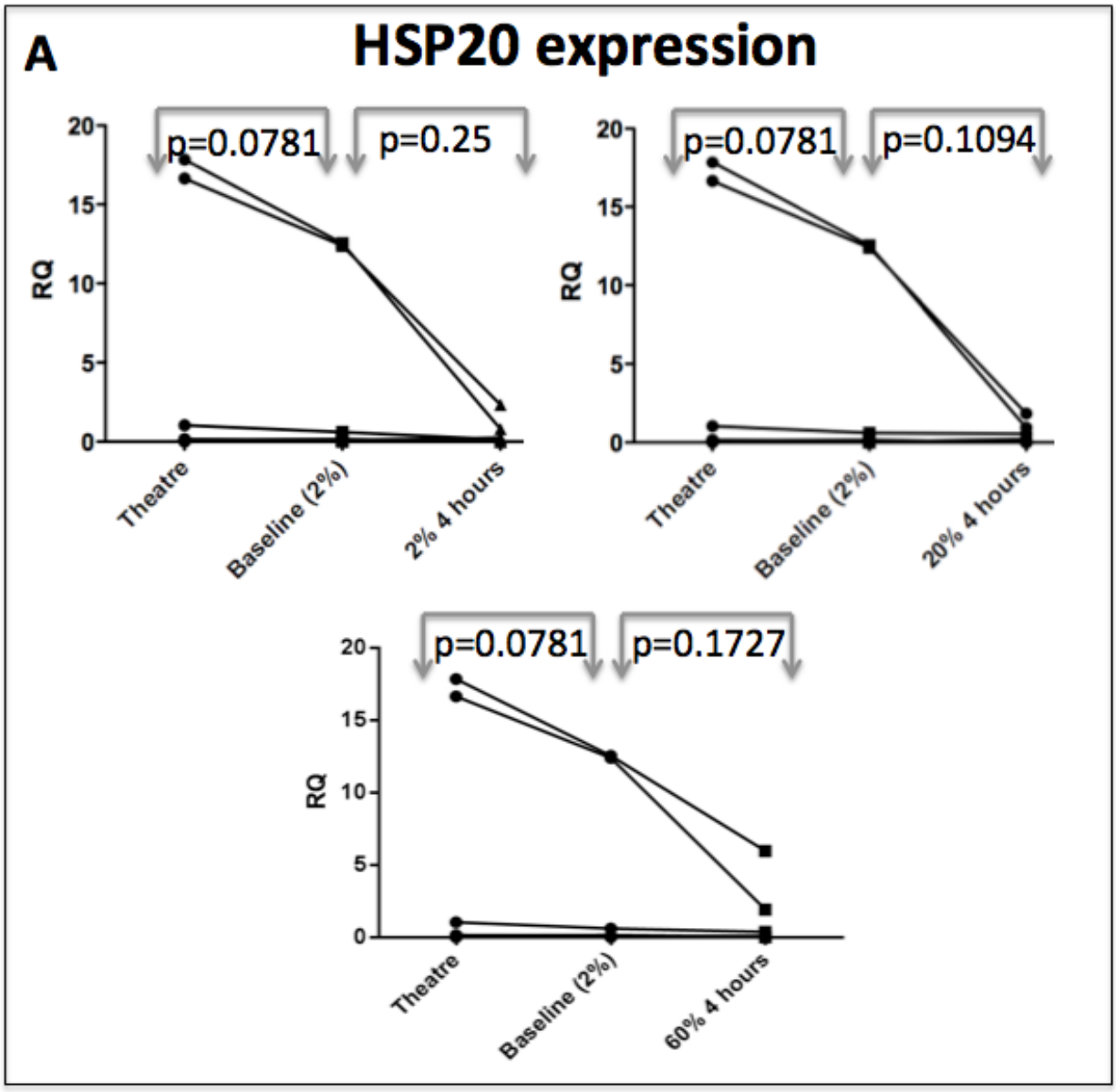
The three points on the x-axis show TLR2 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give relative quantification (RQ) of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-17C. Figure showing the differential expression of MYD88 upon exposure to hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (60% O_2) for 4 hours.



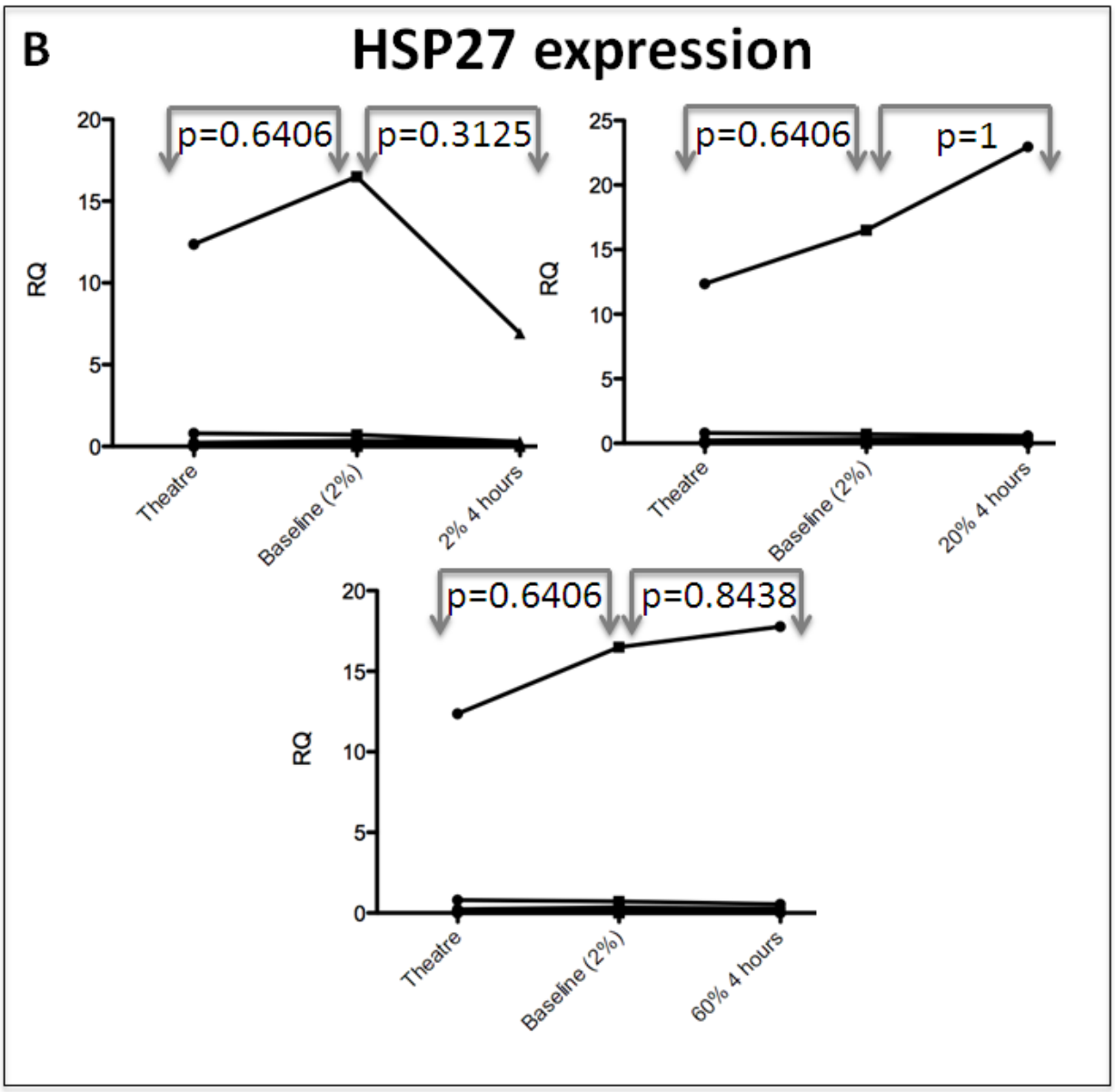
The three points on the x-axis show MYD88 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-18A. Figure showing the differential expression of HSP20 upon exposure to hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (60% O_2) for 4 hours.



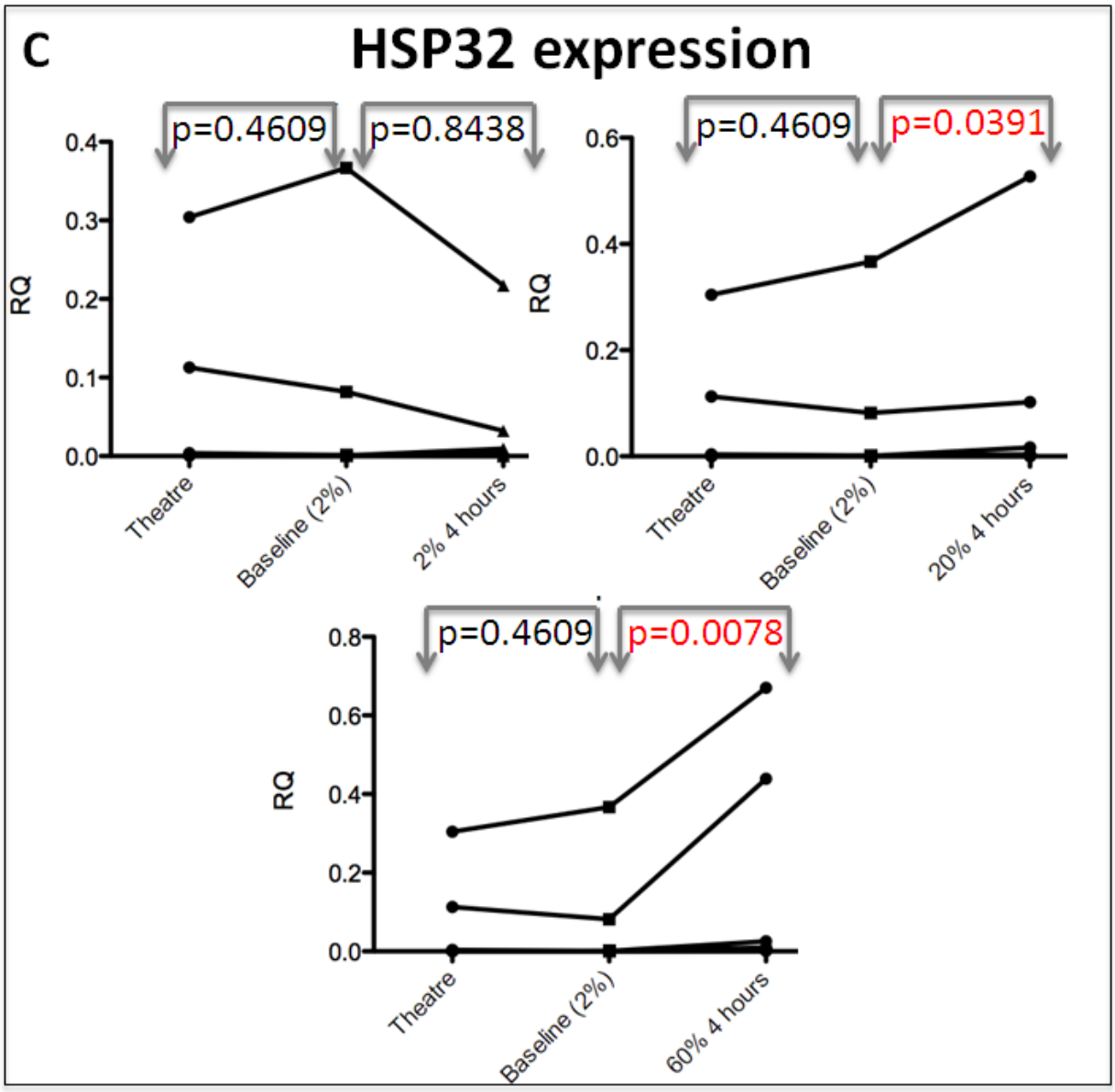
The three points on the x-axis show HSP20 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-18B. Figure showing the differential expression of HSP27 upon exposure to hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (60% O_2) for 4 hours.



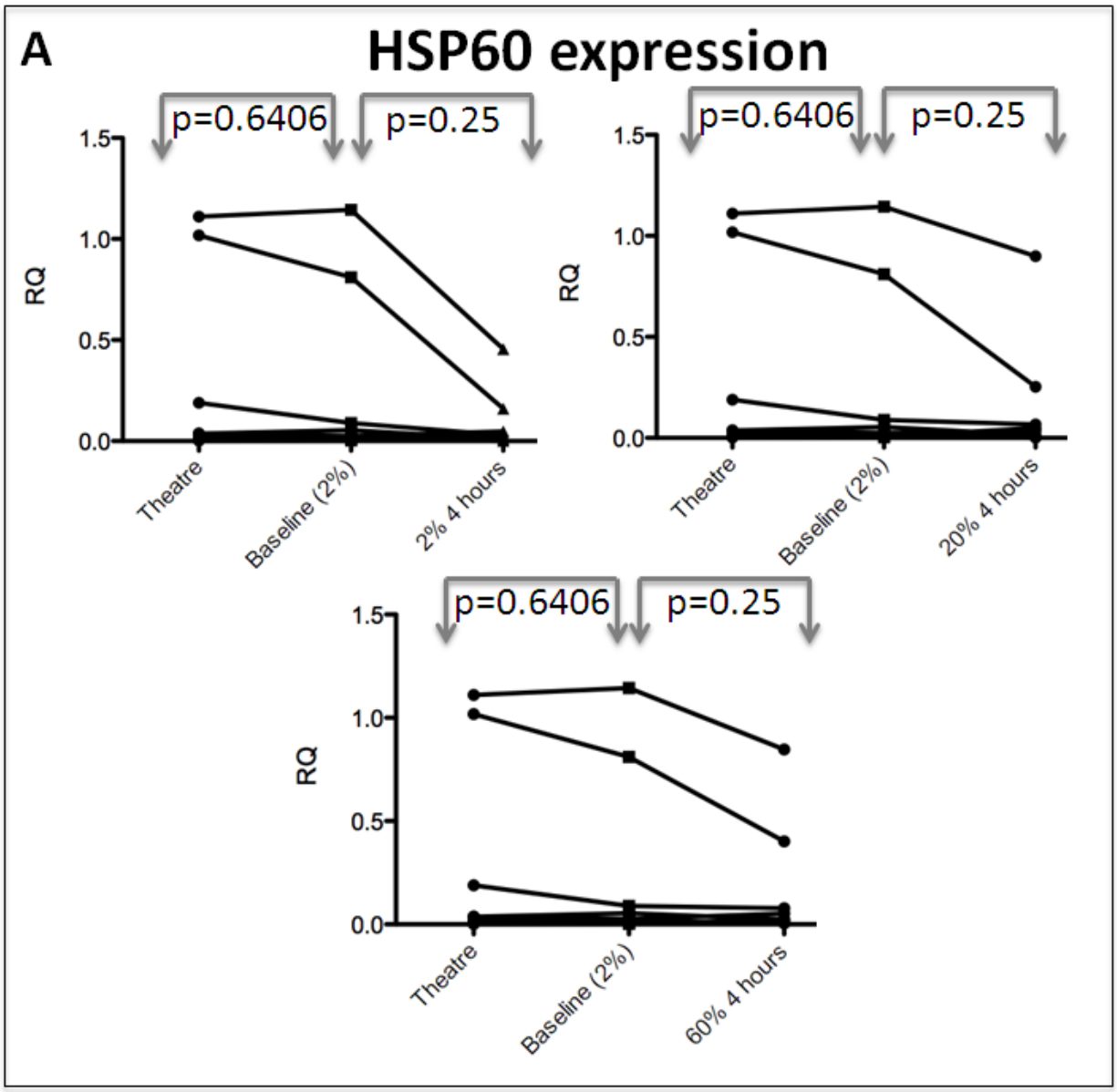
The three points on the x-axis show HSP27 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-18C. Figure showing the differential expression of HSP32 upon exposure to hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (60% O_2) for 4 hours.



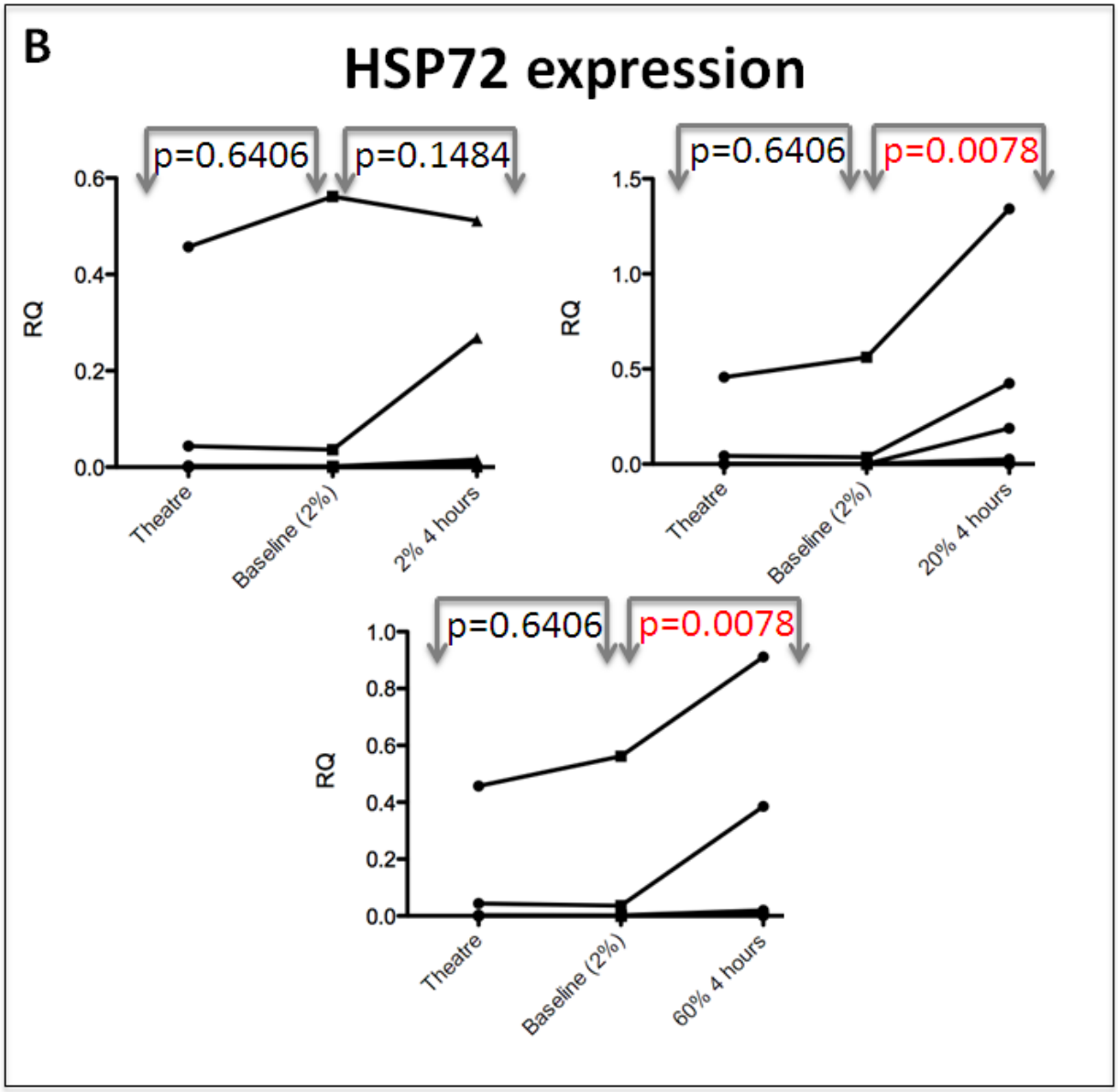
The three points on the x-axis show HSP32 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-19A. Figure showing the differential expression of HSP60 upon exposure to hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (60% O_2) for 4 hours.



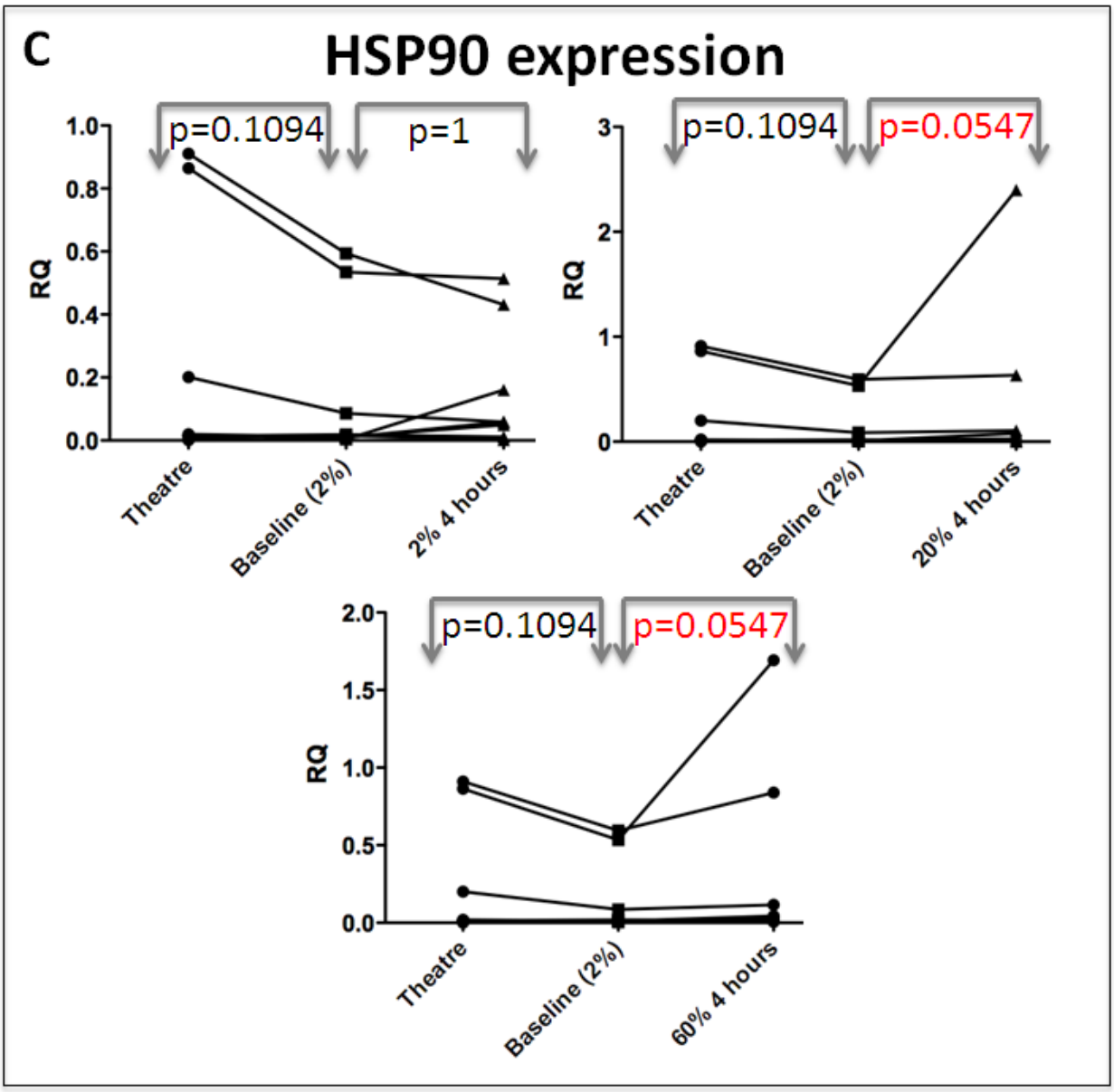
The three points on the x-axis show HSP60 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-19B. Figure showing the differential expression of HSP72 upon exposure to hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (60% O_2) for 4 hours.



The three points on the x-axis show HSP72 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Figure 3-19C. Figure showing the differential expression of HSP90 upon exposure to hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (60% O_2) for 4 hours.



The three points on the x-axis show HSP90 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

The expression of both HSP32 and HSP72 mRNA were increased during 4 hours of normoxic (20% 4 hours) and 4 hours of hyperoxic (60% 4 hours) exposure compared to baseline (baseline 2%). When comparing these two groups for HSP32 (figure 3-20A) using the Wilcoxon matched-pairs signed rank test a significant difference was not found ($p=0.0781$). For HSP72 (figure 3-20B), there was a significant difference found ($p=0.0391$) indicating that exposure to 20% O_2 produced a bigger increase in HSP72 expression than 60% O2 after a period of 4 hours

Figure 3-20 Differential expression of HSP32 (A) and HSP72 (B) in tissue exposed to 4 hours of normoxic (20% 4 hours) and 4 hours of hyperoxic (60% 4 hours).

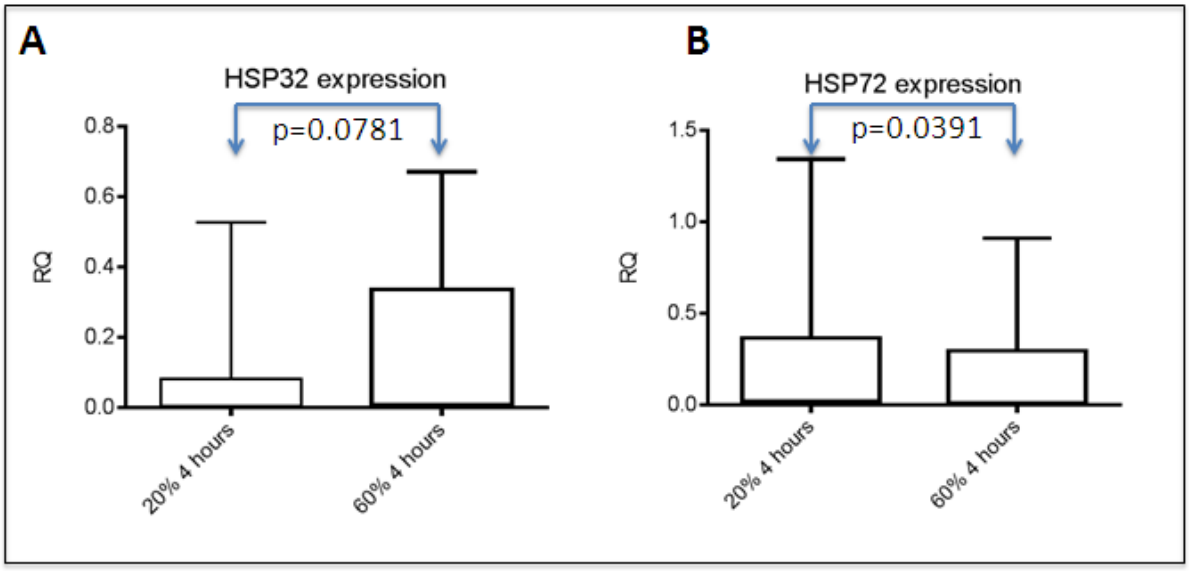


Figure showing the differential mRNA expression of the stress proteins HSP32 (A) and HSP72 (B) in tissue exposed to 4 hours of normoxia (20% 4 hours) compared to tissue exposed to hyperoxia (60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Comparison of cyanotic and acyanotic patients:

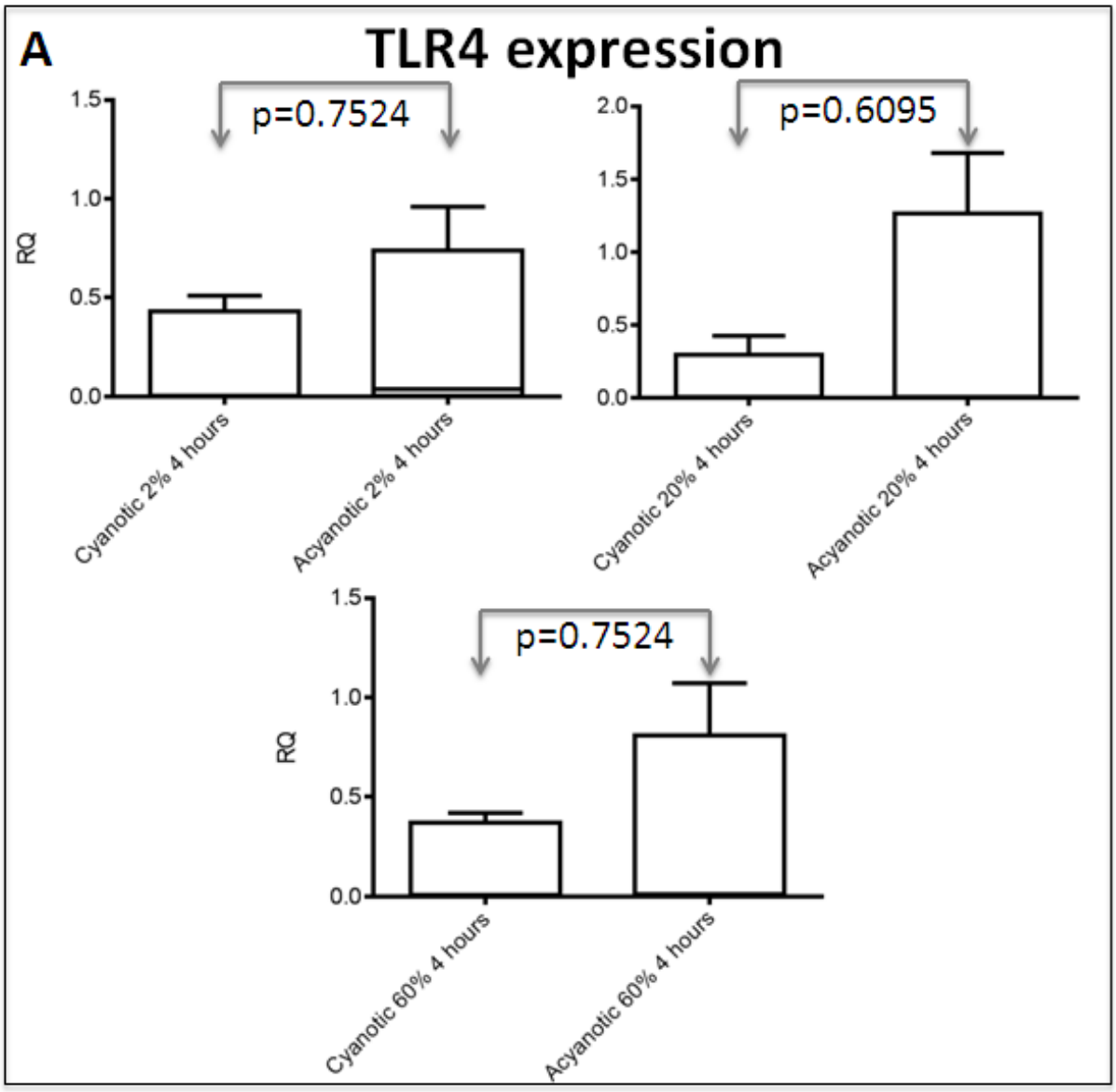
So far the data from patients 38 and 39 has not been used in the analysis because they had no baseline values (see section 3.3.2.2). This has meant that cyanotic and acyanotic patients groups were unable to be compared. There is a full data set for the time points C (2% 4 hours), D (20% 4 hours) and E (60% 4 hours) (see figure 2.2) for patients 38 and 39. It is therefore possible to compare these three groups in cyanotic patients compared to acyanotic patients. The patients recruited were grouped into cyanotic and acyanotic group (table 12).

Table 12. Cyanotic and acyanotic patients exposed to 2%, 20% and E 60% O_2 4 hours.

Patient	Type	Group	Gender	Age
32	PREV TOF	Cyanotic	Male	6 years 0 months
33	TOF	Cyanotic	Male	0 years 10 months
34	TOF	Cyanotic	Female	1 year 4 months
35	TOF	Cyanotic	Female	0 years 6 months
37	TOF	Cyanotic	Male	0 years 11 months
41	COMPLEX TOF	Cyanotic	Male	5 years 0 months
36	REDO TOF (FULLY SATURATED)	Acyanotic	Female	6 years 5 months
38	AORTIC OBSTRUCTION (CTL)	Acyanotic	Male	1 year 3 months
39	TA (CTL)	Acyanotic	Male	0 years 1 month
45	VSD (FULLY SATURATED)	Acyanotic	Female	1 year 2 months

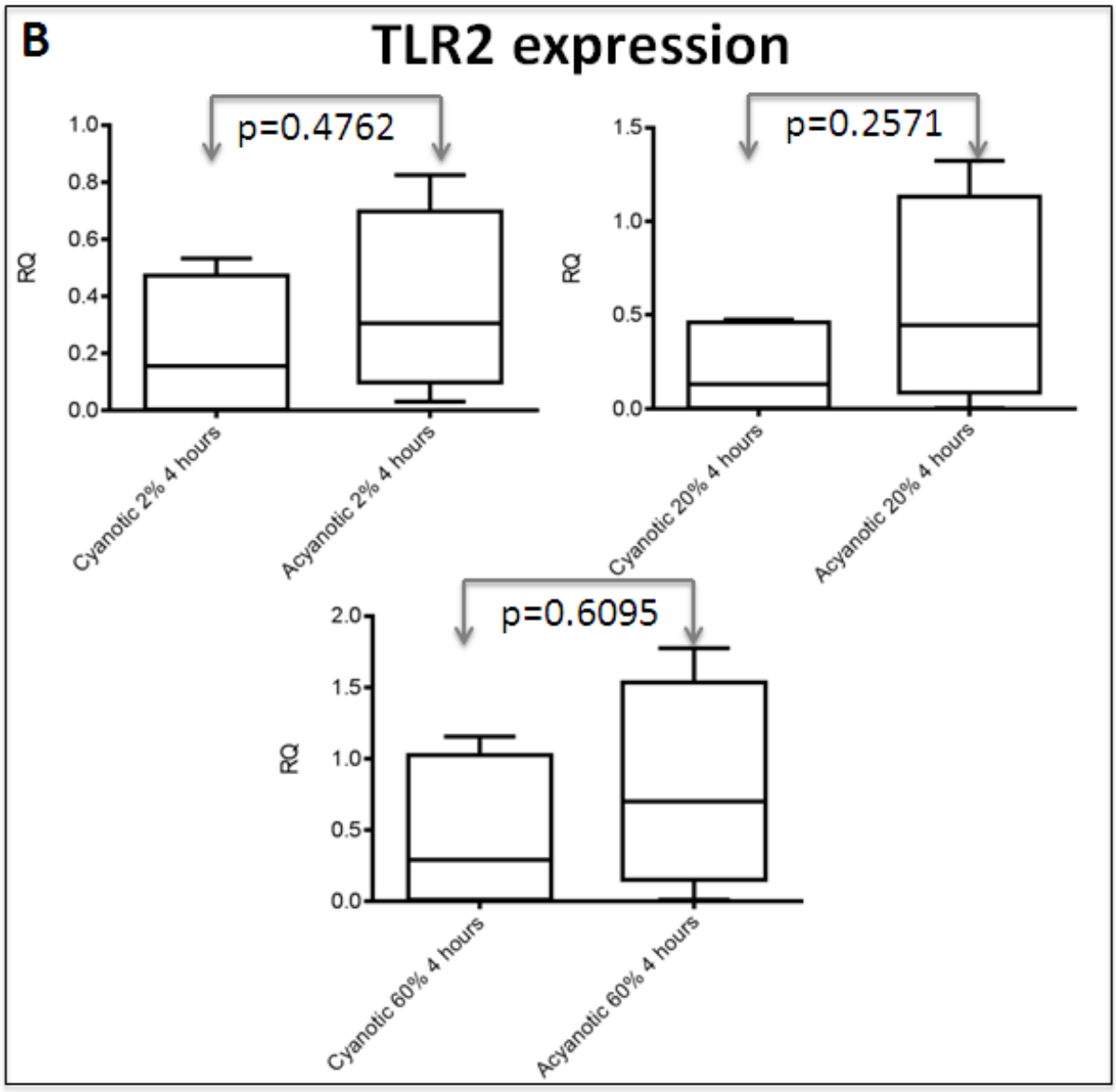
Statistical analysis was performed using GraphPad Prism 6 Software©. The data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. Therefore the test used was the Mann-Whitney U test where data was expressed by the median and interquartile range. A p-value of <0.05 (two-tailed) is considered as statistically significant. This comparison was done for the inflammatory mediators TLR4, TLR2 and MYD88 (figure 3-21) and the stress proteins HSP20, HSP27, HSP32 (figure 3-22), HSP60, HSP72 and HSP90 (figure 3-23). There was no statistically significant difference seen for any genes upon exposure to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours) when comparing the cyanotic group with the acyanotic group.

Figure 3-21A. Differential expression of TLR4 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



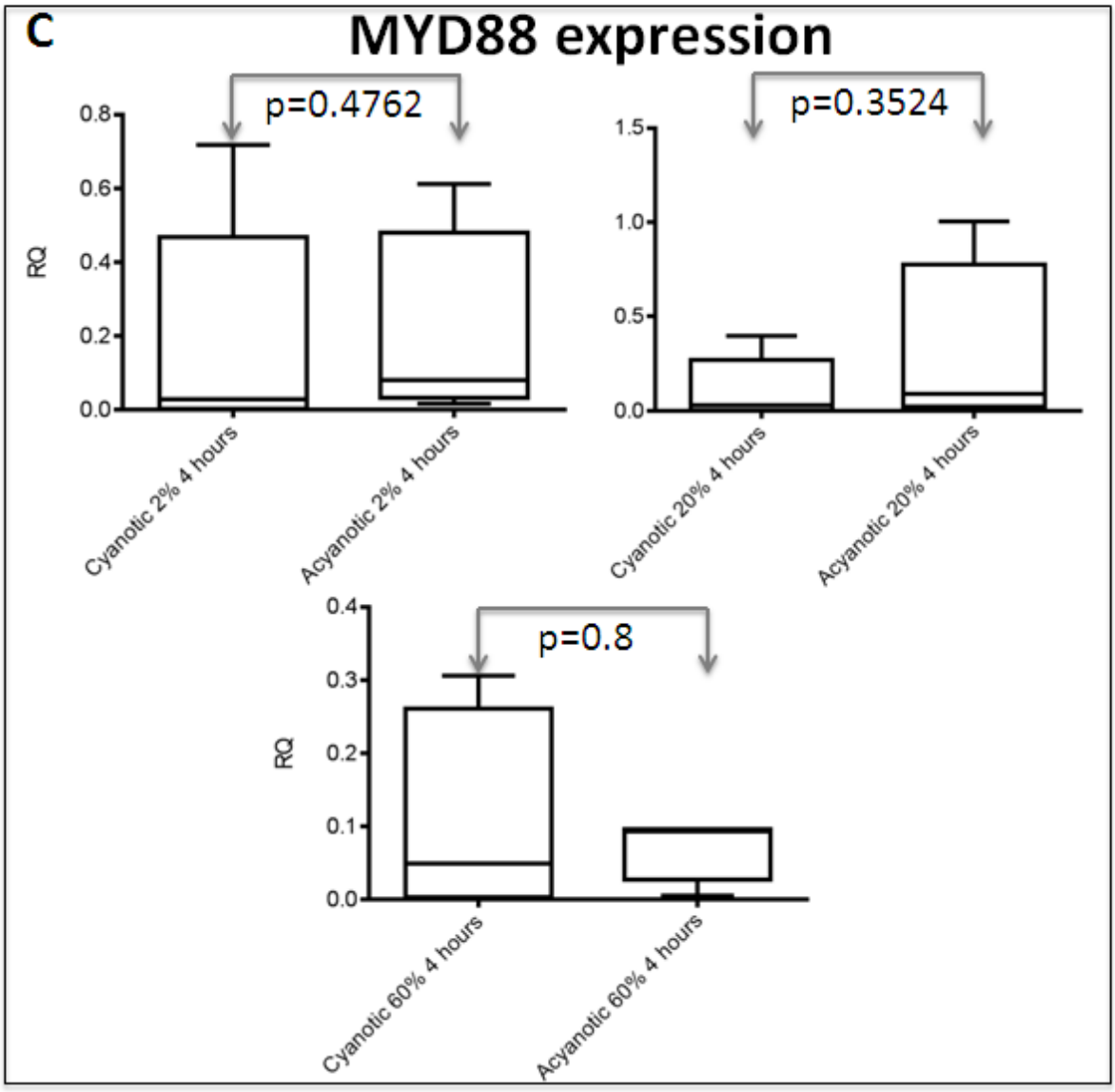
The two points on the x-axis show TLR4 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Figure 3-21B. Differential expression of TLR2 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



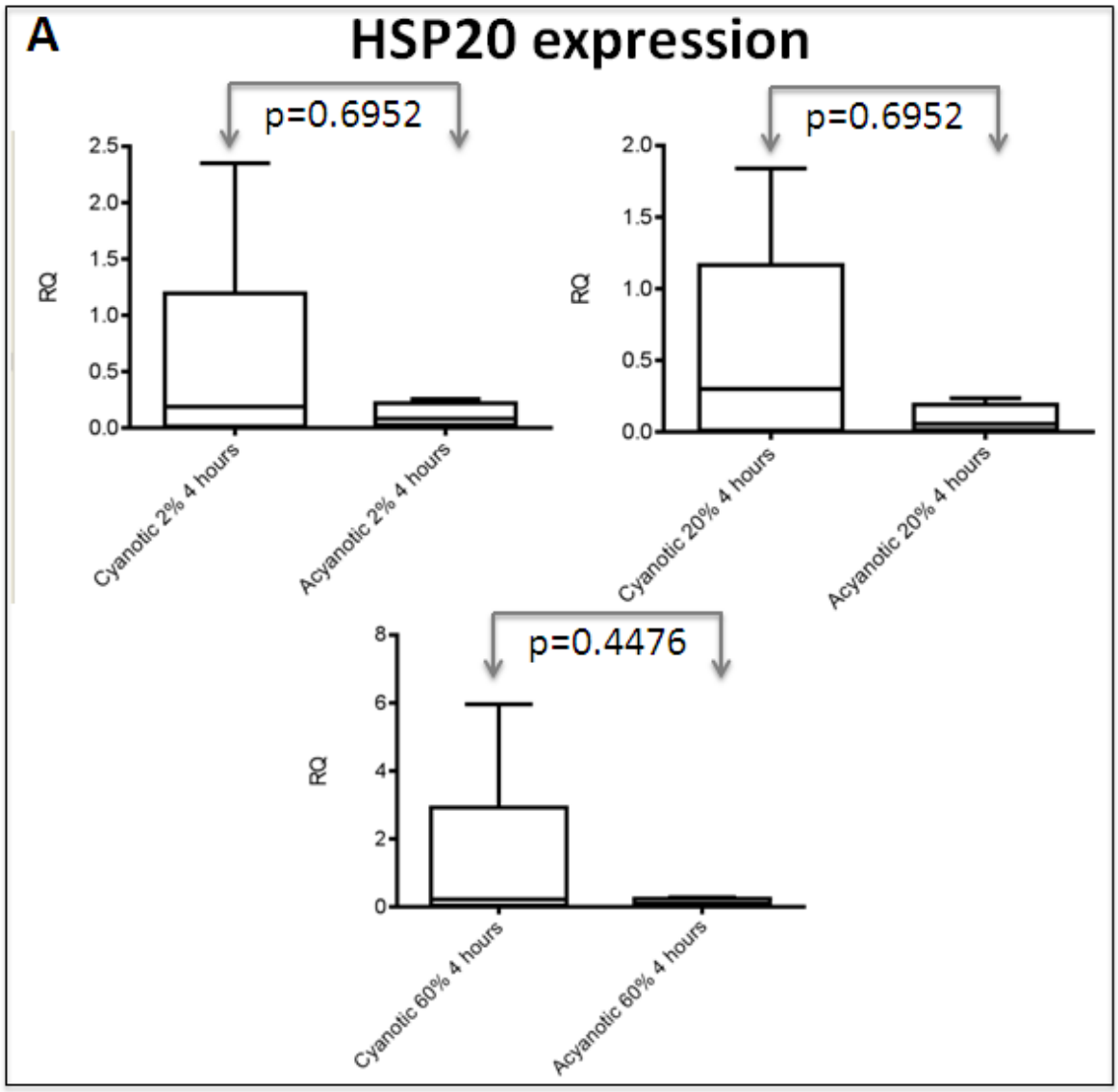
The two points on the x-axis show TLR2 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Figure 3-21C. Differential expression of MYD88 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



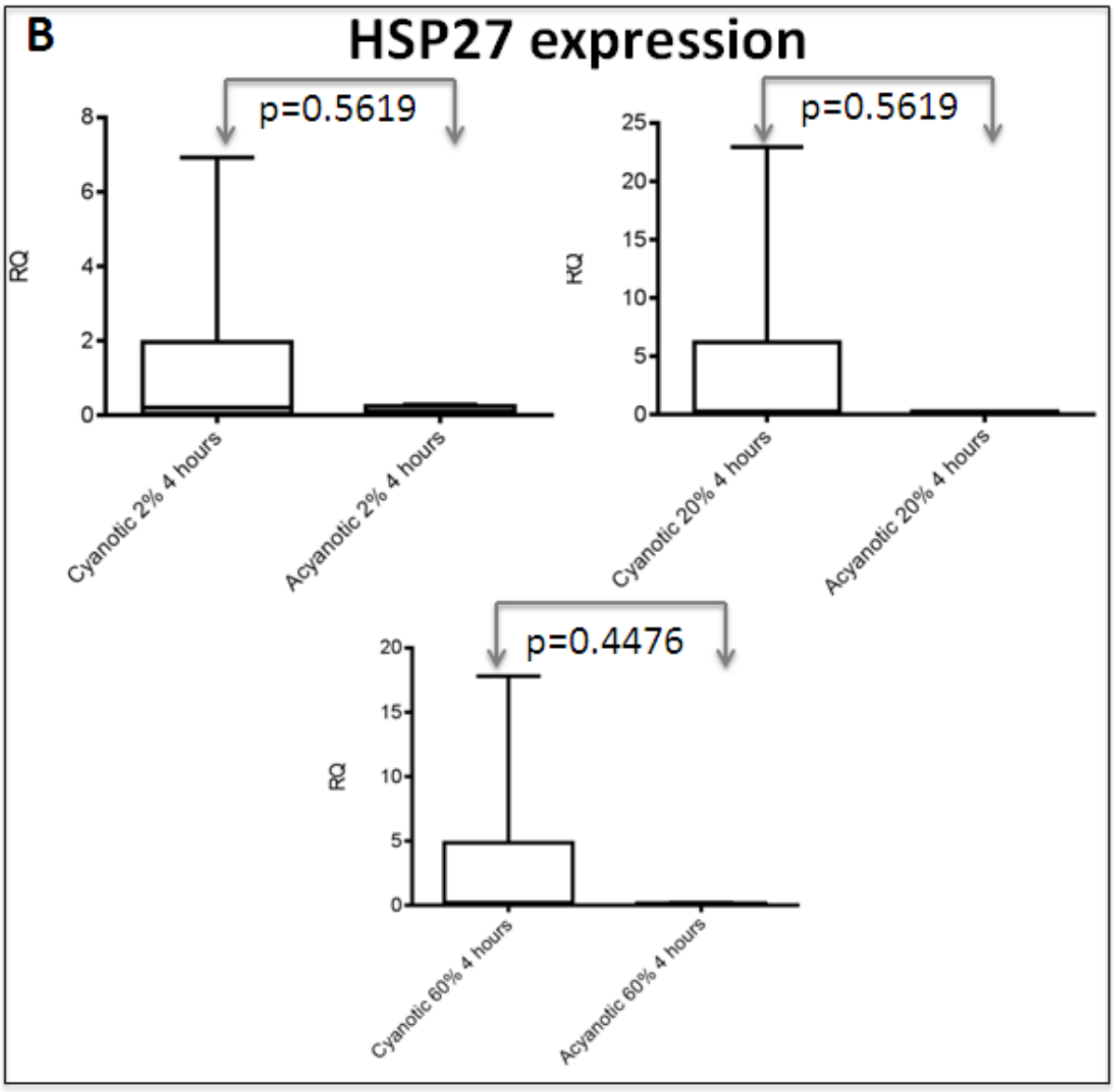
The two points on the x-axis show MYD88 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Figure 3-22A. Differential expression of HSP20 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



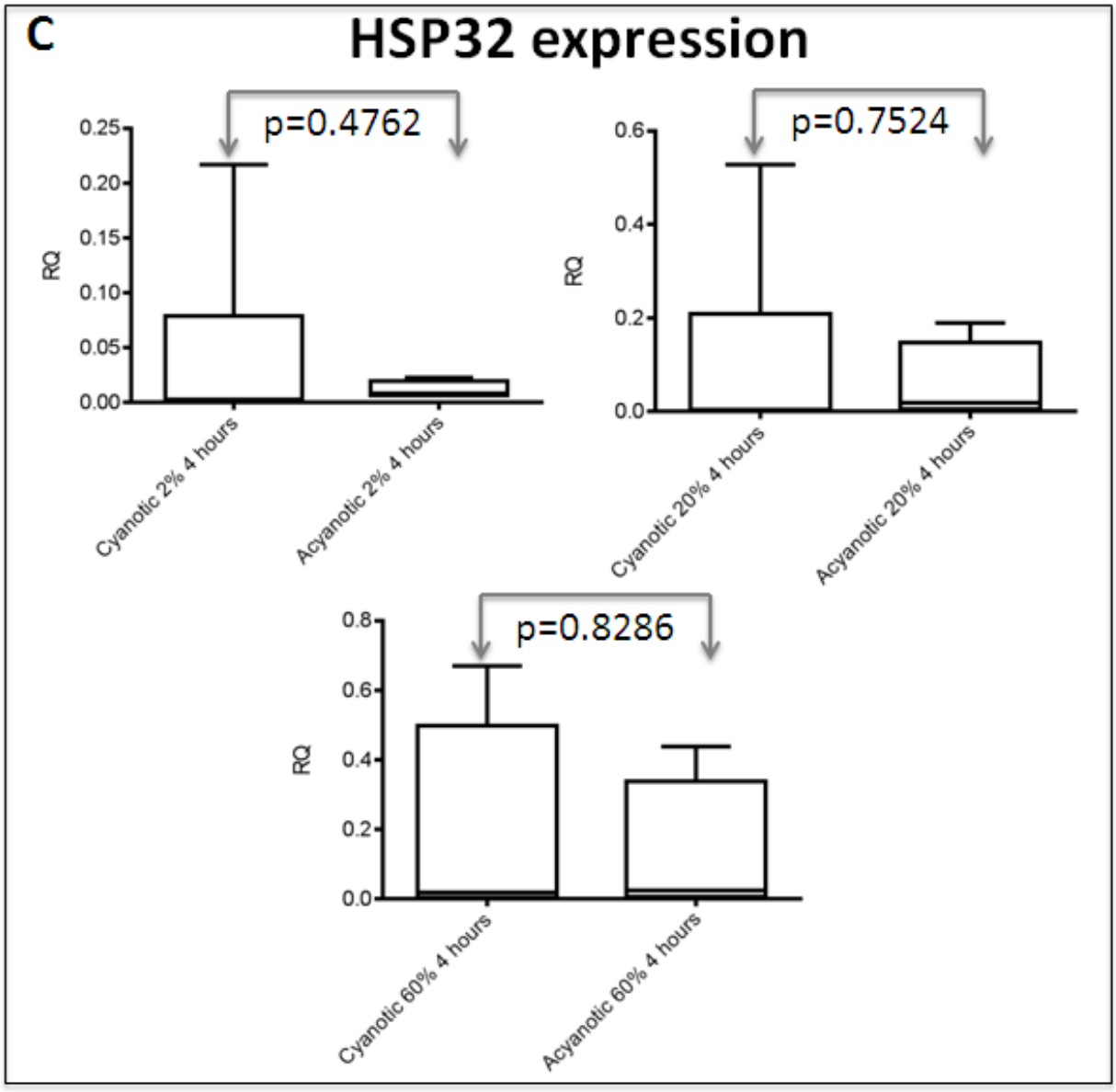
The two points on the x-axis show HSP20 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Figure 3-22B. Differential expression of HSP27 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



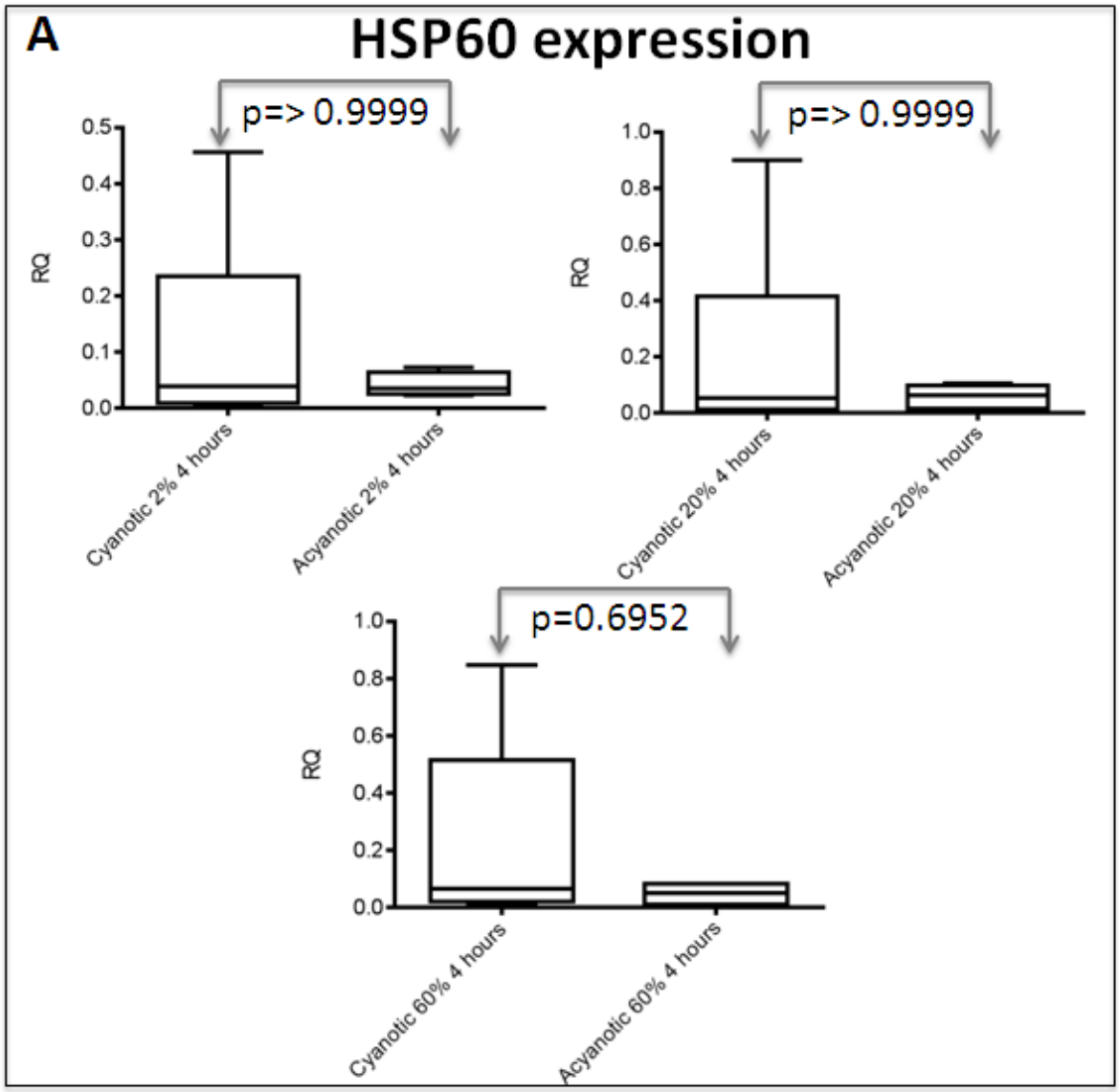
The two points on the x-axis show HSP27 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Figure 3-22C. Differential expression of HSP32 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



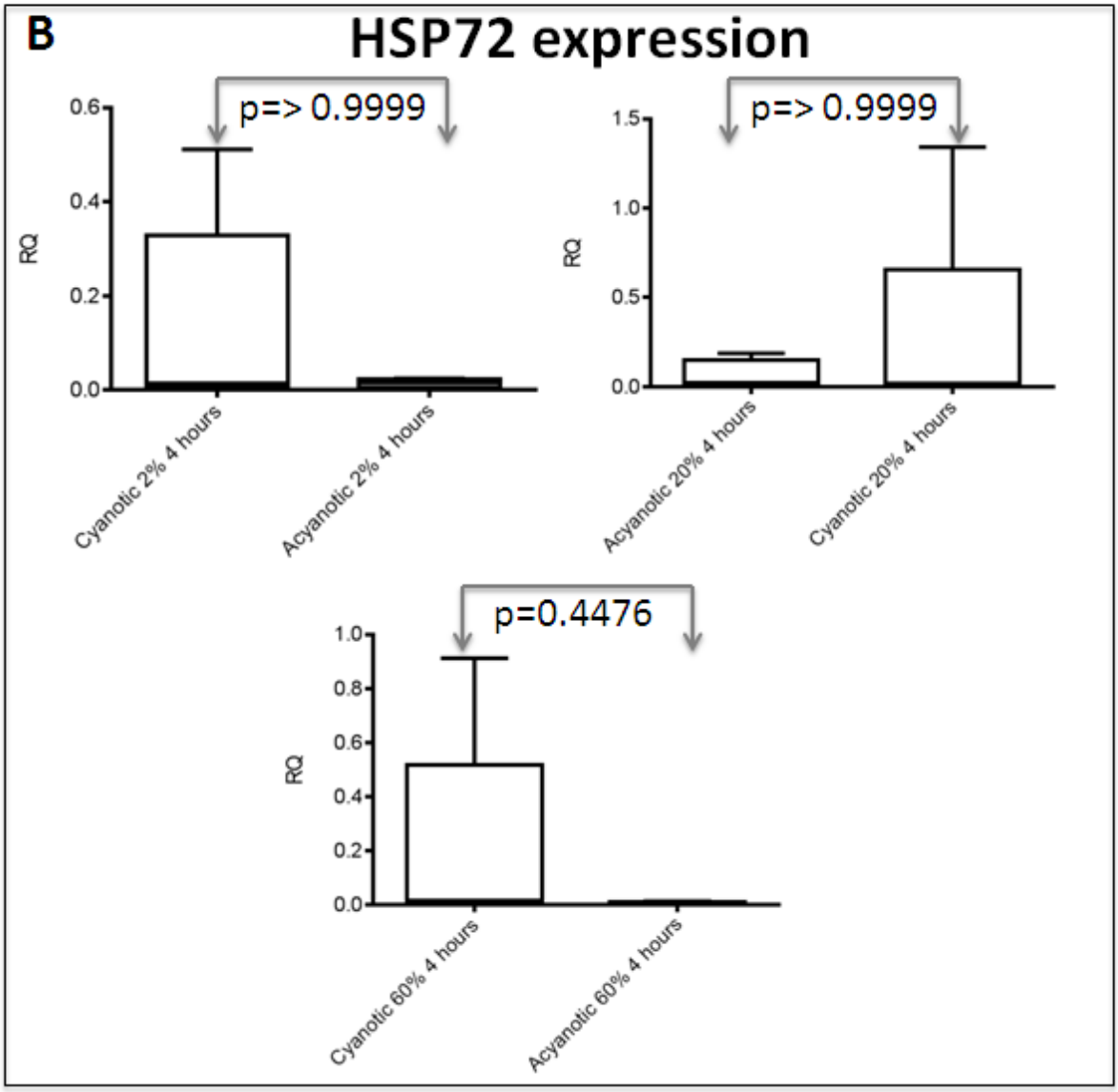
The two points on the x-axis show HSP32 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Figure 3-23A. Differential expression of HSP60 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



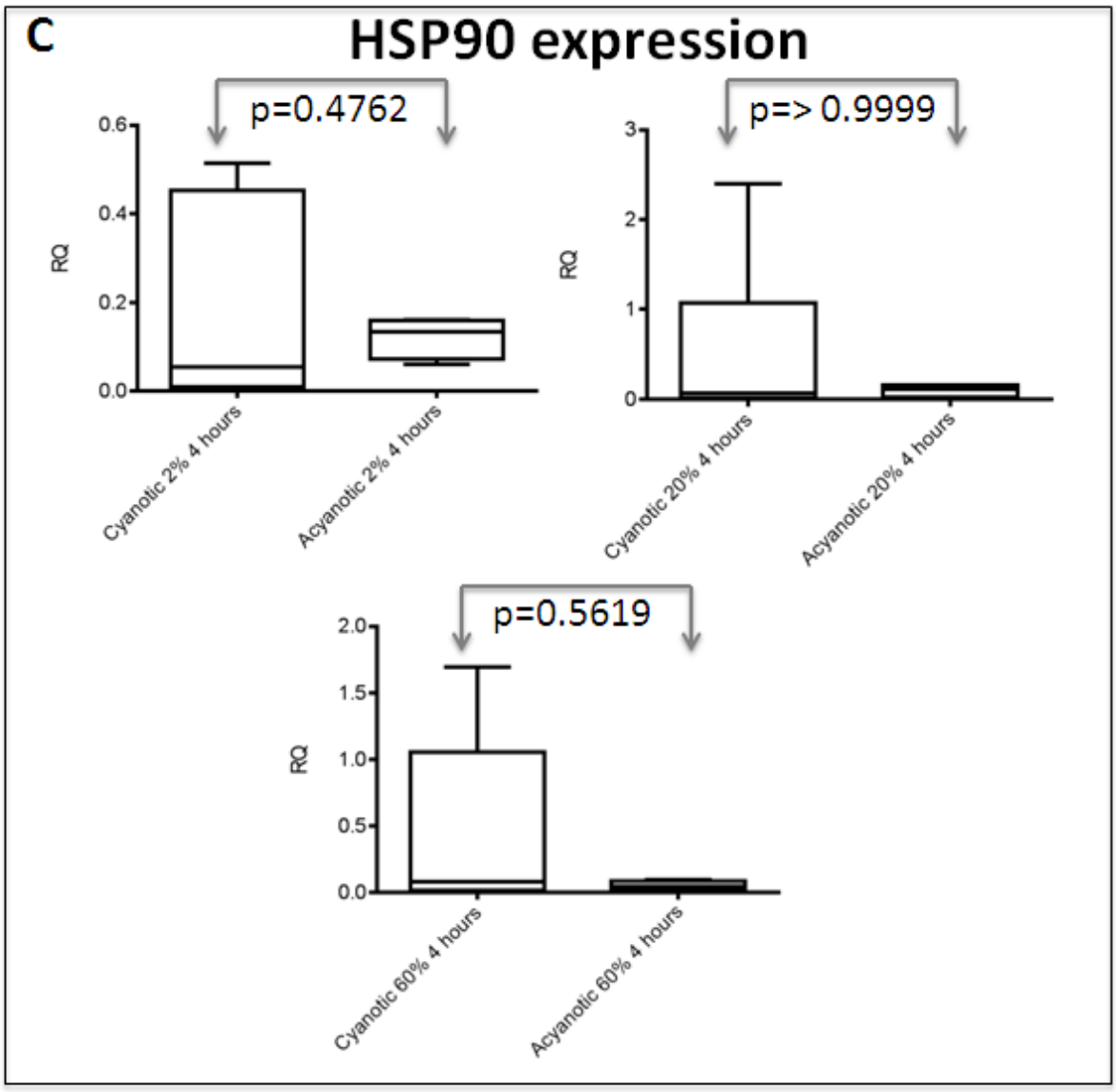
The two points on the x-axis show HSP60 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Figure 3-23B. Differential expression of HSP72 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



The two points on the x-axis show HSP72 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Figure 3-23C. Differential expression of HSP90 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



The two points on the x-axis show HSP90 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Summary of main findings:

- Experiment 1:
  - No significant difference in mRNA expression seen for any genes when exposed to 20% O_2 for 1.5 hours.
- Experiment 2:
  - HSP32 mRNA expression is significantly increased after exposure to 20% ($p=0.0391$) or 60 % ($p=0.0078$) O_2 for 4 hours
  - HSP72 mRNA expression is significantly increased after exposure to 20% ($p=0.0078$) or 60 %($p=0.0078$) O_2 for 4 hours
  - No significant differences in mRNA expression were seen for any genes of interest during 4 hours differential O_2 exposure between the cyanotic and acyanotic patient groups.

3.4 The differential expression of GRP78

3.4.1 Introduction

GRP78 (see section 1.7) is a stress protein that is expressed in response to damage to the cell to either maintain or refold protein structures (Shiu *et al*., 1977; Haas and Wabl, 1983; reviewed in Lee, 1992; reviewed in Lee, 2001). GRP78 is a part of the glucose regulated protein (GRP) family, which are generally located within the ER (Munro and Pelham, 1987; reviewed in Lee, 1992; reviewed in Lee, 2001). When a cell undergoes, for example, nutrient deprivation, the expression of ER regulated stress proteins such as GRP78 are upregulated due to the accumulation of misfolded proteins within the ER (Shiu *et al*., 1977; Li *et al*., 1993; Miles *et al*., 1994; reviewed in Lee, 2001) in a process known as the UPR, which if severe enough can result in apoptosis (Patil and Walter, 2001; Ma and Hendershot, 2001).

There are several studies that show that GRP78 is expressed in the heart under cellular insults such as hypoxic insult and IR injury (Sun *et al*., 2008; Hardy and Raiter, 2010; Xin *et al*., 2011; Martindale *et al*., 2006). Therefore looking at its expression within cardiac tissue in order to investigate if it can protect the myocardium from injury during cardiac surgery with CPB could be beneficial.

3.4.2. Materials and Methods

3.4.2.1 Experiment 1: Screening of differential gene expression in ventricular myocardium excised from cyanotic and control (acyanotic) patient groups.

Ischemic myocardial tissue samples (LV and RV) excisied from cyanotic and control patients that have not been exposed to any type of experimental conditions were screened in

order to establish any differential expression of GRP78 within the myocardium (for primer sequences see section 2.7.3) in both the cyanotic and control patient groups. 12 patients were recruited (please see table 6 in section 3.1.2). Seven patients were cyanotic and were comprised of TOF and FV patients. Five were control (acyanotic) patients, comprising of SAS and TA patients.

qPCR (see section 2.9) was used to investigate changes in expression and any differences seen in gene expression were calculated using the Comparative Ct method (see section 2.9.2) to give RQ of gene expression using the Stepone Software® v2.1 (Applied Biosystems).

Statistical analysis was performed using GraphPad Prism 6 Software©. The data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. Therefore the test used was the Mann-Whitney U test where data was expressed by the median and the interquartile range. A p-value of <0.05 (two-tailed) is considered as statistically significant.

There was no significant difference in GRP78 expression when comparing cyanotic and acyanotic patients (figure 3-24)

Figure 3-24. Figure shows the expression of GRP78 in both the cyanotic and acyanotic patient groups.

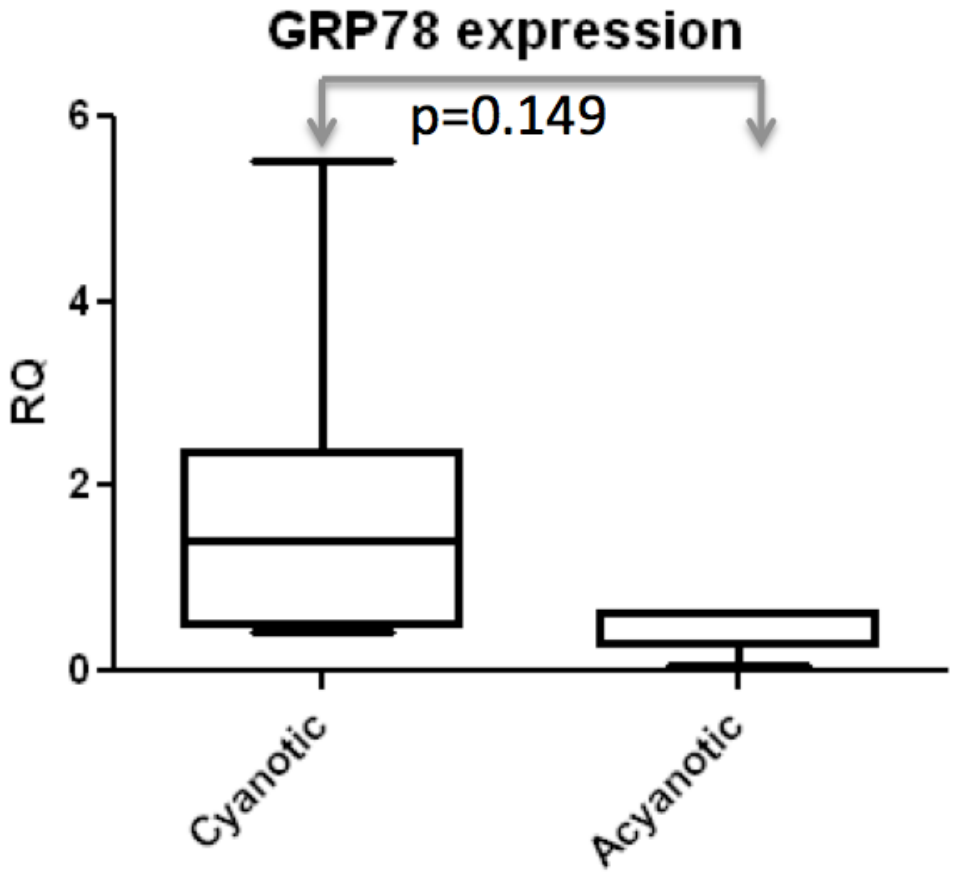


Figure shows comparison in the expression of GRP78 in both the cyanotic (n=7) and acyanotic (n=5) patient groups. Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression using the StepOne Software® v2.1 (Applied Biosystems). Statistical analysis was done using the Mann-Whitney U test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

3.4.2.2 Experiment 2: Reperfusion Experiment - exposing myocardial tissue to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2

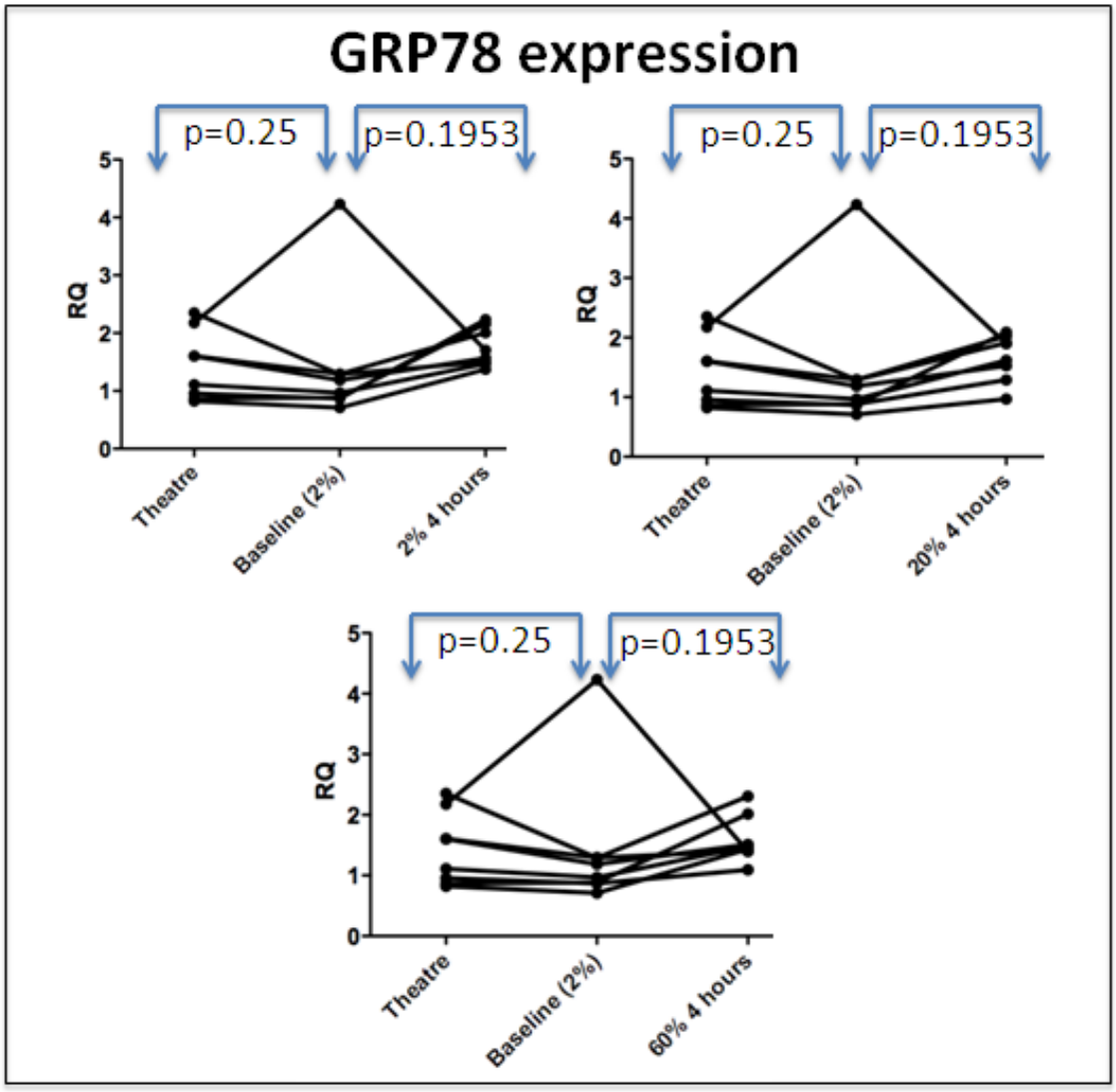
The aim of this experiment was to investigate the effect of exposing ischemic heart tissue, taken from patients undergoing corrective cardiac surgery with CPB for congenital heart defects, to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2 (see section 2.3) in order to recreate reperfusion that occurs in the clinical setting of paediatric open-heart surgery. This was done in order to investigate the effect this had on the differential gene expression of GRP78 using qPCR (see section 2.9). RQ of gene expression was calculated using the comparative C_t method (see section 2.9.2). Ten patients (please see table 11 in section 3.3.2.2) were recruited for this study, consisting of TOF, aortic obstruction and VSD patients.

It should be noted that patients 38 and 39 do not have a theatre (A) or baseline (B) RQ value due to limited tissue availability therefore because paired statistical analysis was carried out, these patient values were not included. All patients recruited for this study were taken as one group.

Statistical analysis was performed using GraphPad Prism 6 Software©. The data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. Therefore the Wilcoxon matched-pairs signed rank test was used to look for any statistical differences in expression for GRP78 due to the exposure to differential O_2 levels. A p-value of <0.05 (two-tailed) is considered as statistically significant.

There was no significant change in the expression of GRP78 upon exposure to 2% (hypoxia), 20% (normoxia) or 60% (hyperoxia) O_2 (figure 3-25).

Figure 3-25. Figure showing the differential expression of GRP78 upon exposure to 2% O_2, 20% O_2 and 60% O_2 for 4 hours.



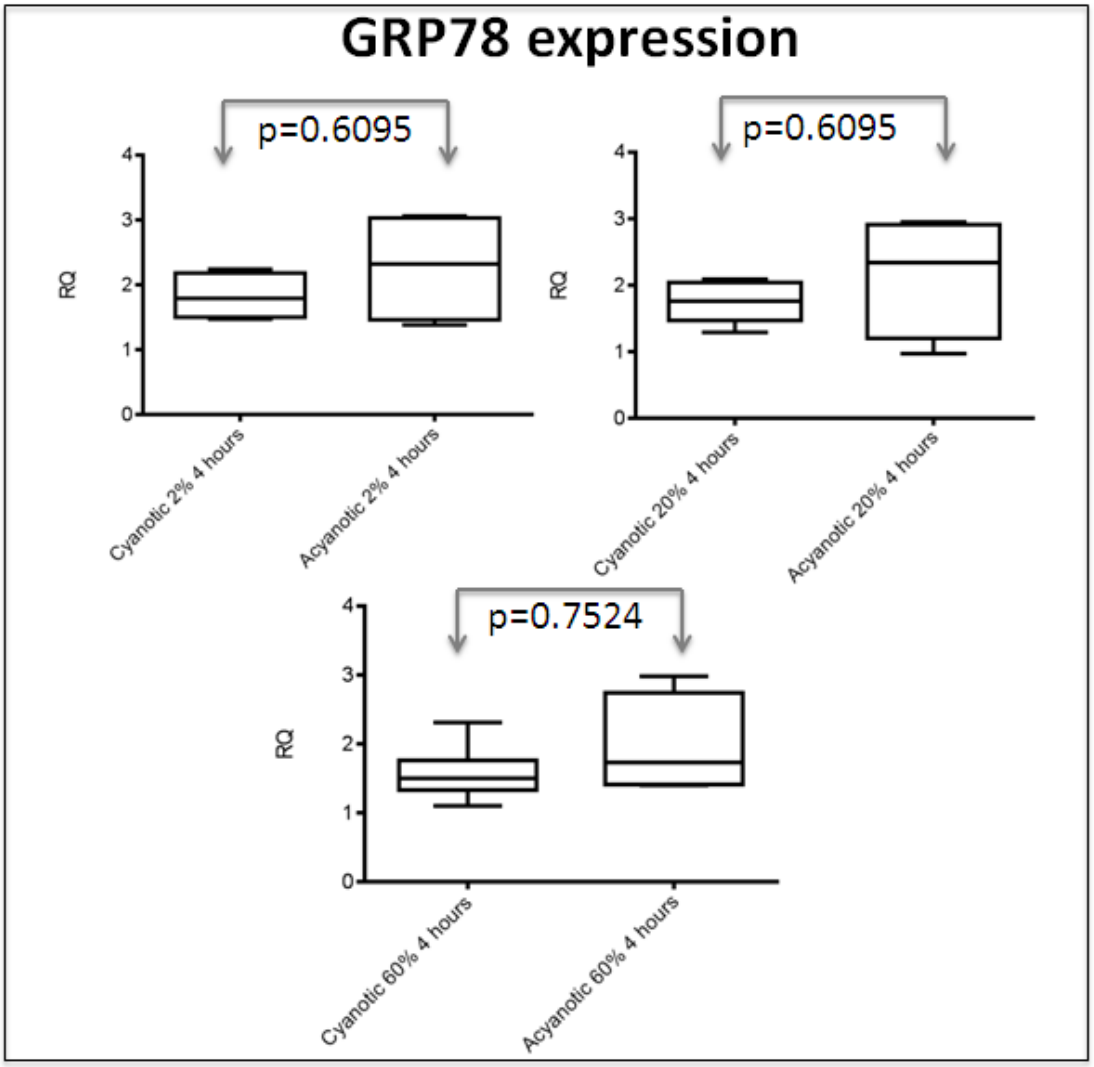
The three points on the x-axis show GRP78 expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Comparison of cyanotic and acyanotic patients:

Data from patients 38 and 39 has not currently been used in the analysis because they have no baseline values (see section 3.3.2.2). This has meant that cyanotic and acyanotic patients groups were unable to be compared. There is a full data set for the time points C (2% 4 hours), D (20% 4 hours) and E (60% 4 hours) (see section 2.2) for patients 38 and 39. It is therefore possible to compare these 3 groups in cyanotic patients compared to cyanotic patients. The patients recruited have been grouped into the cyanotic and acyanotic group (see table 12 in section 3.3.2.2).

Statistical analysis was performed using GraphPad Prism 6 Software©. The data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. Therefore the test used was the Mann-Whitney U test where data was expressed by the median and the interquartile range. A p-value of <0.05 (two-tailed) is considered as statistically significant. This comparison was done for GRP78 (figure 3-26). There was no statistically significant difference seen for GRP78 upon exposure to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours) when comparing the cyanotic group with the acyanotic group.

Figure 3-26. Differential expression of GRP78 seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



The two points on the x-axis show GRP78 expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Summary of main findings:

- Experiment 1:
  - No significant difference in GRP78 mRNA expression between the cyanotic and acyanotic patient groups.
- Experiment 2:
  - No significant difference in GRP78 mRNA expression seen when exposed to 20% or 60% O_2 for 4 hours.
  - No significant differences in GRP78 mRNA expression seen during 4 hours differential O_2 exposure between the cyanotic and acyanotic patient groups.

3.5 The differential expression of APJ and its endogenous ligand Apelin

3.5.1 Introduction

APJ (see section 1.8) is part of the trans-membrane G protein-coupled receptor protein family and its cognate ligand is known as Apelin (O'Dowd *et al*., 1993; Tatemoto *et al*., 1998). There have been some studies that have supported the role of aplein and APJ as vasodilators (Lee *et al*., 2000; Tatemoto *et al*., 2001; Cheng *et al*., 2003). Other studies implicated a hypothalamic role for Apelin (De Mota *et al*., 2000; Reaux *et al*., 2001; Taheri *et al*., 2002; De Mota *et al*., 2004; Reaux-Le Goazigo *et al*., 2004).

APJ and Apelin are expressed in cardiac tissue (Kleinz and Davenport, 2004; Kleinz *et al*., 2005). In relation to cardiac function several studies have been shown them both to help improve cardiac contractibility (Szokodi *et al*., 2002; Berry *et al*., 2004; Ashley *et al*., 2005). This could be beneficial during times of stress that can be caused during cardiac surgery with CPB. Also, there have been several studies that have shown both APJ and Apelin expression play a role in the heart's response to cellular stress and also that they can be cardio-protective (Sheikh *et al*., 2007; Jia *et al*., 2006; *Atluri et al*., 2007). All of this evidence from the literature highlights the fact that APJ was a suitable candidate for investigation in order to help improve the protection of the heart during cardiac surgery with CPB.

3.5.2 Methods and Results

3.5.2.1 Experiment 1: Screening of differential gene expression in ventricular myocardium excised from cyanotic and control (acyanotic) patient groups

Ischemic myocardial tissue samples (LV and RV) excised from cyanotic and control patients that have not been exposed to any type of experimental conditions were screened in order to establish any differential expression of APJ in the myocardium (for primer sequences

see section 2.7.3) in both the cyanotic and control patient groups. 12 patients were recruited (please see table 6 in section 3.1.2). Seven patients were cyanotic and were comprised of TOF and FV patients. Five were control (acyanotic) patients, comprising of SAS and TA patients.

qPCR (see section 2.9) was used to investigate changes in expression and any differences seen in gene expression was calculated using the Comparative Ct method (see section 2.9.2) to give RQ of gene expression using the Stepone Software® v2.1 (Applied Biosystems).

Statistical analysis was performed using GraphPad Prism 6 Software©. The data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. Therefore the test used was the Mann-Whitney U test where data was expressed by the median and the interquartile range. A p-value of <0.05 (two-tailed) is considered as statistically significant.

There was no significant difference in APJ expression when comparing cyanotic and acyanotic patients (figure 3-27)

Figure 3-27. Figure shows the expression of APJ in both the cyanotic and acyanotic patient groups.

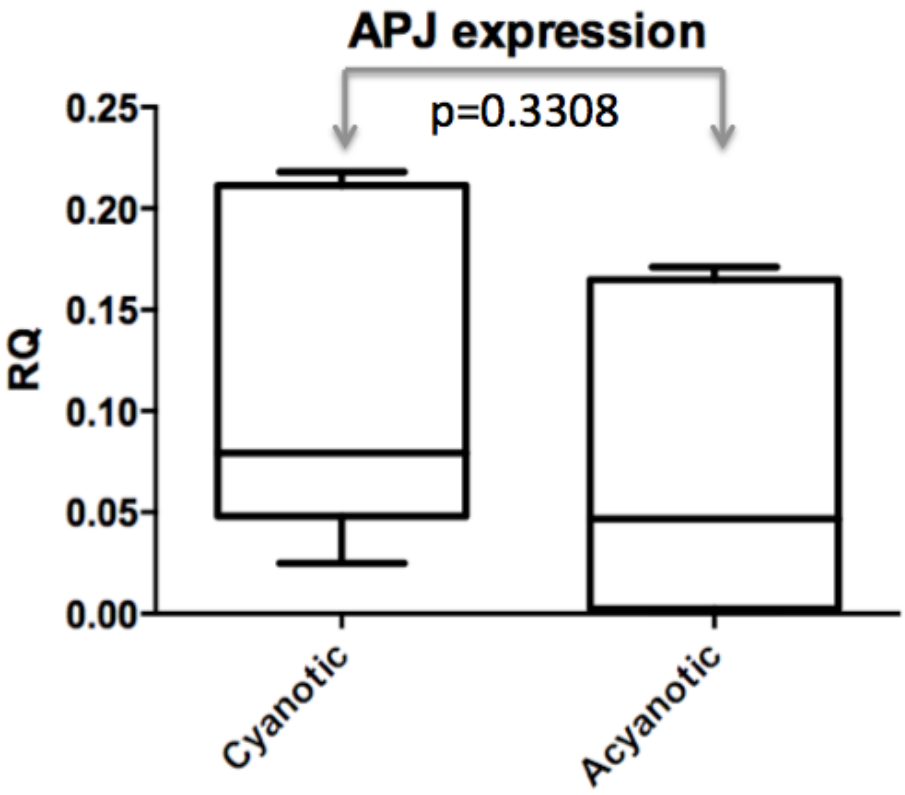


Figure shows comparison in the expression of APJ in both the cyanotic (n=7) and acyanotic (n=5) patient groups. Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give RQ of gene expression using the StepOne Software® v2.1 (Applied Biosystems). Statistical analysis was done using the Mann-Whitney U test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

3.5.2.2 Experiment 2: Reperfusion Experiment - exposing myocardial tissue to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2

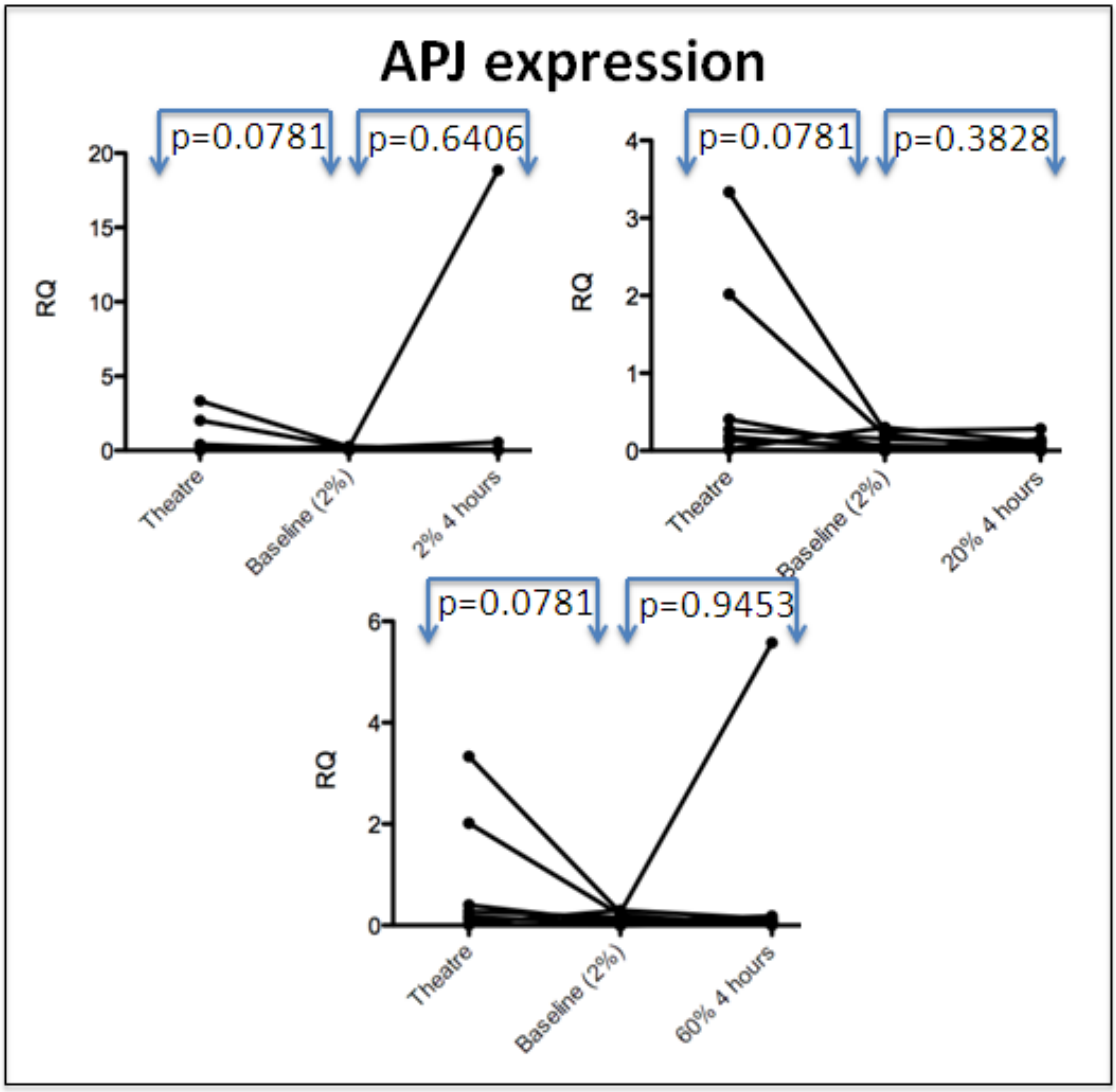
The aim of this experiment was to investigate the effect of exposing ischemic heart tissue, taken from patients undergoing corrective cardiac surgery with CPB for congenital heart defects, to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2 (see section 2.3) in order to recreate reperfusion that occurs in the clinical setting of paediatric open-heart surgery. This was done in order to investigate the effect this had on the differential gene expression of APJ using qPCR (see section 2.9). RQ of gene expression was calculated using the comparative C_t method (see section 2.9.2). Ten patients (please see table 11 in section 3.3.2.2) were recruited for this study, consisting of TOF, aortic obstruction and VSD patients.

It should be noted that patients 38 and 39 do not have a theatre (A) or baseline (B) RQ value due to limited tissue availability therefore because paired statistical analysis was carried out, these patient values were not included. All patients recruited for this study were taken as one group.

Statistical analysis was performed using GraphPad Prism 6 Software©. The data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. Therefore the Wilcoxon matched-pairs signed rank test was used to look for any statistical differences in expression for APJ due to the exposure to differential O_2 levels. A p-value of <0.05 (two-tailed) is considered as statistically significant.

There was no significant change in the expression of APJ upon exposure to 2% (hypoxia), 20% (normoxia) or 60% (hyperoxia) O_2 (figure 3-28).

Figure 3-28. Figure showing the differential expression of APJ upon exposure to 2% O_2, 20% O_2 and 60% O_2 for 4 hours.



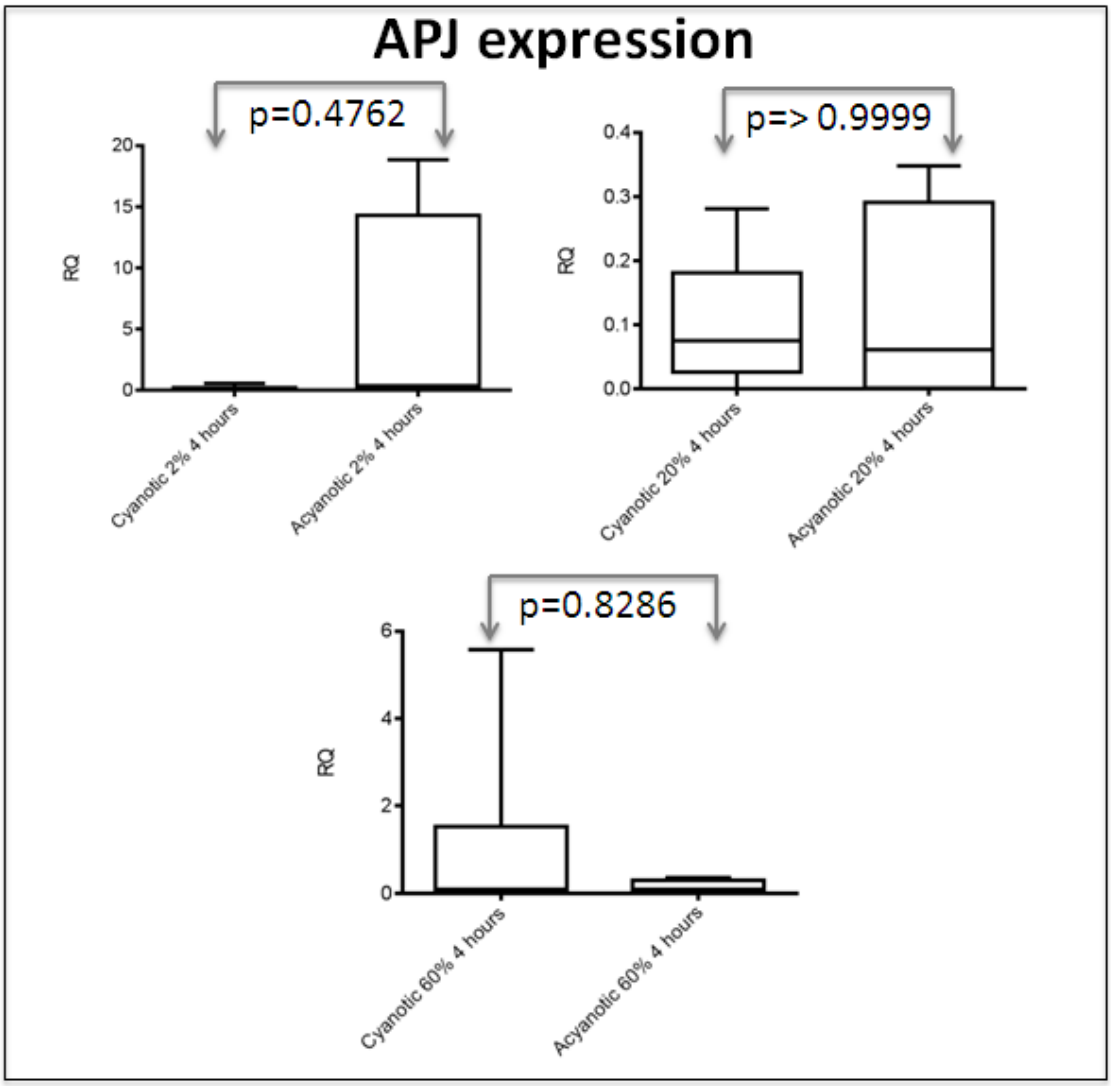
The three points on the x-axis show APJ expression seen through from collection in theatre, in the 2% O_2 media used for transport of tissue from theatre to the laboratory (baseline 2%) and the expression seen after the 4 hour incubation period (2/ 20/ 60% 4 hours). Samples taken from each patient (n=8) were used in each group. Data is shown as line and symbol graphs (each sample's data is plotted separately). Expression was calculated using the Comparative Ct method to give RQ of gene expression. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and a p-value of <0.05 (two-tailed) is considered as statistically significant.

Comparison of cyanotic and acyanotic patients:

Data from patients 38 and 39 has not currently been used in the analysis because they did not have baseline values (see section 3.3.2.2). This has meant that cyanotic and acyanotic patients groups were unable to be compared. There is a full data set for the time points C (2% 4 hours), D (20% 4 hours) and E (60% 4 hours) (see figure 2.2) for patients 38 and 39. It is therefore possible to compare these 3 groups in cyanotic patients compared to cyanotic patients. The patients recruited have been grouped into the cyanotic and acyanotic group (see table 12 in section 3.3.2.2).

Statistical analysis was performed using GraphPad Prism 6 Software©. The data did not follow a normal distribution therefore non-parametric statistical analysis was undertaken. Therefore the test used was the Mann-Whitney U test where data was expressed by the median and the interquartile range. A p-value of <0.05 (two-tailed) is considered as statistically significant. This comparison was done for APJ (figure 3-29). There was no statistically significant difference seen for APJ upon exposure to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours) when comparing the cyanotic group with the acyanotic group.

Figure 3-29. Differential expression of APJ seen in cyanotic patients compared to the acyanotic patients when exposed to hypoxia (2% 4 hours), normoxia (20% 4 hours) and hyperoxia (60% 4 hours).



The two points on the x-axis show APJ expression seen in cyanotic patients (n=6) compared to the acyanotic patients (n=4) group when exposed to hypoxia (2% 4 hours) (top left), normoxia (20% 4 hours) (top right) and hyperoxia (60% 4 hours) (bottom). Data is shown as box and whiskers plots (median, interquartile ranges). Expression was calculated using the Comparative Ct method to give relative quantification (RQ) of gene expression. Statistical analysis was performed using the Mann-Whitney U test (p-value of <0.05 is considered as statistically significant) and p values are shown.

Summary of main findings:

- Experiment 1:
  - No significant difference in APJ mRNA expression between the cyanotic and acyanotic patient groups.
- Experiment 2:
  - No significant difference in APL mRNA expression seen when exposed to 20% or 60% O_2 for 4 hours.
  - No significant differences in APJ mRNA expression seen during 4 hours differential O_2 exposure between the cyanotic and acyanotic patient groups.

3.6 Decay curve experiments

3.6.1 Introduction

The aim of these experiments was to investigate how the expression patterns of certain genes of interest within the infant myocardium were affected by varying incubations times. This was performed in order to illustrate whether changes of expression seen in the results were from the conditions of the experiments or if it was due to the time period of incubation.

3.6.2 Methods and Results

Myocardial tissue (ischemic) samples collected from three cyanotic patients (table 13) were incubated for 1.5 hours, 3 hours, 4 hours and 6 hours at 20% O_2 at 37°C (see section 2.14). In order to establish any differential expression due to incubation time only, the type of patient and O_2 exposure were kept the same. Also, changes in expression were quantified by using qPCR (see section 2.9). RQ of gene expression was calculated using the comparative C_t method (see section 2.9.2). The genes of interest are the inflammatory mediators TLR4, TLR2 and MYD88 and the stress proteins HSP20, HSP27, HSP32, HSP60, HSP72 and HSP90. It was not possible to do statistical analysis for this experiment. It should be noted that for patient 43, there was no 4 hour timepoint because RNA extraction was unsuccessful for this sample. Also, for patient 47 there was no baseline and 3 hour timepoint as there was less tissue available for this patient. Therefore the most important timepoints were chosen, specifically 1.5 hours, 4 hours and 6 hours.

Table 13. Patients recruited for decay curve experiment

Patient	Type	Group	Gender	Age
42	TOF	Cyanotic	Male	1 year 7 months
43	TOF	Cyanotic	Male	2 years 5 months
47	TOF	Cyanotic	Female	0 years 8 months

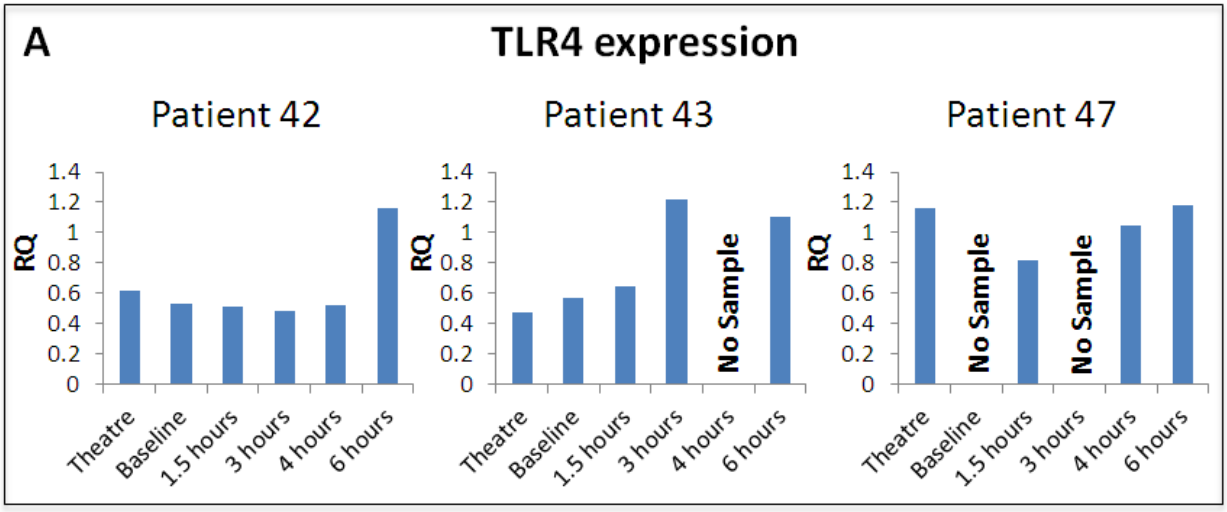
Inflammatory mediators:

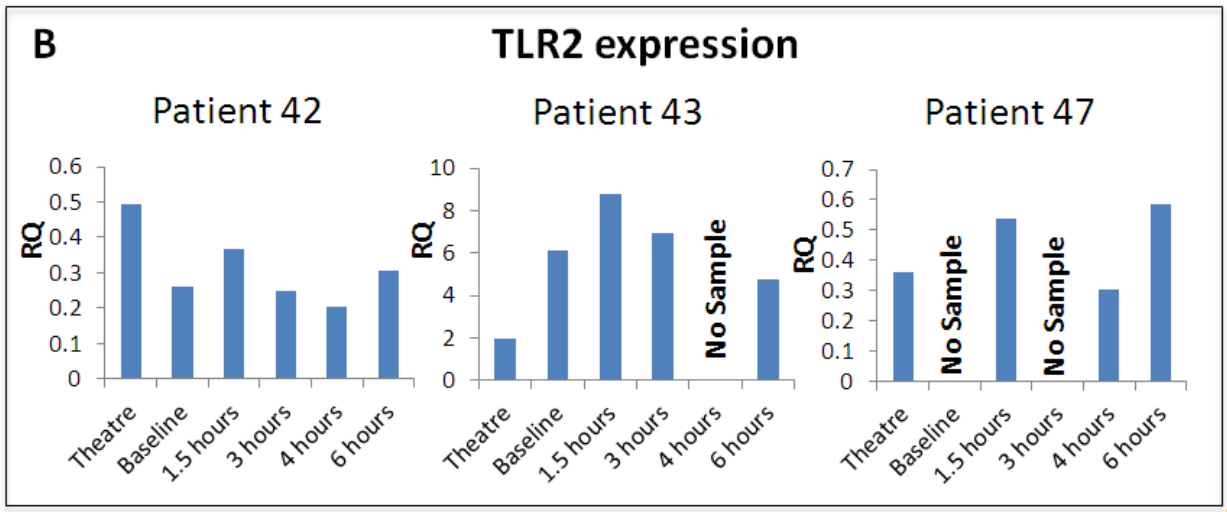
The expression of TLR4, TLR2 and MYD88 were investigated (figure 3-30). There was no obvious pattern in expression over time in the incubator of any of these genes.

Stress proteins:

The expression of HSP20, HSP27, HSP32 (figure 3-31), HSP60, HSP72 and HSP90 were investigated (figure 3-32). HSP20 and HSP27 did not have any particular trends in expression over time. HSP32 appeared to have an increase in expression at about 4-6 hours of incubation. HSP72, HSP72 and HSP90 all appeared to have slight trend of increase as time of incubation increased. Such changes seen did not appear to be significant. The observational nature of these results and the fact only 3 patients were used, some of which had absent time points, means statistical analysis was not possible.

Figure 3-30. Figure showing the differential expression of inflammatory mediators during differential incubation times. A shows TLR4, B shows TLR2 and C shows MYD88.





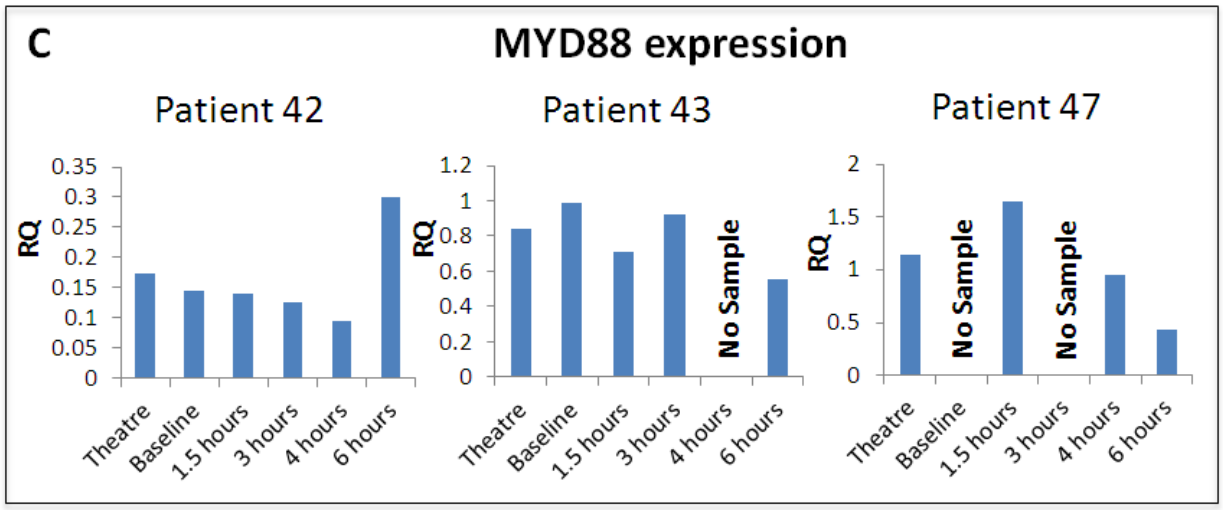
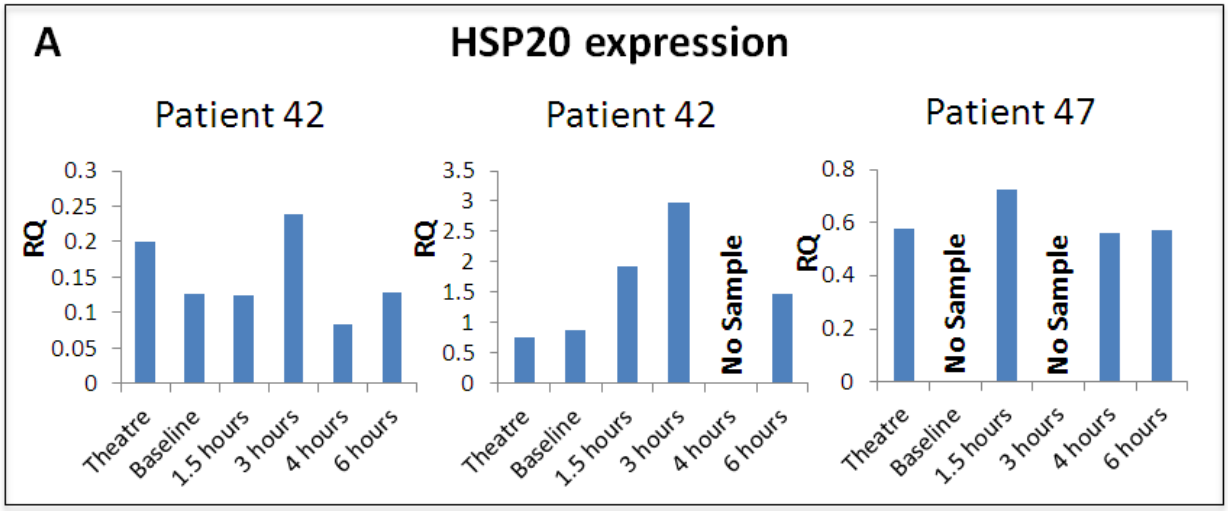
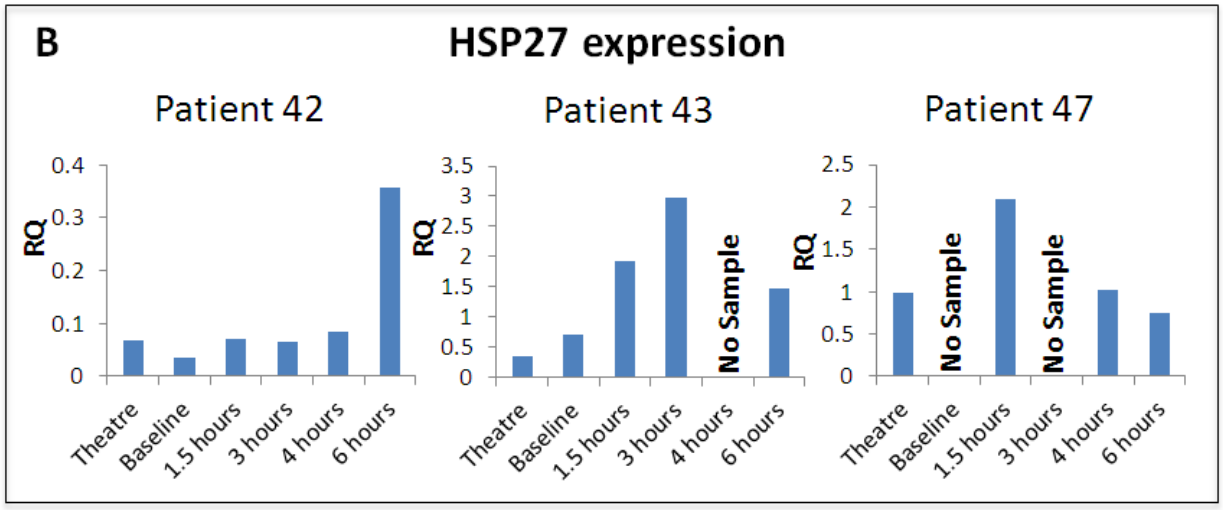


Figure showing the differential expression of TLR4 (A), TLR2 (B) and MYD88 (C) when infant myocardium (n=3) is exposed to differential incubation times. Data is shown as bar graphs. The graphs show the expression seen at the time of collection in theatre (theatre), at the time of the start of the incubation back in the laboratory (baseline), after 1.5 hours incubation (1.5 hours), after 3 hours incubation (3 hours), after 4 hours incubation (4 hours) and after 6 hours incubation (6 hours). Expression was calculated using the Comparative Ct method to give RQ of gene expression.

Figure 3-31. Figure showing the differential expression of stress proteins during differential incubation times. A shows HSP20, B shows HSP27 and C shows HSP32.





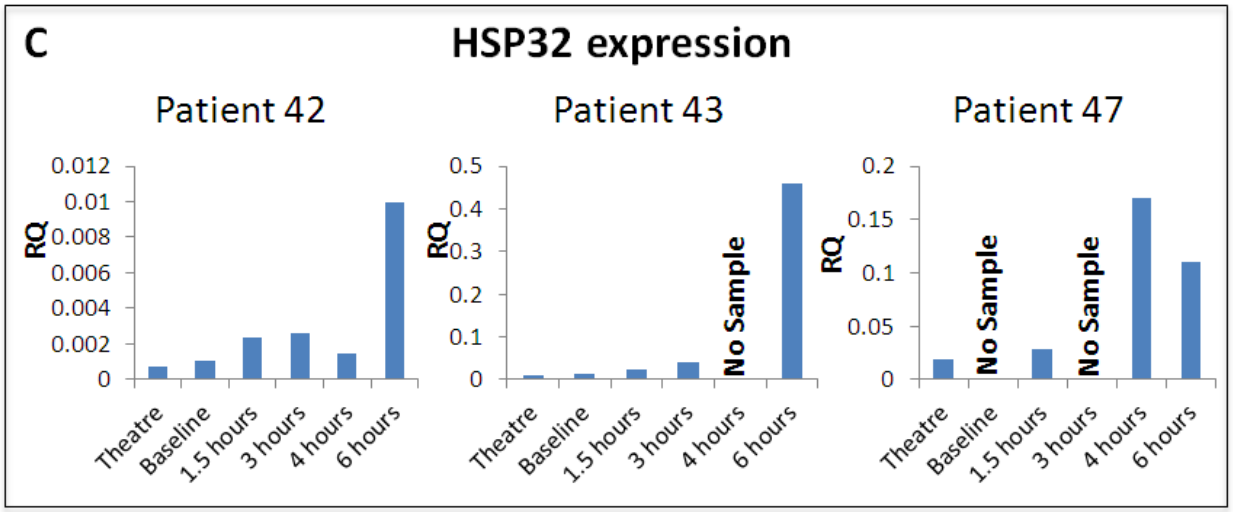
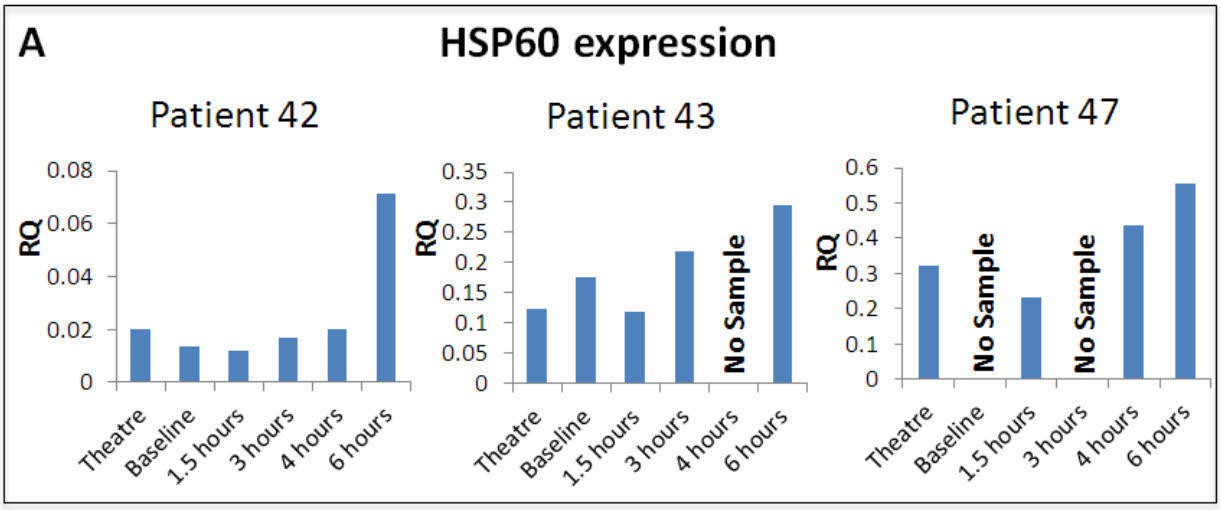
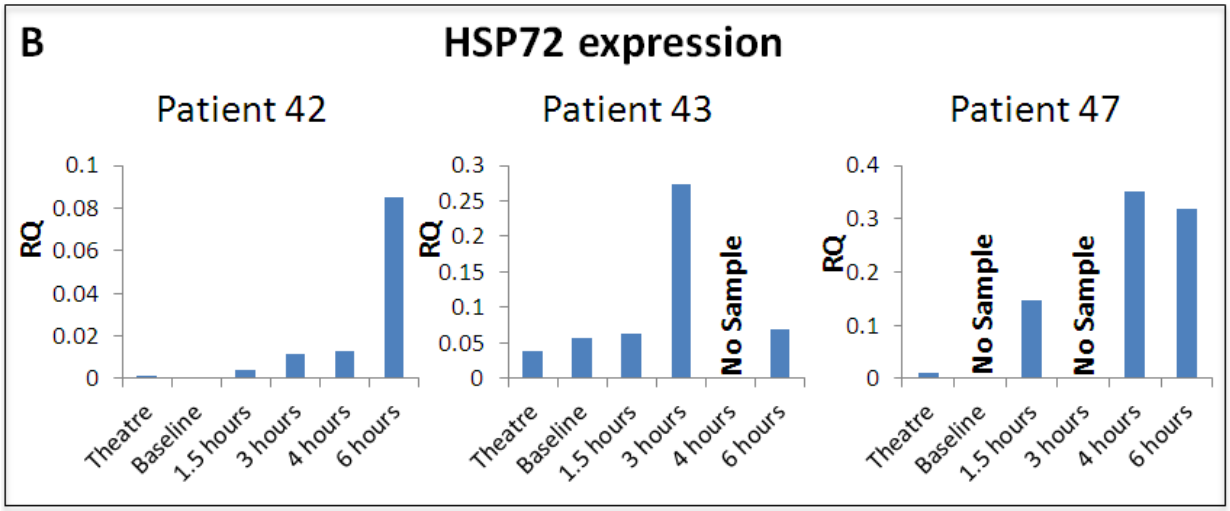


Figure showing the differential expression of HSP20 (A), HSP27 (B) and HSP32 (C) when infant myocardium (n=3) is exposed to differential incubation times. Data is shown as bar graphs. The graphs show the expression seen at the time of collection in theatre (theatre), at the time of the start of the incubation back in the laboratory (baseline), after 1.5 hours incubation (1.5 hours), after 3 hours incubation (3 hours), after 4 hours incubation (4 hours) and after 6 hours incubation (6 hours). Expression was calculated using the Comparative Ct method to give RQ of gene expression.

Figure 3-32. Figure showing the differential expression of stress proteins during differential incubation times. A shows HSP60, B shows HSP72 and C shows HSP90.





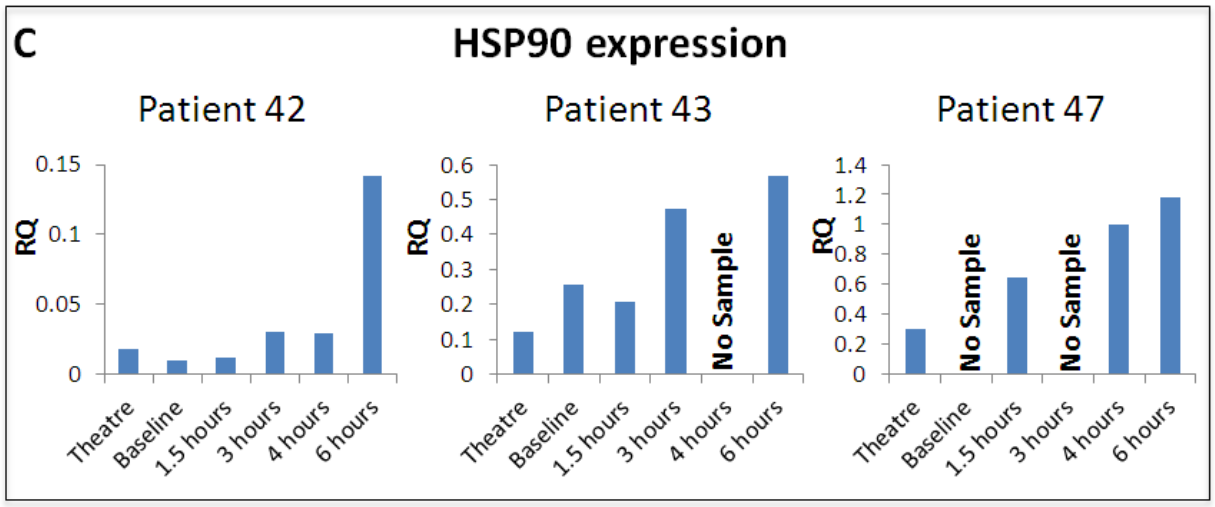


Figure showing the differential expression of HSP20 (A), HSP27 (B) and HSP32 (C) when infant myocardium (n=3) is exposed to differential incubation times. Data is shown as bar graphs. The graphs show the expression seen at the time of collection in theatre (theatre), at the time of the start of the incubation back in the laboratory (baseline), after 1.5 hours incubation (1.5 hours), after 3 hours incubation (3 hours), after 4 hours incubation (4 hours) and after 6 hours incubation (6 hours). Expression was calculated using the Comparative Ct method to give RQ of gene expression.

Summary of main findings:

- Experiment 1:
  - Only mRNA expression pattern of note was seen for HSP32, but this was not significant enough to cause a problem.

3.7 Association of HSP-27 expression with improved right ventricle function suggests a protective effect in cyanotic Tetralogy of Fallot.

3.7.1 Introduction

TOF (see section 1.1.3) is the most common cyanotic congenital heart disease with an incidence of 4 per 10,000 live births. Complete repair can be performed with a low mortality risk (<2%) (Starr, 2010). Early outcome can be determined by RV function in the immediate post-operative period**.** In the 20 years after repair, 10% to 15% of patients will require a further operation on their RV outflow tract, most for limited exercise capacity, ventricular dysrhythmias or more severe symptoms of heart failure (Nollert *et al*., 1997). TOF is an example of a congenital heart condition in which the RV is exposed to chronic ischemia and

pressure overload. Patients have an increased risk of RV dysfunction following corrective surgery. The causes include residual haemodynamic load from PS or regurgitation, conduction abnormalities, residual RV outflow tract obstruction and direct surgical trauma. How adequate the myocardium is protected during surgical repair has been an area of concern. Pre-operatively the ventricle is exposed to increased systemic pressure. Furthermore CPB exposes the myocardium to ischemic stress/ hypoxia (during bypass) and potential reperfusion injury when O_2 is restored at super-physiological levels. Whether the chronically hypoxic (cyanotic) myocardium is more or less tolerant of injury compared to normoxic (non-cyanotic) patients is unclear. Free radical injury has been reported during surgery as well as a non-homegeneous distribution of cardioplegia in what is a hypertrophied RV mass (Imura *et al*. 2001).

The heart adapts to an adverse physiological environment by altering gene expression (Ghorbel *et al*. 2010). Cyanotic TOF is associated with higher mRNA expression of genes associated with apoptosis and remodeling and reduced expression of genes associated with myocardium contractility and function as well as genes that protect against re-oxygenation injury compared with non-cyanotic TOF (Willis and Patterson, 2010) and this could limit the ability of the RV to protect itself against ischemic injury. An increase in ventricular mass will increase the susceptibility to IR injury further. In TOF, very young patients are subjected to complex open-heart surgery along with CPB. The ability of this "infant" myocardium to protect itself against ischemic (and reperfusion) injury during aortic cross-clamping will determine post-operative outcome.

HSPs (see section 1.6) are a family of proteins that can be constitutively expressed in cells or induced by a variety of stress stimuli such as hypoxia, ischemia and pressure overloading (Willis and Patterson, 2010). The best described role for HSPs is maintaining the correct folding of proteins (Willis and Patterson, 2010). Although studied in other fields of medicine, the role of HSPs before, and following pediatric cardiac surgery, is not well understood. However, it is becoming clear from adult human and animal studies that HSPs may have an important role in protecting the damaged heart, which accumulates misfolded proteins (Schmitt *et al*., 2002; Taggart *et al*., 1997; Jayakumar *et al*., 2001; Hollander *et al*., 2004; Yet *et al*., 2001; Aloy *et al*., 2008; Franklin *et al*., 2005; Efthymiou *et al*., 2004 and (Knowlton *et al*., 1998).

Only a few of the smaller HSPs, including HSP27 (see section 1.6), are increased in cells subjected to stress (Efthymiou *et al*., 2004). HSP27 is involved in protein folding and in the apoptotic/ programmed cell death pathway. Only a small number of studies on HSP27 have been performed on the heart. These have been restricted mainly to animal studies and a few to adult disease. They do suggest that HSP27 is protective for the heart (Vander Heide, 2002).

The role of HSP27 in the infant heart in TOF or during CPB, which itself is an IR insult, is not known. Thus the aim of this study was to determine the expression of HSP27 protein in myocardium resected from both cyanotic and non-cyanotic TOF patients during surgical repair and to determine its relationship to RV function and clinical outcome.

3.7.2 Materials and Methods

3.7.2.1 Patients recruited

20 patients with TOF who underwent surgical repair at Royal Hospital for Sick Children, Glasgow were recruited for the study. Patients were divided into a cyanotic group (n=10) and non-cyanotic group (n=10) based on preoperative saturations at rest <90% and >90% at room air respectively. A separate control group of age-matched children (n=15) presenting with an innocent murmur (median age 27.1, interquartile range 4.0-47.2 months versus TOF patients, p=0.62) was recruited to obtain normal reference values for tissue Doppler myocardial velocities. Glasgow West Research Ethics Committee approved this study. Parental consent was obtained for all patients.

3.7.2.2 Peri-operative tissue Doppler Echocardiography

Tissue Doppler echocardiography was performed under general anesthesia as described previously (Peng *et al*., 2011) before skin incision and repeated on post-operative day one (POD1) in the intensive care unit (ICU) and then one week later. Pulsed-wave tissue Doppler velocity measurements at tricuspid annulus, basal septum and mitral annulus were obtained from apical 4-chamber view to quantify systolic myocardial velocity (S_m cms^{-1}). Three or more loops were stored for subsequent offline analysis. The presence of restrictive RV physiology was defined as antegrade flow across the pulmonary artery coincident with atrial systole in all respiratory cycles (parasternal short axis RV outflow tract (RVOT) view)

(Cullen *et al*., 1995).

3.7.2.3 Surgical repair and Myocardial Protection

Standard repair was performed as described previously (Peng *et al*., 2011) by VSD closure and resection of RV infundibular muscle via trans-tricuspid and trans-pulmonary routes using moderate hypothermia and blood cardioplegia. Prior to CPB, all patients received O_2 supplementation and fractional inspired O_2 was adjusted to maintain adequate oxygenation whilst avoiding hyperoxia (median P_aO_2 8.0kPa in the cyanotic group versus 12.1kP_a in the non-cyanotic group (p=0.2). Epicardial echo was routinely performed to exclude any residual lesion at the end of the procedure. Direct RV and LV pressures were measured intra-operatively post-repair.

3.7.2.4 Western Blot Analysis

All myocardium resected from the RV outflow tract, as part of the repair, was collected for protein analysis. Muscle was snap-frozen immediately in liquid N_2 following resection and stored at -70°C until required. Pieces of muscle were grouped according to the ischemic time point at collection. Protein concentrations of all samples collected were calculated using the Bradford protein assay described in section 2.10.2 and differential protein expression analysis of HSP27 was done by Western blot analysis as described in section 2.11. The primary antibody (HSP 27, New England Biolabs, number 2402, mouse monoclonal) was used at 1:1000). The secondary antibody was horseradish peroxidase conjugated donkey anti-mouse secondary antibody (Abcam, cat. no. ab6820, 1:1000).

The beginning of ischemic time was defined as the time when aortic cross-clamping was initiated. The ischemic baseline period was defined as 0-15 minutes after aortic cross-clamp and before repeat cardioplegia administration. Baseline HSP27 protein density was calculated from mean HSP27 expression in the RV myocardium resected during this period. This was considered to represent as close to the pre-operative HSP27 expression in the myocardium as possible. Subsequent muscle resected after this period was grouped together to determine any effect of ischemic time on HSP 27 expression.

3.7.2.5 Post-operative monitoring

Post-operatively, serum lactate level, mixed venous oxygen saturation (S_vO_2,%) and

Troponin-I were measured concurrently with echocardiography. S_vO_2 is a function of systemic arterial oxygen saturation (S_aO_2), which can be affected by various factors including right-to-left intra-cardiac shunt, lung pathology therefore oxygen extraction ratio [$OER=(S_aO_2-S_vO_2)/S_aO_2$] represents a better indicator of systemic perfusion (Martin and Shekerdemian, 2009]. O_2 saturation was measured from concurrent blood sampling taken from the peripheral arterial line and the central venous catheter in the superior vena cava (Martin and Shekerdemian, 2009). Inotrope score was calculated (Davouros *et al*., 2006) and duration of ventilation and length of ICU stay were recorded.

3.7.2.6 Statistical Analysis

Statistical analysis was performed following advice from a professional statistician using the Minitab® Statistical Software Release 14.2 (MinitabInc, Pennsylvania, US). Non-parametric data (expressed in median + interquartile range, Q1-Q3) was analysed using the Mann-Whitney U-test, Wilcoxon Matched Pair or Kruskal-Wallis test for continuous variables and Fisher exact test for categorical variables. The strength of relationship between two variables was tested using Spearman rank correlation (rho). $p < 0.05$ was considered to be statistically significant.

3.7.3 Results

Table 14 summarises the peri-operative characteristics of the two groups. There were no significant differences between the cyanotic and the non-cyanotic group with regard to age, gender and body weight. The cyanotic group had a significantly increased haematocrit level, lower O_2 saturation and thicker interventricular septal wall thickness. Cross-clamp time and bypass time were significantly increased in the cyanotic group however ventilation time and length of stay in critical care were similar.

Table 14. Peri-operative characteristics between Cyanotic and non-Cyanotic group.

	Cyanotic group (n=10)	**Non-cyanotic group (n=10)**	**p-value**	**95% CI of median difference**
PRE-OPERATIVE FACTORS				
Age (months)	22.1 (11.8-24.3)	16.4 (6.8-23.4)	0.36	(-3.8,16.3)
Gender (n, %)	6 males (60%)	5 males (50%)	1.00	-
Weight (kg)	9.2 (8.6-12.2)	10.5 (7.9-11.0)	0.94	(-2.2,3.2)
Haematocrit level %	47.0 (40.8-50.0)	38.1 (37.0-40.9)	0.01	(1.9,11.3)
O_2 saturation %	76 (68-86)	93 (91-97)	0.0002	(-26,-10)
BT shunt - BTS (n, %)	6, 60%	2, 20%	0.17	-
RV wall thickness (cm)	0.70 (0.58-0.83)	0.65 (0.57-0.82)	0.91	(-0.13,0.16)
IVS wall thickness (cm)	0.85 (0.70-0.94)	0.66 (0.54-0.75)	0.01	(0.08,0.34)
Sm - tricuspid annulus, cm/s	10.01 (7.28-11.55)	7.85 (7.49-10.13)	0.31	(-1.12,3.64)
Sm - basal septum, cm/s	4.93 (4.18-6.03)	4.69 (3.73-6.10)	0.53	(-1.52,0.82)
Sm - mitral annulus, cm/s	4.90 (3.33-6.23)	4.83 (3.95-5.63)	0.96	(-1.47,1.72)
INTRA-OPERATIVE FACTORS				
Cross-clamp time, minute	100.0 (75.0-130.0)	67.5 (61.8-76.0)	0.004	(-58.0,-8.0)
Bypass time, minute	218.5 (180.8-310.0)	114.5 (98.8-130.3)	0.003	(63.0,182.0)
Transannular Patch use (n, %)	6.60%	4. 40%	0.67	-
Subvalvar patch (n, %)	5.50%	2.20%	0.35	-
RV to systemic pressure ratio	0.79 (0.69-1.11)	0.72 (0.62-0.88)	0.41	(-0.12,0.35)
HSP 27 VALUES				
Median HSP 27 in baseline first 15 minute period (R.O.D.)	0.79(0.68-1.6)	0.71 (0.5-0.85)	0.03	
Median HSP 27 after the first15 minute period (R.O.D.)	0.83 (0.7-1.0)	0.72 (0.53-0.88)	0.48	
Median HSP 27i from XC period (R.O.D.) (<15+>15min)	0.82 (0.68-1.4)	0.72 (0.5-0.85)	0.01	
POST-OPERATIVE FACTORS (DAY 1)				
Troponin-I (ng/ml)	16.5(11.7-51.0)	11.1 (7.6-13.5)	0.04	(0.1,37.6)
Inotrope score	14.0 (9.5-16.3)	6.5 (0.8-14.9)	0.05	(0.0,13.0)
Haemoglobin (g/dl)	12.2 (11.4-14.0)	11.5 (10.4-13.6)	0.41	(-1.2,2.1)
SvO_2 %	65.4 (55.5-71.3)	66.5 (61.8-68.3)	0.91	(-9.0,7.0)
Oxygen extraction ratio, OER	0.33 (0.28-0.42)	0.32 (0.27-0.35)	0.68	(-0.1, 0.1)
Oxygen excess factor, Ω	3.1 (2.4-3.6)	3.1 (2.9-3.7)	0.68	(-0.9, 0.6)
Lactate	1.6 (0.9-1.9)	1.2 (0.88-1.35)	0.26	(-0.2,0.9)
Sm - tricuspid annulus, cm/s	3.60 (2.50-4.60)	4.08 (2.87-4.77)	0.85	(-1.56,0.93)
Sm - basal septum, cm/s	3.21 (2.39-4.48)	2.71 (2.13-4.66)	0.52	(-0.83,1.53)
Sm - mitral annulus, cm/s	5.21 (3.94-6.46)	3.98 (3.61-5.15)	0.10	(-0.24,2.46)
Restrictive RV physiology	2, 20%	3, 30%	1.00	-
Ventilation time hours	141.0 (58.7-195.2)	82.0 (19.1-121.3)	0.14	(-7.7,122.7)
Critical care stay days	7.8 (5.0-11.2)	5.0 (3.5-8.3)	0.27	(-2.3,6.1)

All data expressed in median (interquartile range). BTS (Blalock-Taussig shunt), RV (right ventricle), IVS (interventricular septum), Sm (systolic myocardial velocity, XC (cross clamp), SvO_2 (mixed venous oxygen saturation), ROD (relative optical density).

When compared to the control group with innocent murmur, TOF patients had significantly reduced systolic annular velocity pre-operatively at tricuspid (8.13 v 12.67 cms^{-1} $p<0.0001$), septum (4.80 v 7.58 cms^{-1} $p<0.001$) and mitral (4.83 v 7.52 cms^{-1} $p<0.001$). Pre-operative annular velocities were similar in the cyanotic and non-cyanotic TOF groups. At post-operative day 1, systolic myocardial velocity was reduced further in the tricuspid and basal septum in both the cyanotic group ($p<0.001$ and $p=0.02$ respectively) and non-cyanotic group ($p<0.001$ and $p=0.02$ respectively) compared to pre-operative velocities. No differences in post-operative annular velocities or the presence of restrictive RV physiology were found between the cyanotic and non-cyanotic group. No association was found between post-operative RV velocities (tricuspid and septal) and pre-operative O_2 saturation, haematocrit, cardiopulmonary bypass time, troponin release, surgical method, degree of pulmonary regurgitation and residual right ventricle outflow tract obstruction.

All TOF patient annular velocities improved one week after surgery compared to day 1 (tricuspid $p=0.01$, septum $p=0.004$, mitral $p=0.02$) but remained below the baseline velocities of healthy children.

3.7.3.1 Postoperative Troponin-I release and HSP-27.

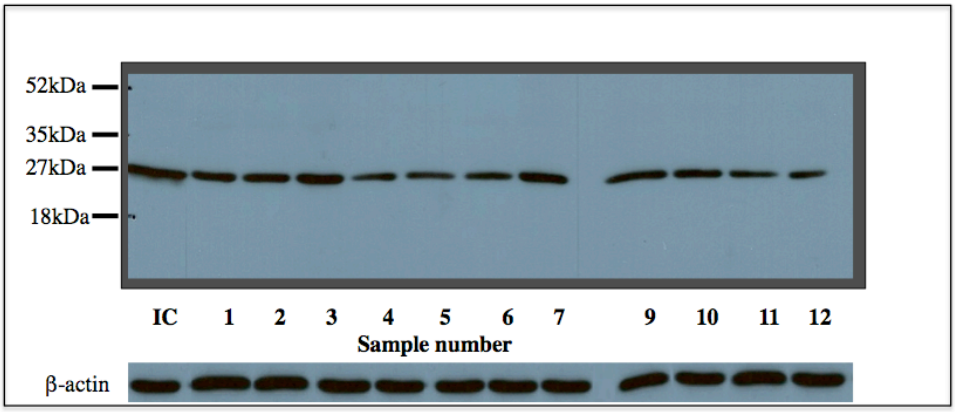
The cyanotic group released significantly more Troponin-I on post-operative day 1 compared to the non-cyanotic group (Table 14). Higher Troponin-I release was associated with a higher pre-operative haematocrit level ($p=0.003$), longer aortic cross-clamp time ($p=0.02$) and CPB time ($p=0.02$). There was no correlation between Troponin I and HSP27 or the weight of the total muscle harvested from each group.

3.7.3.2 HSP27 expression in tissue resected from RV outflow tract obstruction.

There was no statistical difference between the amount of ventricle muscle resected from the cyanotic compared with the non-cyanotic group (748.7 mg versus 583.6 mg) ($p=0.08$). Figure 3-33 is a Western blot showing HSP27 expression in samples resected from two patients (numbered 9 and 19) at different times following initiation of cross-clamp (patient 9 (lanes 1-7) and patient 19 (lanes 9-12). All muscle samples collected during the aortic cross-clamp period expressed HSP27. Also shown is the internal control and the

molecular weight markers used to confirm the HSP27 protein molecular weight was the expected 27 kDa.

Figure 3-33. Representative Western blot showing HSP27 expression in 2 different patients



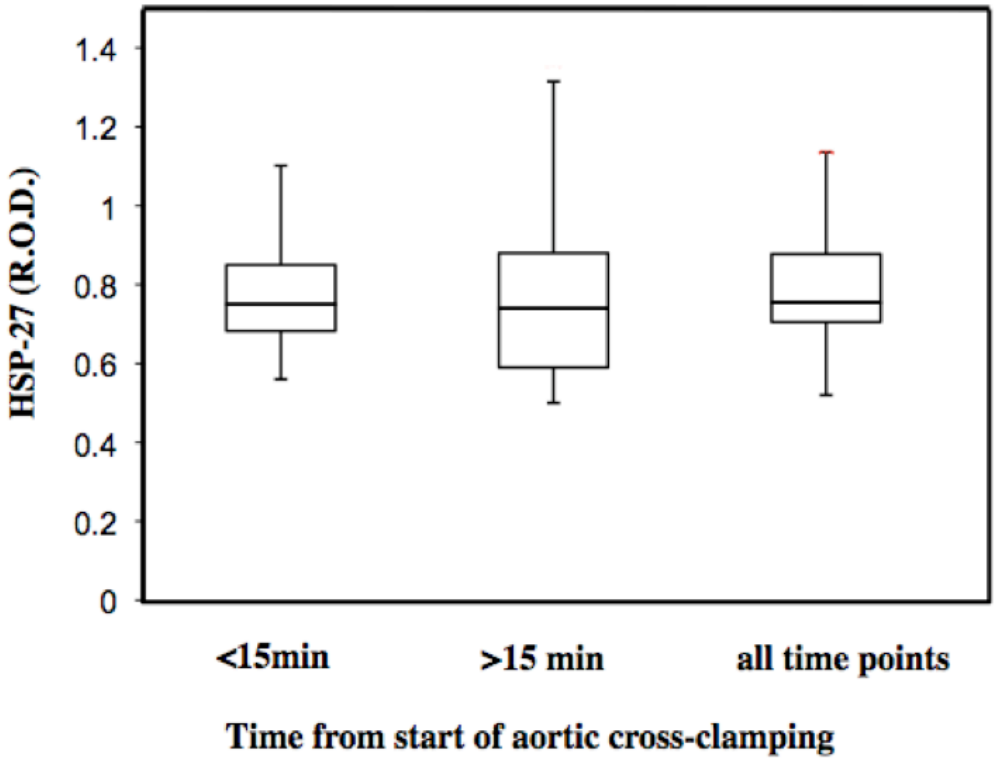
Western blot showing HSP27 expression in 2 different patients (patient 9 (lanes 1-7) and patient 19 (lanes 9-12) at time intervals from the start of cross-clamping. Patient 9 lane: 1, 10-15 minutes; 2, 15-20 minutes; 3, 20-25 minutes; 4, 30-35 minutes; 5, 35-40 minutes; 6, 40-45 minutes; 7, 45-50 minutes). Patient 19 lanes: 9, 5-10 minutes; 10, 10-15 minutes; 11, 15-20 minutes; 12, 20-25 minutes. IC (internal control human placenta tissue known to express HSP27). Molecular weight markers are shown on the left. 31.25μg total myocardial protein was loaded per lane.

When both cyanotic and non-cyanotic groups were combined and analysed for HSP27 levels before 15 minutes (baseline) or after 15 minutes there were no differences between any groups (Figure 3-34). The results for median and interquartile range for HSP27 expression were as follows: (i) for all samples collected during the first 15 minutes of aortic cross-clamp time were 0.75 (0.64-0.93), for samples collected after the first 15 minutes were 0.74 (0.58-0.93) and for all time points together 0.74 (0.61-0.93).

Next a separate analysis was performed for the cyanotic and non-cyanotic groups separately. The results are shown in Table 14 and Figure 3-35. There was a significant increase in HSP27 levels in the combined <15 minutes and >15 minutes cyanotic groups compared to the combined <15 minutes and >15 minutes non-cyanotic groups (p=0.01). When the <15 minutes and >15 minutes time points were analysed separately there was a significant

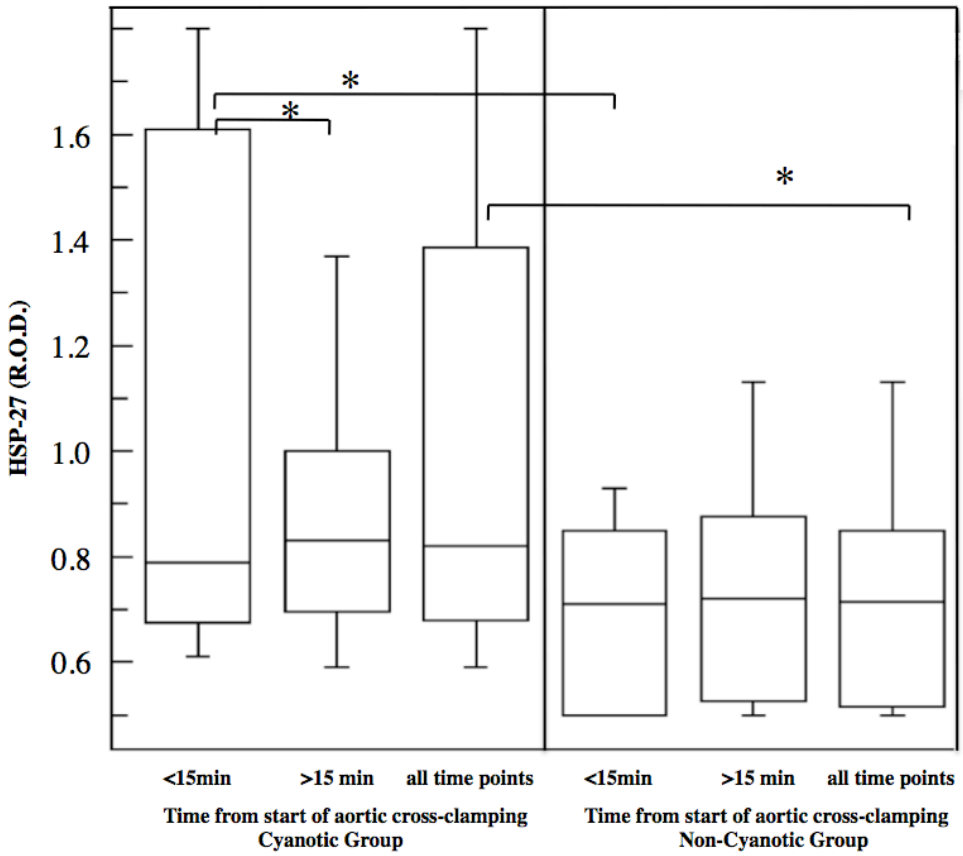
increase in HSP27 levels in the <15 minutes cyanotic compared to the <15 minutes non-cyanotic group (p=0.03). In the cyanotic group, HSP27 expression <15 minutes was significantly lower when compared to HSP27 experssion > 15 minutes (p<0.05). This was not seen in the non-cyantoic group. There was no significant relationship between baseline HSP27 levels and pre-operative variables i.e. age, weight, oxygen saturation, haematocrit and right ventricular wall thickness.

Figure 3-34. HSP27 expression (ROD) expression in all patients (cyanotic and non-cyanotic)



Box and whiskers plot (median, interquartile-range) of HSP27 (ROD) expression in all patients (cyanotic and non-cyanotic) grouped by time interval collected during aortic cross-clamping time (p>0.05).

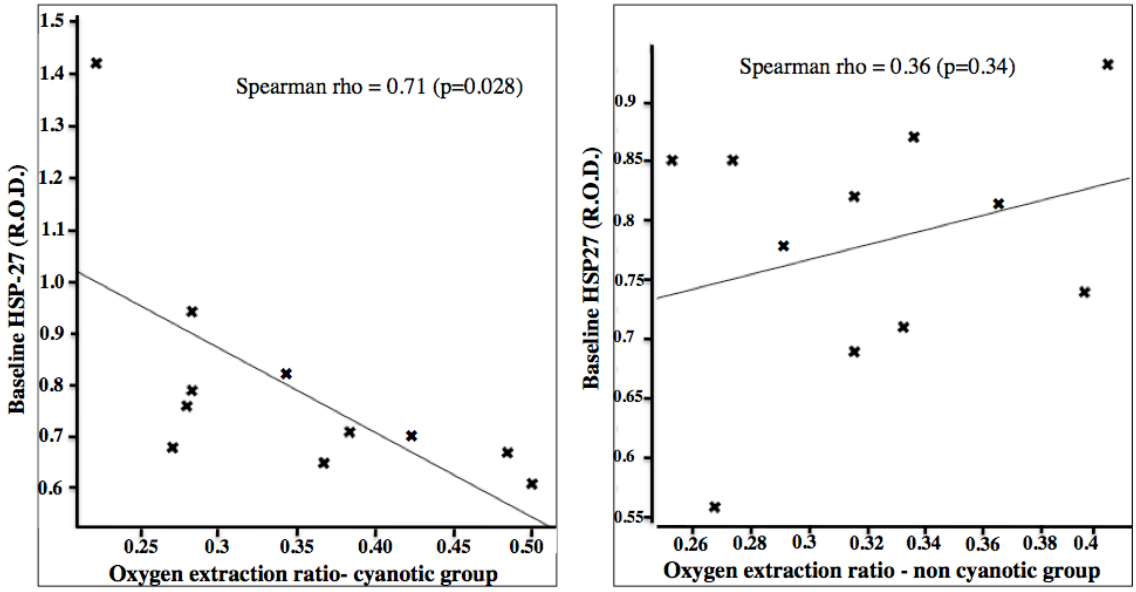
Figure 3-35. HSP27 expression (ROD) expression in the cyanotic (left panel) and non-cyanotic patients (right panel)



Box and Box and whiskers plot (median, interquartile-range) of HSP27 (ROD) expression in the cyanotic (left panel) and non-cyanotic patients (right panel) grouped by time interval collected during aortic cross-clamping time. (*$p<0.05$)

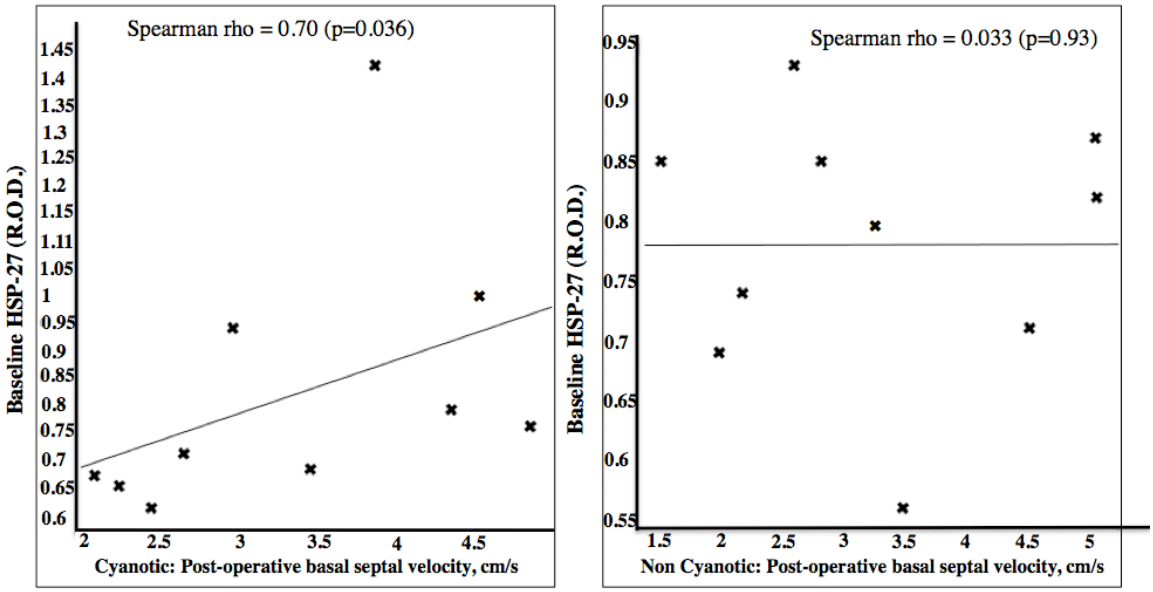
In the cyanotic group baseline HSP27 levels (tissue collected during the first 15 minutes of aortic cross clamp (and the closest representation of pre-operative status) correlated highly with oxygen extraction ratio. The results are shown in Figure 3-36 (p=0.028). In contrast this relationship was not seen in the non-cyanotic group (p=0.34). In the cyanotic group baseline HSP27 levels also significantly correlated with post-operative basal septal velocity (Figure 3-37, p=0.036) and again this relationship was not seen in the non-cyanotic group (p=0.93). There was no correlation of HSP27 with tricupsid or mitral annular velocity. Figure 3-38 shows that HSP27 also significantly correlated with better mixed venous oxygen saturation in the cyanotic group (p=0.02) but not the non-cyanotic group (p=0.93). None of the above associations were found when the analyses were performed on tissues collected after 15 minutes from the start of cross-clamping time. No other associations were found.

Figure 3-36. Oxygen extraction ration and baseline HSP27 expression



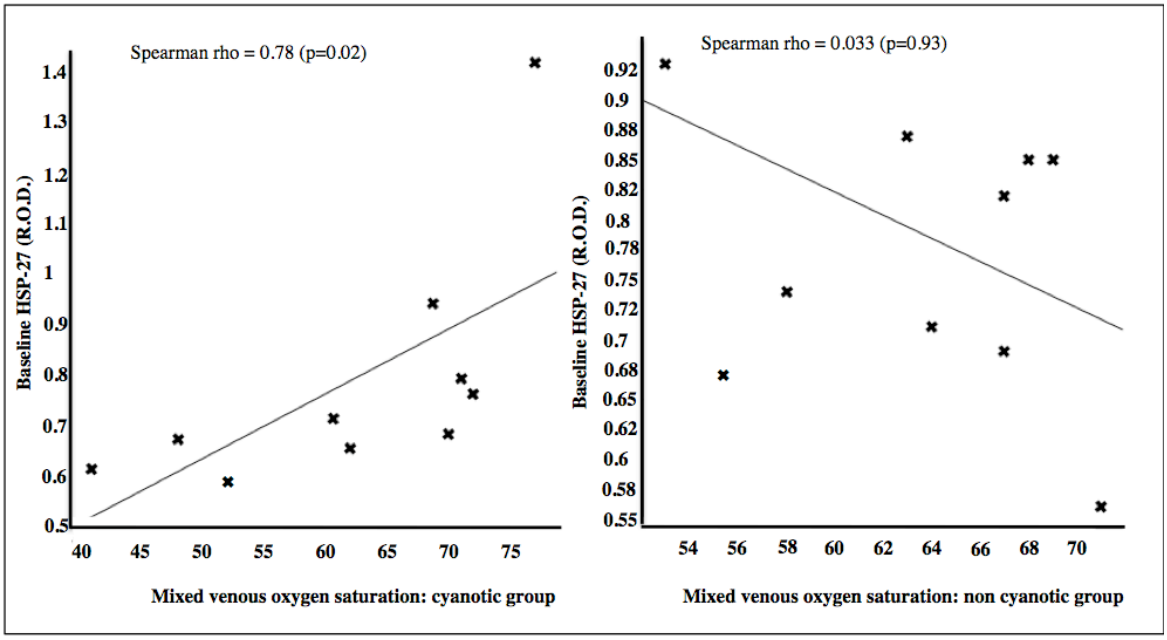
Scatter plot of oxygen extraction ration and baseline HSP 27 in cyanotic patients (left) and non-cyanotic (right).

Figure 3-37. Post-operative (Day 1) basal septal velocity and baseline HSP27 expression



Scatter plot of Post-operative (Day 1) basal septal velocity and baseline HSP 27 in cyanotic patients (left) and non-cyanotic (right).

Figure 3-38. Mixed venous oxygen saturation and baseline HSP27 expression



Scatter plot of mixed venous oxygen saturation and baseline HSP 27 in cyanotic patients (left) and non-cyanotic (right).

Summary of main findings:

- There was a significant increase in HSP27 protein expression levels in the combined <15 minutes and >15 minutes cyanotic groups compared to the combined <15 minutes and >15 minutes non-cyanotic groups (p=0.01).
- When the <15 minutes and >15 minutes time points were analysed separately there was a significant increase in HSP27 protein expression levels in the <15 minutes cyanotic compared to the <15 minutes non-cyanotic group (p=0.03).
- In the cyanotic group, HSP27 baseline protein expression (<15 minutes) was significantly lower when compared to HSP27 protein expression after 15 minutes ($p<0.05$).
- In the cyanotic group baseline HSP27 protein expression levels (tissue collected during the first 15 minutes of aortic cross clamp (and the closest representation of pre-operative status) correlated highly with oxygen extraction ratio (p=0.028).
- In the cyanotic group baseline HSP27 protein expression levels also significantly correlated with post-operative basal septal velocity (Figure 3-36, p=0.036).
- HSP27 also significantly correlated with better mixed venous oxygen saturation in the cyanotic group (p=0.02).

3.8 Association of APJ expression with improved right ventricle function suggests a protective effect in cyanotic TOF

3.8.1 Introduction

The APJ receptor and its cognate ligand and their function within the heart have been discussed previous (see section 1.8). To date, Apelin and the APJ receptor have never been identified in the infant myocardium. The aim was to look for the presence of the APJ receptors and therefore Apelin activity in the infant heart. A further aim was to determine whether the ischemic stress of surgery for correction of TOF, involving CPB, results in an increase in the expression of the Apelin APJ receptor in the infant myocardium as part of a cardio-protective mechanism. A further aim was to establish if a relationship existed between levels of APJ receptor expression in the infant myocardium in relation to clinical outcome both pre and post-operatively. The function of Apelin-APJ could be advantageous in the post-operative myocardium for two reasons. The well-established functions of Apelin (intropy, vasodilation, diuretic) reduce physiological strain of the recovering post-operative heart and the molecular protection of the cardiomyocytes to ischemic stress can limit the damage inflicted by the use of CPB and reduce IR injury.

3.8.2 Materials and Methods

A similar study was performed for the differential protein expression of HSP27 (see section 3.7). Patient recruitment (section 2.7.2.1), Tissue Doppler echocardiography (section 3.7.2.2), surgical repair (section 3.7.2.3), post-operative monitoring (section 3.7.2.5) and statistical analysis (section 3.7.2.6) were performed as described in section 3.7.2. Western blotting was used to assess APJ differential protein expression. This was performed as previously described (section 3.7.2.4) except for the following factors: The primary antibody used was rabbit polyclonal anti-APJ (Phoenix Pharmaceuticals) at a concentration of 1/1000 after this was deemed suitable after antibody optimisation. The secondary antibody used was Donkey anti-rabbit (Abcam, 1/3000).

For the purposes of analysis, the samples were grouped by time according to how long after the initiation of surgery the tissue was taken i.e. after application of the aortic clamp. The samples near the start of the surgery (taken during first 10 minutes) were taken as the closest

possible indicator of preoperative expression, as the myocardium would not have been subject to high levels of ischemic damage due to the short time of cardioplegia. All samples taken after 10 minutes until surgical resection was complete were combined and grouped separately (>10 minutes) and represent samples with increasing exposure to ischemic injury.

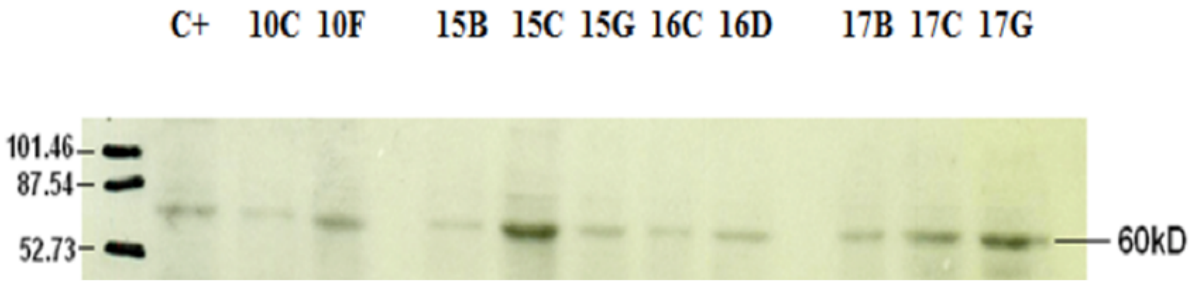
3.8.3 Results

Table 14 (see section 3.7) summarises the peri-operative characteristics of the two groups. Comparisons of these traits between the cyanotic, non-cyanotic group and the separate control group are already described (see section 3.7.3).

Samples were placed into two groups depending on how long after the initiation of surgery the tissue was taken. The samples near the start of the surgery (taken during first 10 minutes) were taken as the closest possible indicator of preoperative expression. All samples taken after 10 minutes until surgical resection was complete were grouped separately (>10 minutes).

Western blot analysis showed phosphorylated APJ receptor expression in all the samples examined although the level varied between patients. Figure 3-39 shows a typical Western blot. A separate analysis with β-Actin antibody (see figure 3-33) showed that the amount of protein loaded was consistent between each sample.

Figure 3-39 Representative Western blot showing APJ expression in different patients

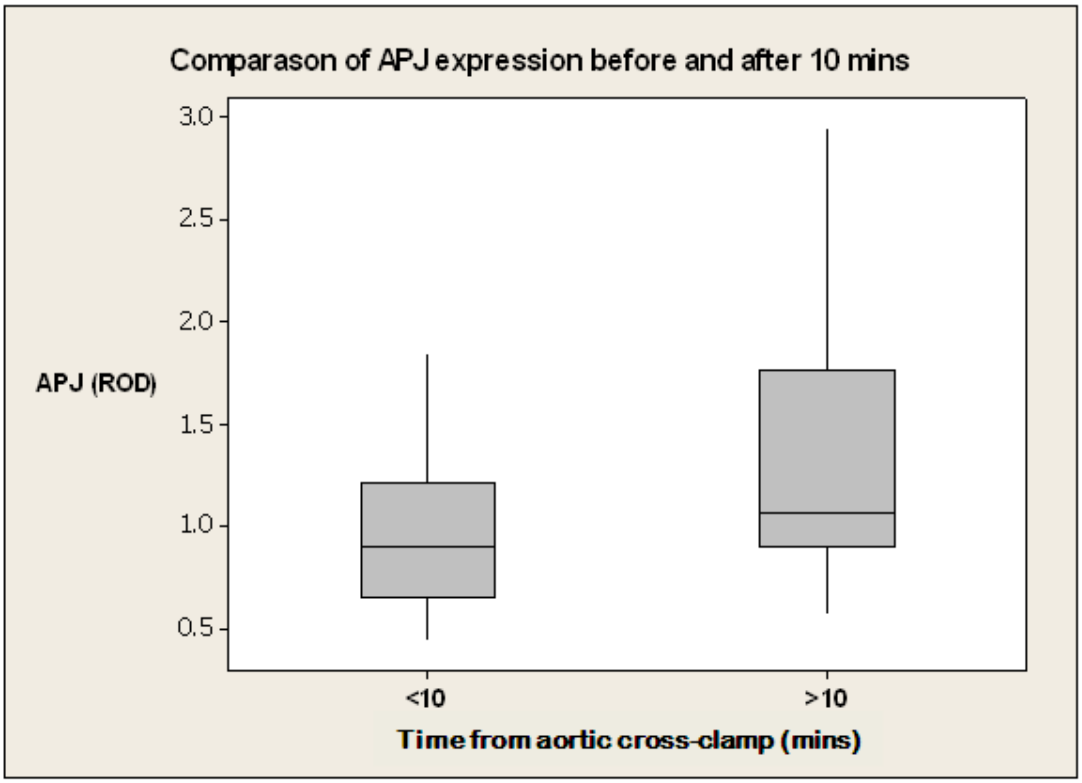


Western blot showing the Apelin receptor (APJ) in different patient samples. APJ receptor antibody NBP1-30869. All samples showed APJ receptor expression with variable amounts of expression between different patients.

When comparing expression levels using the parameters described above, a significant difference was found when all of the samples were analysed together (cyanotic group and non-cyanotic group combined) (figure 3-40): expression of phosphorylated APJ was found to be increased after 10 minutes when compared to values in the first 10 minutes of surgery ($p<0.05$), therefore as time of cardioplegia and ischemia increased, expression of phosphorylated APJ receptor increased.

When the cyanotic and non-cyanotic groups were analysed separately, no statistical difference was found between the groups before or after 10 minutes (data not shown, $p>0.05$).

Figure 3-40. APJ expression (R.O.D) expression in all patients (cyanotic and non-cyanotic)

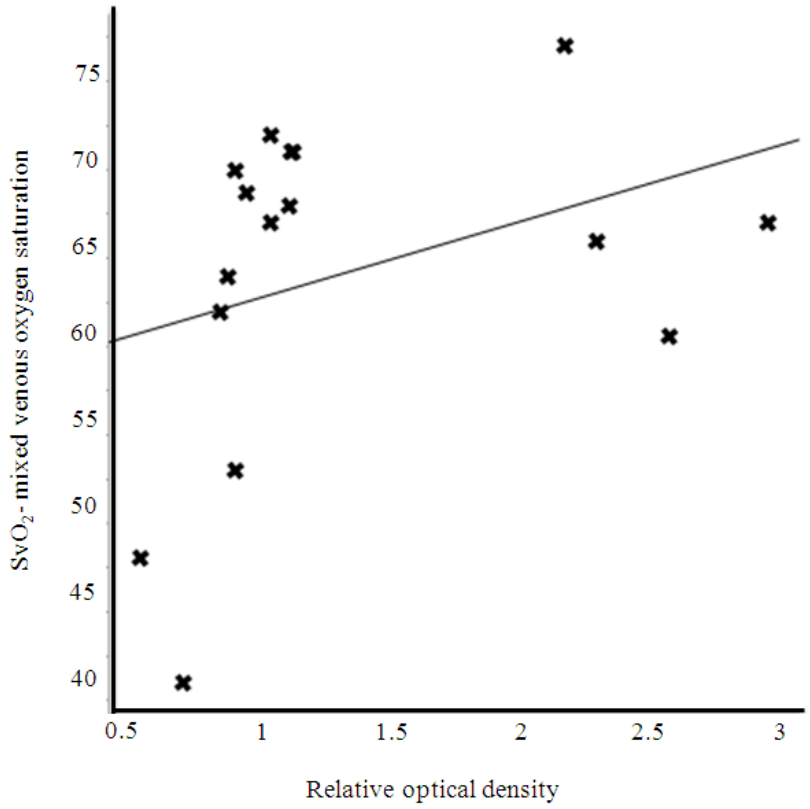


Box and whiskers plot (median, interquartile-range) of APJ (relative optical density (R.O.D) expression in all patients (cyanotic and non-cyanotic) grouped by time interval collected during aortic cross-clamping time ($p<0.05$).

Troponin is a standard serum marker of cardiac damage. Expression levels of phosphorylated APJ were also correlated to serum troponin levels (measured post-operative day 1). In both the cyanotic and non-cyanotic patient groups, whether analysed separately or combined, levels of APJ expression did not significantly correlate to post-operative troponin levels before or after the 10 minute time point or in all time points combined (data not shown, $p>0.05$).

Mixed venous oxygen saturation (SvO_2) is a measure of the O_2 saturation of the venous blood taken from one of the pulmonary arteries and therefore is representative of O_2 saturation for the entire venous circulation. This figure can be a measure of O_2 uptake from tissues in the periphery as well as arterial O_2 and cardiac output. Phosphorylated APJ receptor expression (in samples collected from the cyanotic and acyanotic patient group after 10 minutes) positively correlated with SvO_2 measured post operatively on day 1 ($p<0.05$) (figure 3-41). This association was not present when the APJ receptor expression was correlated with samples collected <10 minutes (data not shown, $p>0.05$).

Figure 3-41. Mixed venous oxygen saturation and APJ expression



Scatter plot of mixed venous oxygen saturation and APJ expression in in samples collected from the cyanotic and acyanotic patient group after 10 minutes

Summary of main findings:

- The protein expression of phosphorylated APJ was found to be increased after 10 minutes when compared to values in the first 10 minutes of surgery ($p<0.05$).
- Phosphorylated APJ receptor protein expression (in samples collected from the cyanotic and acyanotic patient group after 10 minutes) positively correlated with SvO2 measured post operatively on day 1 ($p<0.05$).

CHAPTER 4 – DISCUSSION

4.1 Introduction

Despite the various measures employed to protect the myocardium during CPB (see section 1.2.2) during surgical repair of congenital heart defects there is still an increased incidence of post-operative low cardiac output (Babu *et al*., 2012). Children with TOF often have cyanotic damage due to the chronic exposure of the RV myocardium to insufficiently oxygenated blood shunted from the right to the left side of the heart through the VSD (see section 1.1.3). Such myocardium is thought to be more vulnerable to injury (Merante *et al*., 1998; Najm *et al*., 2000; Corno *et al*., 2002).

The use of bypass is associated with various forms of insult (see section 1.3). In this thesis, the main one of interest is localized myocardial ischemic-reperfusion (IR) injury (1.3.2). During both paediatric and adult cardiac surgery with CPB, high levels of O_2 are often used. Therefore, the systemic systems of the body are exposed to high O_2 tensions throughout the procedure. The myocardium is exposed to ischemia upon application of the cross-clamp and is then reperfused (aortic-clamp release) with these high O_2 levels resulting in myocardial IR injury (Ihnken *et al*., 1998; reviewed in Allen *et al*., 2001; reviewed in Morita, 2012).

Surgical correction of congenital defects accompanied by cyanosis is, as already described, carried out on bypass using hyperoxic levels of O_2 (usually 250 to 400 mm Hg) (reviewed in Morita, 2012). A collection of studies performed using a pig animal model illustrated that cyanotic myocardium (PaO_2 of 25mmHg) is especially vulnerable to re-oxygenation injury at high levels of O_2 (PaO_2 400mmHg). Such damage is characterized by oxidative damage (ROS), a reduction in antioxidant reserve, oxidative degradation of lipids, cardiac contractile impairment and a reduction in cardiac output (Ihnken (i) *et al*., 1995; Ihnken (ii) *et al*., 1995; Morita (i) *et al*., 1995; Morita (ii) *et al*., 1995). This has also been shown in children with cyanotic damage who are undergoing corrective surgery on bypass. Hyperoxic re-oxygenation caused an increase in the level of troponin (marker of cardiac dysfunction), oxidative damage (ROS), depletion in antioxidants and a rise in pro-inflammatory cytokines (Modi *et al*., 2002; Bulutcu *et al*., 2002). This topic has been very recently reviewed (reviewed in Morita, 2012).

There have been several different methods employed in order to protect cyanotic myocardium from hyperoxic re-oxygenation injury. The first is the use of a lower O_2 tension. Using normoxic levels during bypass is known to produce less damage than hyperoxic re-oxygenation during adult heart surgery (Ihnken *et al*., 1998; reviewed in Morita, 2012). In relation to paediatric corrective heart surgery, several studies noted above, which used a pig animal model of re-oxygenation injury of cyanotic myocardium (about 25 mm Hg), demonstrated that using a moderate O_2 tension (100 mmHg) rather than hyperoxic O_2 levels (400 mmHg) reduced levels of ROS, reduced CK levels (marker of tissue damage), maintained antioxidant levels and improved cardiac dysfunction (Morita (i) *et al*., 1995). This reduction in damage by using a lower O_2 tension using a pig animal model was demonstrated in various ways in other studies (Ihnken (i) *et al*., 1995; Ihnken (ii) *et al*., 1995). The protective effects of using moderate O_2 levels during surgery on bypass were also shown in studies involving cyanotic patients undergoing paediatric corrective heart surgery (Bulutcu *et al*., 2002; Caputo *et al*., 2009).

There is also evidence that controlling re-oxygenation during CPB for patients that have congenital defects with pre-operative cyanotic damage will reduce post-operative injury. This was also demonstrated using a pig animal model. Cyanotic myocardium (about 25 mmHg) was either exposed to hyperoxic O_2 tension (400 mmHg) immediately upon initiation of bypass or exposed to a gradual increase of O_2 tension from ambient O_2 levels up to hyperoxic levels (400 mmHg). Controlling the O_2 level resulted in a reduction in oxidative damage, the maintenance of antioxidant reserve, less oxidative degradation of lipids, and an improvement of cardiac output (Morita (ii) *et al*., 1995). Similar findings were found in another study (Ihnken (ii) *et al*., 1995). This was also demonstrated during paediatric cardiac surgery, where 31 children with cyanotic damage were recruited and put into 2 different groups. In the first group, bypass was initiated with very low O_2 levels that were gradually increased for 5 minutes until levelling out at about 200 to 300 mmHg. In the other group, bypass was initiated at very high levels of O_2 for 5 minutes then again levelled out to 200 to 300 mmHg. Patients that had controlled re-oxygenation experienced significantly lower levels of creatine-phosphokinase-MB (marker of cardiac damage) and ventilation time (Babu *et al*., 2012). This was shown in a similar study by another research group (Zhu (i) *et al*., 2005).

The molecular mechanism at work within in the myocardium in adults undergoing corrective heart surgery has been investigated. Such investigation into children undergoing corrective heart surgery for congenital heart defects is limited. Some genes of interest that have been investigated include aspects of the immune system, such as TLRs, and cardio-protective proteins such as HSPs. By looking at the differential expression of such genes and how they behave in the myocardium under such stresses as IR injury could allow new and novel protective methods to be highlighted. As such, this was one of the main aims of this project. Another aim was to determine if there was any difference in the molecular mechanisms that occurs in the myocardium of children with pre-operative cyanotic damage during insult when compared to appropriate controls.

A novel aspect of this project was the fact that the tissue being used to analyse these molecular mechanisms involved using human tissue from paediatric patients undergoing surgery for congenial heart defects. This type of clinical investigation has not yet been undertaken.

4.2 Screening of differential gene expression in ventricular myocardium excised from TOF and control patient groups

The main aim of these experiments (see section 3.3) was to investigate the differential expression of inflammatory markers and stress proteins within ventricular myocardium excised from both cyanotic and acyanotic patients. Such patients undergo corrective surgery with CPB in order to repair a congenital cardiac defect. Patients who are cyanotic due to chronic exposure of ischemia include those with TOF (see section 1.1.3) and patients who are acyanotic include those with SAS (see section 1.1.4).

Damage done to the myocardium and the systemic organs of the body by cardiac surgery with CPB has already been discussed (see section 1.3) (Kirklin *et al*., 1983; Menasche, 1995; reviewed in Kozik and Tweddell, 2006). The protection of the myocardium during such procedures is very important. This is thought to be even more important for patients with pre-operative cyanosis, who are thought to be more vulnerable to further insult (Merante *et al*., 1998; Najm *et al*., 2000; Corno *et al*., 2002).

By looking at the expression of various genes at the molecular level, it is possible cyanotic patients have a different expression profile than the acyanotic patients. Such differences could help improve the knowledge of the molecular mechanisms at work within the myocardium under cyanosis. Therefore the first experimental investigation carried in this thesis was to look at the difference in the expression of inflammatory mediators (TLRs) and stress proteins (HSPs) in the myocardium from cyanotic and acyanotic patients.

4.2.1 Experiment 1: TLR screening by endpoint PCR

The aim of this experiment (see section 2.8.1) was to look at the expression profile of all 10 human TLRs (inflammatory mediators) and secondly to determine if there was any difference in their expression between the acyanotic and cyanotic patient groups. This helped to determine which TLRs were expressed in the tissue being used in this work but also to see if cyanosis had an affect on their expression. RNA was extracted from the ventricular myocardium and a kit that had primers for all 10 human TLRs (Invivogen) was used to investigate mRNA gene expression using PCR in all relevant patients from both groups (see section 3.1.2.1).

In the cyanotic group (figure 3-1), TLR4 was the most highly expressed. TLR1 was not expressed in any of these patients. The rest of the TLRs were all expressed to varying degrees in all 7 cyanotic patients. In the acyanotic group (figure 3-2), TLR4 was expressed at the highest level, except for sample 6(1) where TLR8 was also highly expressed. Also, for sample 18A, TLR9 and TLR10 were highly expressed. TLR1 was not expressed at all. The other TLRs were expressed in all 5 acyanotic patients to varying degrees. There was no noticeable difference in the TLR gene expression profile of cyanotic patients compared to control patients.

The initial reason this experiment was performed was to provide evidence of TLR expression, with emphasise on TLR2 and TLR4, within the infant ventricular myocardial tissue that was being used for all the analysis within this project. Some examples of relevant evidence of TLR expression within the literature is described below, but the expression of TLRs within the infant myocardium has not been widely investigated.

The expression of TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 was demonstrated in both adult human and mice cardiomyocytes. However, only TLR2, TLR4 and TLR5, when induced with their cognate ligand, produced pro-inflammatory cytokines (Boyd *et al*., 2006). Another study demonstrated the expression of TLR4 in both normal and abnormal myocardium from human and mice (Frantz *et al*., 1999). Most other studies within the literature only investigate the expression of one TLR, usually TLR4, and some examples are noted later in this section. This thesis, for the first time, both looked at and demonstrated the expression profile of all 10 human TLRs in infant myocardium.

TLR4 is one of the best characterized TLRs in the literature in relation to both the innate immune system and the heart. The fact that there are so many studies that demonstrate the role of TLR4 in inflammation caused by IR injury could suggest that TLR4 is the most important to cardiac function (for examples see section 1.4). This could suggest why it is the most highly expressed in both the cyanotic and acyanotic group.

TLR4 is expressed on the plasma membrane and can be activated by extracellular signals such as PAMPs and DAMPs. Cardiac surgery can result in various forms of injury. This can cause both cell damage and/ or cell death that can cause the release of DAMPs, such as HSPs. TLR4 was the most highly expressed TLR in both patient groups. All patients had gone through cardiac surgery with CPB. Therefore all patients would have been exposed to cellular insult, which could have initiated an inflammatory response. This could have caused the high TLR4 expression in both patient groups. Also, most of the studies in the literature that look at the activation of TLRs by DAMPs mostly focus on TLR4 and to some extent TLR2 (see section 1.4). Therefore it may be that these TLRs are the ones mostly activated by extracellular DAMPs and the reason why TLR4 was the most highly expressed within the two groups.

On the premise that cyanotic patients have the potential for pre-operative myocardial injury because of exposure to chronic hypoxia, it may be expected that the expression of TLRs would be higher in the cyanotic group, in particular TLR4. This is because TLRs are activated by DAMPs and can initiate an inflammatory response (see section 1.4). The work in this thesis indicated that there was no difference in TLR expression between the two patient groups.

TLR1 was not apparent in any patient samples from either of the patient groups. This may be because it is expressed at very low levels and was therefore not detectable by the assay. The primers used in the kit resulted in PCR products of unknown sizes. Despite this, it was apparent that the PCR product produced by the TLR1 primer pair was the largest.

This experiment had some limitations. The first was the use of endpoint PCR that is only semi-quantitative and therefore any observations on the differential expression of the genes of interested will be limited. Also, the commercial kit used for this experiment presented some obstacles. Initially, only the positive control provided within the kit produced a PCR product with all 10 TLR primer pairs. No PCR products were produced with the mRNA extracted from the myocardial tissue. After weeks of troubleshooting, it was discovered that the PCR protocol given by the company was incorrect. The elongation step during each cycle of the PCR reaction should have been 2 minutes. The original protocol had stated 30 seconds. After this was rectified through the trouble shooting system, the PCR worked correctly.

If there had been time, it would have been ideal to have more patient samples. The nature of the patient groups that tissue was obtained from is not common. Therefore, although limited in the number of samples I could obtain, this study is unique in being the first to attempt to do investigative work of this nature.

The expression of all 10 human TLRs (except TLR1) was demonstrated in this experiment. This allowed for the TLRs of most interest to be identified for further investigation. TLR2 and TLR4 were chosen. Based on the fact qPCR allows a more quantitative analysis of differential expression, it was used in the next experiment of this section to further the observations described above.

4.2.2 Experiment 2: Screening the differential expression of several genes of interest by qPCR

The aim of this experiment was to look at the differential expression of inflammatory mediators and stress proteins and to determine if there were any differences in their expression between the acyanotic and cyanotic patient groups. The last experiment only looked TLRs. TLR4 and TLR2 were selected for experiment 2 based on the observations highlighted. MYD88 is involved in the downstream signalling of both these TLRs therefore was also

selected. HSPs were not investigated in experiment 1 and are known stress proteins and were thought good candidates for investigation. Using RNA extracted from the ventricular myocardium and appropriate primers, mRNA gene expression was therefore investigated for all genes of interest using qPCR in all relevant patients from both groups.

Inflammatory mediators:

The expression of TLR4, TLR2 and MYD88 were investigated (figure 3-3). It was found that both TLR4 (0.51 vs. 0.35; p=0.0303) and TLR2 (1.23 vs. 0.68; p=0.0177) had significantly higher expression in the cyanotic group when compared to the acyanotic patient group. There was no such difference seen for MYD88.

A direct comparison of the expression of TLR4, TLR2 and MYD88 between cyanotic and acyanotic myocardium has not been performed before in either adult or infant hearts. That makes this experiment novel. No change in TLR2 and TLR4 expression was seen between the 2 patient groups in experiment 1. This contradicts findings in experiment 2. Endpoint PCR is only semi quantitative, while qPCR (see section 2.9) looks at the differential expression within every sample being tested in real time, allowing for a more accurate and informative assessment of the differential expression of the gene being investigated. Therefore the differences observed in experiment 1 and experiment 2 could be explained because qPCR is more sensitive.

Both TLR4 and TLR2 are expressed on the plasma membrane and can cause an inflammatory response when activated by DAMPs (danger signals released during times of cell damage and/ or death) (Gallucci *et al*., 1999; Shi *et al*., 2000; Asea *et al*., 2002). Patients with cyanotic heart disease are known to have pre-operative hypoxic injury. Therefore, it is possible that this could have resulted in the elevated levels of both TLR2 and TLR4 seen in the cyanotic patient group.

MYD88 is an adapter protein that is involved with the downstream signalling of both TLR4 and TLR2. It would have been expected that the mRNA for this gene would also be higher in the cyanotic patient group. Looking at the expression of MYD88 (figure 3.3), a trend can be seen where its expression in the cyanotic group is higher than in the acyanotic group but this did not reach statistical significance.

TLR4 can also result in the activation of the IRF3 via a MYD88 (TRIF) independent pathway. This results in the production of IFN-β and the activation of its associated pathways **(**Fitzgerald *et al*., 2003; Yamamoto (ii) *et al*., 2002; reviewed in Chao *et al*., 2009). Therefore an increase in the expression of TLR4 would not necessarily result in an increase in the expression of MYD88 at the same time. This would not be applicable for TLR2 since it only works through the MYD88 pathway. Another possibility for an elevation in TLR4 expression could be its involvement in apoptosis (Ward *et al*., 2005; Goyal *et al*., 2002; Lombardo *et al*., 2007).

Stress proteins:

The expression of HSP20, HSP27, HSP32, HSP60, HSP72 and HSP90 was investigated (figure 3-4). Of the six HSPs, only HSP27 showed a significant difference i.e. HSP27 expression was significantly higher (0.12 vs. 0.05; p=0.0303) in the cyanotic group when compared to the acyanotic group. When looking at the expression of the other HSPs (figure 3-4), especially at HSP20, HSP60 and HSP90, there is a trend for higher expression in the cyanotic group when compared to the acyanotic group but these did not reach statistical significance.

HSP27, like other HSPs, is a stress protein and its expression is induced by cellular stress (Landry *et al*., 1989; reviewed in Salinthone *et al*., 2008). HSP27 is mainly intracellular where it will carry out its function as a protein chaperone. This ensures the maintenance or restoration of the structure of damaged cellular proteins (reviewed in Noble *et al*., 2008; reviewed in de Jong *et al*., 2009; reviewed in Horvath *et al*., 2008; reviewed in Liberek *et al*., 2008; reviewed in Latchman, 2001). Cyanotic patients that suffer from congenital defects such as TOF have pre-operative hypoxic damage, which is a cellular insult. This could explain the elevated expression of HSP27 in these patients. Also, HSP gene expression has a novel trait known as pre-conditioning (reviewed in Latchman, 2001). This is where an initial cellular insult increases HSP expression that protects against a subsequent insult. This could be an adaptive measure used by the body to try and protect itself again the ischemic damage caused by such congenial defects. HSP27 is also known to be involved in the regulation of the apoptotic pathway (Bruey *et al*., 2000; Charette *et al*., 2000). If the elevation in the expression of HSP27 was because of this pathway, it could also potentially be beneficial for patients with

cyanosis. This is because it could help prevent cell death and maintain cellular integrity within the heart under such ischemic stress.

It has also been shown that HSPs, including HSP27, can be found outside the cell where they interact with cells of the immune system. (Bassan *et al*., 1998; Basu *et al*., 2000; reviewed in Calderwood (i) *et al*., 2007; reviewed in Horvath *et al*., 2008). Such interaction can also involve the use of receptors such as TLR4 (Asea *et al*., 2000). Perhaps both TLR4 and HSP27 expression were elevated in the cyanotic group because they were involved in inflammation caused by the chronic ischemic exposure sustained in the cyanotic group. Such an interaction has been shown in a study that investigated the expression of HSPs in skin in relation to occurrence of allergies (Yusuf *et al*., 2009).

HSP32, HSP72 and HSP90 are well known stress proteins and are well characterized in the literature (see section 1.6). They are known to be induced by cellular stress therefore it might have been expected that a change in their expression would have been observed between the two patient groups but this was not the case.

The majority of work within this thesis used qPCR. Its use has many benefits but as with most methods in science there are some limitations. The type of qPCR assay I used was the GoTaq® qPCR Master Mix with appropriate primers. The dye this assay contains binds indiscriminately to any double stranded DNA. In order to ensure a single discreet product is detected, great care was taken with primer design and melt curve analysis was used (see section 2.9 for more detail). Also, because the correct size (bp) of amplicon is known for each primer pair when they are designed (see section 2.7.1), the size (bp) of a qPCR product can be established by gel electrophoresis (see section 2.10) (reviewed in Smith and Osborn, 2009). All these steps ensured the results obtained were a true reflection of change in expression of the genes of interest.

The comparative C_t method (see section 2.9.2) was used to look at the differential expression of all genes of interest in all samples. This method is a RQ method (for more detail see section 2.9.2). Many methods are used to minimise intra experimental variation that could affect resulting RQ values. It is ensured that the expression of the endogenous control is uniform in all samples. For this work the endogenous control was β-Actin, which has been

demonstrated as a suitable endogenous control in this laboratory. Also, a standard amount of RNA (100ng) is used for each sample in every RT reaction (see section 2.6), standardising the amount of cDNA used in the subsequent PCR/ qPCR. The concentration (ng/ μl) of RNA is determined on a NanoDrop®. The ratio of absorbance at 260 nm and 280 nm gives a measurement of RNA purity (see section 2.5). A value of 1.8-2.1 indicates optimal purity. Using high purity RNA during RT helps ensure good quality cDNA is used in the subsequent PCR/ qPCR. The use of the same arbitrary reference sample in all qPCR reactions helps minimised inter experimental variation and allows RQ values produced from various different qPCR reactions to be compared (see section 2.9.2 for more detail). This reference sample works with all primers used and goes through all procedures experienced by other samples therefore it also acts as a positive control for this assay. All these checks helped ensure the results obtained herein reflected true differences in gene expression for all qPCR experiments carried out for this thesis.

The overall results of experiment 1 and 2 provided evidence that the genes of interest in this study were expressed in the myocardium. Also, it was found that several of them had differential expression between the 2 patient groups: these were TLR4, TLR2 and HSP27. This demonstrated that molecular mechanisms within the myocardium are affected by the presence of pre-operative ischemic damage present in the cyanotic patient group. Therefore, further investigation of the expression these genes could lead to better methods of protection of the myocardium. This is especially important for the cyanotic patient group, which was shown to have elevated expression of both inflammatory mediators and stress protein in this thesis.

The expression of both inflammatory mediators and stress proteins has been demonstrated in the myocardium of both patient groups. The next important step was to see if the stress proteins could be induced in the tissue used for the work within this thesis.

4.3 HSP induction experiments

The aim of these experiments (see section 2.4) was to investigate whether or not HSPs could be induced within infant myocardium. Induction was attempted using heavy metals, which are classic inducers of stress proteins. Therefore the myocardial tissue was exposed to $NaAsO_2$ and $CdCl_2$ and any differential HSP expression was investigated. This investigation

was performed at both the mRNA (see section 3.2.2.1) and the protein level (see section 3.2.2.2). The inflammatory mediators were also investigated but only at the mRNA level. These findings are discussed in the following sections.

4.3.1 mRNA level gene expression in response to heavy metal exposure

The aim of this experiment (see section 3.2.2.1) was to look at the differential expression of stress proteins in infant myocardium exposed to heavy metals. This was to see if these proteins could be induced in this tissue under cellular stress. Inflammatory mediators were also investigated. Using RNA extracted from the ventricular myocardium of patients undergoing corrective surgery for congenital defects and appropriate primers, mRNA gene expression was investigated using qPCR in all relevant patients for all the genes of interest.

Stress proteins:

The expression of HSP20, HSP27, HSP32, HSP60, HSP72 and HSP90 were investigated. When looking at the differential expression of all six HSPs when exposed to either $NaAsO_2$ or $CdCl_2$, no statistically significant differences were observed (see table 8). It is possible that the low patient numbers for each chemical inducer (only 3 patients) meant that any significant difference in expression present was not identified. This is unlikely because previous studies have shown that if heavy metals are effective then the response is usually very large (Barber *et al*., 1999).

The differential expression of HSPs induced by both the heavy metal inducers combined was then analysed. HSP27 (figure 3-6B) and HSP60 (figure 3-7A) showed no significant differences when exposed to heavy metals for 6 hours. There are several examples of heavy metal exposure inducing the expression of HSP27 and HSP60. For example, exposure of various types of human endothelial cell types to $CdCl_2$ for 3 hours caused an increase in the expression of both these HSPs (Wagner *et al*., 1999). However, another study that investigated the effect of $NaAsO_2$ exposure on cultured human urothelial cell line UROtsa showed that the expression of HSP27 and HSP60 were unaffected at the mRNA level by such exposure (Rossi *et al*., 2002). 6 hour induction with heavy metals was chosen based on previous work performed in this laboratory (Barber *et al*., 1999). Induction of HSP27 and HSP60 mRNA expression might have occurred before the 6 hour timepoint because our

published studies looked at HSP protein levels only. However, the fact some other HSP mRNAs increased (see later) suggested it is not the time of incubation that is the reason. Also, the studies mentioned above do not look at their expression within the heart; therefore it is possible that their expression is not inducible in this tissue.

HSP20 (figure 3-6A) showed a significant decrease in expression upon exposure to heavy metals. This was not seen in the control (media only). There is very little mention of HSP20 expression in relation to heavy metal exposure in the literature. One study involving HSP20 expression in airway smooth muscle indicated that exposure to 20 μM $NaAsO_2$ did not affect the expression of HSP20 (Ba *et al*., 2009). The decrease in expression of HSP20 seen during this experiment could have been because exposure to heavy metals for 6 hours caused damage to RNA within the tissue that encodes for HSP20 or somehow inhibited steady state synthesis. The fact that the tissue in media only did not produce the same result supports the fact it was the heavy metal and not the 6 hour incubation period that caused the potential deterioration of RNA. Interestingly, this was not observed for the expression of other HSPs. Therefore, it is likely something specific about HSP20 and its RNA transcript that was affected by the heavy metal exposure. In future experiments it would be interesting to look at other time points to investigate this further.

HSP32 (figure 3-6A), HSP72 (figure 3-7B) and HSP90 (figure 3-7C) all showed a significant increase in expression when exposed to heavy metals for 6 hours. This was also true for the tissue exposed to media only for 6 hours (control). The increase in expression due to exposure to heavy metals for 6 hours was significantly higher (figure 3-8) when compared to the increase in the expression seen for the controls. Therefore the increase due to heavy metals was greater. There are many examples of HSP72 expression being induced by both $NaAsO_2$ and $CdCl_2$ (Mirkes *et al*., 1994; Wagner *et al*., 1999; Wirth *et al*., 2002; Rossi *et al*., 2002). This is not surprising as HSP72 is the best characterized HSP in the literature. HSP32 has also been shown to be induced by these heavy metal inducers (Wagner *et al*., 1999; Malstrom *et al*., 2004; Wijeweera *et al*., 2001). Studies that focused on HSP90 expression upon heavy metal exposure are fairly limited (Rossi *et al*., 2002). Of those it was shown that exposure to $NaAsO_2$ did not significantly affect HSP90 expression (Rossi *et al*., 2002). Therefore the work in this thesis for HSP32 and HSP72 is supported. For HSP90, this thesis shows it can be induced by heavy metals in infant myocardium.

All these studies generally show that HSPs can be induced by heavy metals. No previous studies looked at their expression in infant myocardium. Therefore the demonstration of the inducement of the expression HSP32, HSP 72 and HSP90 in this tissue by heavy metals is a novel finding. The fact that all 3 HSPs were also induced significantly in the control after 6 hours is also worth noting. This suggests that tissue being in the incubator itself caused a degree of cellular stress, therefore inducing their expression.

Inflammatory mediators:

The expression of TLR2, TLR4 and MYD88 was investigated. When looking at the differential expression of all inflammatory mediators when exposed to either $NaAsO_2$ or $CdCl_2$, there were no statistically significant differences observed (see table 8).

The differential expression of these genes induced by both the heavy metal inducers combined was then analysed. TLR2 and TLR4 (figure 3-5) showed no significant differences in expression when exposed to heavy metals for 6 hours. This was the same for the controls. MYD88 (figure 3-5C) also did not show a change in expression upon exposure to heavy metals for 6 hours, but showed a significant decrease in the control for 6 hours. Perhaps when tissue was left in media only, the RNA encoding for MYD88 was especially vulnerable to degradation by being in the incubator for 6 hours. This seems unlikely due to the fact that this same decrease in expression was not seen for the tissue exposed to heavy metals. Based on these results, heavy metals seem to not induce an inflammatory response. This can only be said for exposure of these genes within the myocardium and for the 6 hour time point used in the experiment.

Although heavy metals have been used in the literature as classic inducers of HSPs, there have not been studies showing them as inducers of inflammatory mediators. However, since we are interested in inflammatory mediators as well as HSPs, and we also had sufficient cDNA available it was possible to easily investigate whether heavy metals also induced inflammatory mediators in this context. Also, exposure to heavy metals will cause cellular stress, which may result in inflammation. The lack of expression caused by heavy metals in the work of this thesis and the lack of studies on the subject suggest that heavy metals are not appropriate inducers of the inflammatory mediators. There was one study that indicated that $NaAsO_2$ exposure inhibited NFkB activation, suggesting it could inhibit an inflammatory

response in beta-stimulated Caco-2 cells (Hershko *et al*., 2002). However, this study did not look at TLR2, TLR4 or MYD88 and did not involve the myocardium.

Overall, heavy metal induction of HSPs has been well documented. Such induction in the heart however has not. This study clearly demonstrates that HSPs are expressed in the infant myocardium and that several of them can be induced within these tissues by classic HSP inducers. Therefore, this experiment acted as a control for the reperfusion experiments as it showed that HSPs were expressed but also that they could be induced upon cellular stress in infant myocardium. While investigating mRNA HSP expression, we also investigated HSP expression at the protein level. This is described in the next section.

4.3.2 Protein level gene expression in response to heavy metal exposure

The aim of this experiment (see section 3.2.2.2) was to look at the differential protein expression of HSPs in infant myocardium exposed to heavy metals taken from patients in the cyanotic patient group. This was to supplement the mRNA HSP expression data just discussed.

Using protein extracted from the ventricular myocardium and appropriate antibodies, protein expression of HSP27, HSP32 and HSP72 were investigated using Western blotting. It should be noted that due to small patient samples used for this experiment, analysis was performed by looking at expression caused by the combination of both heavy metals. It was therefore not possible to look at the differential expression caused by both heavy metals separately with valid statistical analysis. Also, as protein level expression analysis required more tissue from each patient than mRNA gene expression analysis, there are no baseline samples (except for patient 44) in this dataset. Therefore the only comparison possible for this part of this work of the thesis was to compare the level of HSP expression produced in tissue exposed to heavy metals for 6 hours to the expression in tissue kept in media only for 6 hours (control).

Differential expression of HSP32:

HSP32 expression was investigated in infant myocardium but no detectable levels of the protein were demonstrated within this tissue using the chosen antibody (see section 2.12.4). To ensure it was not the assay at fault, an appropriate positive control was used. This produced a positive result for HSP32 (see figure 3-9). It could be that HSP32 was not detectable within the limits of the assay or that it was not detectable in the tissue being used for the experiment. HSP32 was shown in this tissue at the mRNA level, therefore it was expected that it would be detectable at the protein level as well. As such, due to time constraints it was decided to limit the optimisation period for this protein and no further investigation was performed.

Differential expression of HSP72 and HSP27:

The expression of HSP72 (figure 3-10) and HSP27 (figure 3-11) were investigated by Western blotting (see section 2.12). There was no significant difference in the expression of HSP27 and HSP72 (figure 3-12) seen between tissue that had been incubated with a chemical inducer for 6 hours and tissue that had been incubated in media only for 6 hours (control).

At the mRNA level, there was no difference in HSP27 expression (figure 3-5A) when exposed to heavy metals. This is agreed upon at the protein level. HSP27 expression has been shown to be unaffected by exposure to heavy metals, which supports these findings (Rossi *et al*., 2002). Whether other time points would have produced a change is not known but 6 hours is the normal time one might expect any indication to occur based on previously published studies on HSP induction (Barber *et al*., 1999).

For patient 44, a baseline sample was collected as this patient yielded more tissue than expected. This patient's tissue was exposed to $NaAsO_2$. It appeared that expression of HSP27 at the start of incubation was higher than that seen in samples exposed to heavy metals or media only (control) for 6 hours (figure 3-11). A heart control sample is also included in these experiments (patient 1, TOF). This patient sample was not exposed to any experimental conditions. The expression of HSP27 in this sample is higher than that seen in the samples exposed to 6 hours in the incubator. This is also seen for the placenta control sample used. It suggests that protein stability of HSP27 in the incubator after 6 hours is severely reduced,

resulting in reduced HSP27 expression. There was only one baseline sample from one patient exposed to one heavy metal. Therefore such a suggestion is only observational.

HSP72 mRNA expression (figure 3-7B) was induced by heavy metal exposure after 6 hours compared to tissue in media only (control). This was not repeated at the protein level (figure 3-10) where no statistical difference was observed (figure 3-12). The elevated expression of HSP27 in the baseline (patient 44) and heart/ placental control samples when compared to the tissue exposed to 6 hours incubation (heavy metals or media only) was not seen for HSP72. There are several studies that show that HSP72 is induced by heavy metals at both the mRNA and protein level (Mirkes *et al*., 1994; Wagner *et al*., 1999; Wirth *et al*., 2002; Rossi *et al*., 2002; Kuboki *et al*., 2007). This means induced HSP72 expression by heavy metals may have been expected. These studies did not investigate HSP72 expression within the myocadium. Therefore, it may be that such changes in HSP72 expression by heavy metals is not seen in the infant myocardium at the protein level. Or it may be that such change would be apparent at a different time point than 6 hours, so although unlikely, it might be beneficial for future studies to examine other time intervals.

Heavy metal exposure with recovery period:

An additional experiment was performed where tissue from a TOF patient (patient 40: TOF, cyanotic, male) was exposed to $NaAsO_2$ for 4 hours then allowed to recover in media only for 20 hours (see section 2.4.3). Control tissue was incubated in media only for 24 hours. This was performed because a number of other published studies that examined induced HSP expression allowed for a period of recovery to determine if HSP expression was caused after an initial exposure to heavy metals (Kim *et al*., 2001; Rossi *et al*., 2002; Somji *et al*., 2002). The expression seen for HSP27 and HSP72 in this one patient seemed to follow all the trends seen in the tissue exposed to heavy metal exposure without a period of recovery. HSP27 expression (see figure 3-13) was again reduced within the tissue that had been incubated when compared to controls. There was no difference seen between heavy metal tissue exposure (4 hour exposure with 20 hour recovery) and media only tissue exposure (control for 24 hours). HSP72 expression (see figure 3-13) was not different for any of the samples either. Although this small investigation only used one patient and one type of heavy metal inducer, a recovery period did not seem to make a difference.

Overall, HSP32 and HSP72 protein expression has been demonstrated in the infant myocardium but such expression seems unaffected by exposure to heavy metals. This is in contradiction to mRNA level expression seen in similar tissue, which did increase. Reasons for this have already been described.

The number of clinical samples used in both the molecular and protein analysis of this experiment was limited. This is because the types of sample used were rare and hard to obtain. Also, due to the problems encountered setting up the hyperoxic reperfusion system, other avenues of investigation were followed in order to help to establish the experimental system used throughout this project. Despite this, the study does provide pilot data from which future studies can be based.

HSPs are induced at the mRNA level in the myocardium by classic inducers. They are also present at the protein level despite not being chemically induced. Also, mRNA TLR expression within the myocardium was shown in earlier work within this thesis. The presence of the genes of interest has been demonstrated in the tissue used for all experimental work herein. Therefore, it was now possible to investigate the effect of re-oxygenation injury on these genes within myocardium. Also, it was possible to investigate if chronic ischemic damage resulting in cyanosis further modified their expression.

4.4 Differential gene expression in RV myocardium excised from TOF and control patient groups exposed to differential oxygen levels

The main aim of the work within this thesis was to investigate the effect of re-oxygenation injury on the expression of inflammatory mediators and stress proteins within ischemic infant myocardium. Surgical correction on bypass can have adverse effects on post-operative cardiac output. During such procedures, the myocardium is exposed to high O_2 tensions (Ihnken *et al*., 1998; reviewed in Allen *et al*., 2001; reviewed in Morita, 2012). There are some concerns that this could cause adverse post-operative complications. When this involves patients with TOF, which due to chronic ischemic insult results in cyanosis, it is thought a bigger concern as they are thought to have more vulnerable to re-oxygenation injury (Modi *et al*., 2002; Bulutcu *et al*., 2002).

Therefore, during the surgical correction of congenital heart defects of children from both the cyanotic and acyanotic patient groups, ventricular ischemic myocardial tissue was removed. This tissue was exposed to differential O_2 exposure resulting in a model of localised myocardial IR injury. IR injury has been shown to cause inflammation and cellular stress. By looking at the affect this had on the expression of both inflammatory mediators and stress genes within the myocardium, the molecular mechanisms at play could be investigated. Having a better understanding of this would have the potential to help identify new and novel methods of myocardial protection.

4.4.1 Experiment 1: exposing myocardial tissue to 2% (hypoxia) and 20% (normoxia) O_2

The aim of this experiment was to look at the differential expression of inflammatory mediators and stress proteins within ischemic infant myocardium and to see if they are affected when exposed to 20% O_2 for 1.5 hours (see section 3.3.2.1). This re-enacts re-oxygenation injury of the myocardium *in vitro* and allows a better understanding of its affect on the molecular mechanisms within the myocardium. Using RNA extracted from the ventricular myocardium and appropriate primers, mRNA gene expression was investigated using qPCR in all relevant patients for all the genes of interest.

Inflammatory mediators:

The expression of TLR4, TLR2 and MYD88 were investigated (figure 3-14). Exposure of the ischemic myocardium to 2% O_2 for 1.5 hours acted as an experimental control. There was no significant difference in expression observed for any of these genes. Exposure to 20% O_2 was performed in order to re-enact re-oxygenation injury i.e. exposure of ischemic tissue to 20% O_2 for 1.5 hours. This did not result in any significant changes in expression.

The original idea had been to expose the tissue to 60% O_2, mimicking the hyperoxic exposure seen during correction of congenital heart defects. Setting up this experimental system was problematic and took several months to achieve as the original incubator bought for the experiment did not work and was returned to a company overseas. During this period it was decided that exposure to 20% O_2 would be investigated as a change from ischemia to 20% O_2 was still technically a hyperoxic shock. The time period of 1.5 hours was chosen because it is the length of time the myocardium is reperfused after ischemia (aortic-clamp release) during cardiac surgery on bypass.

TLR4 and TLR2 are trans-membrane proteins that once activated by PAMPs or DAMPs result in the activation of the innate immune system and the production of pro-inflammatory cytokines. MYD88 is an adapter protein that is involved in the downstream signalling of both TLR4 and TLR2. 20% O_2 is approximately the level of O_2 in ambient air. It could be that this level of O_2 exposure for 1.5 hours is not sufficient to cause an inflammatory response within the myocardium. This could explain why there was no significant difference in the expression of the inflammatory mediators during the work herein, despite the hyperoxic shock. It might also be that there was no change in expression at the 1.5 hour timepoint used during this experiment. It might have been different if more time points were included in the study but this was not possible due to the limited amount of tissue available from each patient. Future studies could address this.

The effect of IR injury on the expression of these inflammatory mediators has been described in the literature. There are several studies that have used animal models, usually involving the occlusion of blood to the heart for a period of time then allowing the flow to return. The size of the resulting MI was then measured as an indicator of the level of injury. In one study involving knockout TLR4 mice and appropriate controls, a model of IR injury was created, with the LV being exposed to 1 hour of ischemia then reperfused for 2 hours. This insult resulted in a MI. It was found that infarct size was larger in the wild type mice than in the mutant TLR4 mice. This suggested that the lack of TLR4 expression reduced the size of infarction (Chong *et al*., 2004). This reduced size of MI was observed in other similar studies involving mutant TLR4 mice and IR injury (Oyama *et al*., 2005; Shimamoto *et al*., 2006; Hua *et al*., 2007). In relation to TLR2 expression, several studies showed that TLR2 null mice resulted in a reduction in MI formation due to IR injury compared to controls (Favre *et al*., 2007; Arslan *et al*., 2010). For MYD88 expression, a study using MYD88 mutant mice and appropriate controls underwent a model of IR injury. They were exposed to 30 minutes of ischemia and then 24 hours of reperfusion. MI size was measured in both types of mice, with the mutant MYD88 having a smaller infarction size (Feng *et al*., 2008). This finding was again supported in a similar study (Hua *et al*., 2005).

The fact that a lack of expression of these genes seems to reduce the size of MI formation suggested that exposure of the ischemic myocardium to re-oxygenation would cause an increase in their expression. This was not the case in the work herein. The work in this

thesis involved the isolation of ischemic myocardial tissue that was exposed to differential levels of O_2 in culture media to determine its effect on gene expression. These animal studies removed the expression of these genes then evaluated the end result i.e. MI formation caused. It makes the assumption that the difference of MI formation was because of the lack of these genes. The methodology is different therefore comparison of these studies to the work within this thesis is not appropriate.

A number of studies involving animal models examined TLR4 expression during cardiac dysfunction (Tavener *et al*., 2004; Fallach *et al*., 2010; Baumgarten *et al*., 2006; Binck *et al*., 2005). The main question raised was whether it was TLR4 expression on cells of the immune system or TLR4 expression within the heart that caused cardiac dysfunction. There was no consensus reached as these studies all came to different conclusions. A similar problem was raised for TLR2 in relation to IR injury (Favre *et al*., 2007; Arslan *et al*., 2010). The work herein only looked at expression of defined genes within the myocardium itself. Perhaps re-oxygenation injury during corrective surgery causes inflammation within the systemic circulation rather than within the myocardium. This could explain the lack of change in the inflammatory mediators seen in this thesis. Therefore it suggests it might be worth investigating the systemic response in the future.

TLR expression has been investigated during cardiac surgery within the literature. One study showed that TLR2 and TLR4 expression on circulating monocytes were elevated after adult cardiac surgery with CPB (Dybdahl *et al*., 2002). This study did not look at the myocardial expression of TLR4 or TLR2. Another study was performed that used the TLR4 inhibitor eritoran. Patients undergoing adult CABG and/or cardiac valvular surgery were administered with either eritoran (prevents TLR4 expression) or a placebo pre-operatively. There were no significant differences seen when looking at systemic inflammation or organ dysfunction. This study will relate more to the damage sustained during CPB as a whole more than damage only done locally within the myocardium. Still it suggested that damage sustained during cardiac surgery does not cause a TLR4 initiated inflammatory response (Bennett-Guerrero *et al*., 2007). These studies did not investigate the expression of inflammatory mediators within the myocardium. Therefore the work of this thesis provided a novel opportunity to investigate such expression. Also, the work herein looked at the expression of genes within the myocardium in isolation upon re-oxygenation rather than the

effect of surgery on bypass as a whole. Therefore methodologies employed were different than those used in the above studies (Dybdahl *et al*., 2002; Bennett-Guerrero *et al*., 2007).

The use of CPB means that the blood of the patients comes into contact with the extra-corporeal unit of the CPB machine. This foreign surface initiates the priming of components of the immune system within the systemic blood circulation. Once this blood returns to the body, it causes a systemic inflammatory response. In this experiment in this thesis, the ischemic tissue was exposed to normoxic levels of O_2 in media only and therefore the tissue was not exposed to an active circulation. The media lacks several of the physiological components present within the blood e.g. cells of the immune system. Therefore, the lack of expression of these inflammatory mediators in this thesis may have been due to the absence of exposure to these factors present during surgery. The work herein highlights that inflammation within the myocardium in isolation is not caused by re-oxygenation injury at 20% O_2 for 1.5 hours. As it has been shown in other work that TLR expression within the blood circulation is affected by surgery on bypass, investigating expression of these genes within the active circulation could be the next logical step.

TLR4, TLR2 and MYD88 expression (see figure 3-14) within the myocardium was not changed during the 1.5 hours exposure to either hypoxia or normoxia. This indicates that such exposure does not cause an inflammatory response via the TLR mediated signalling pathway. This is supported by several studies that have shown normoxic O_2 exposure during cardiac surgery with CPB is less harmful than using hyperoxic exposure (Ihnken *et al*., 1998; Caputo *et al*., 2009). The comparison between normoxia and hyperoxia will be discussed in experiment 2.

The evidence within the literature, mostly involving animal models, does support the role of TLR4, TLR2 and MYD88 in being involved with inflammation caused during IR injury. Despite this, the methodology used is different than those used within this thesis. Evidence that looked at the expression of these genes during cardiac surgery is sparse and inconclusive. Also, such studies do not look at myocardial expression. This experiment indicated that normoxic reperfusion of ischemic myocardium does not cause the expression of TLR4, TLR2 and MYD88. Therefore, it is quite likely that it is the expression of circulating

inflammatory mediators responsible for myocardial inflammation caused during cardiac surgery rather than those in the myocardium. These findings are therefore novel.

Stress proteins:

The expression of HSP20, HSP27, HSP32 (figure 3-15), HSP60, HSP72 and HSP90 were investigated (figure 3-16). Exposure of the ischemic myocardium to 2% O_2 for 1.5 hours acted as an experimental control. There was no significant difference seen in the expression of the HSPs investigated. This was also true when the ischemic tissue was exposed to 20% for 1.5 hours.

HSPs are molecular chaperones that help maintain protein conformation and are induced by a variety of stressors including IR injury (reviewed in de Jong *et al*., 2009). The lack of HSP expression in the ischemic myocardium after 1.5 hours of 20% O_2 exposure suggests that exposure to normoxia does not cause enough stress to induce HSP expression. Also, the fact that only one time point was looked suggests that expression may have occurred at another time. However, 1.5 hours was chosen as it represented the time frame the myocardium is exposed to reperfusion (after release of cross-clamp) during surgical repair.

HSP expression during IR injury has been investigated in many studies within the literature using a variety of methods. There are several studies that have shown that overexpression of both HSP27 and HSP72 in rat cardiac cells reduced damage caused by exposure to ischemia and IR injury (Mestril *et al*., 1984; Liu *et al*., 2007; Kwon *et al*., 2007; Lu *et al*., 2008). These studies show prior induction of HSPs protects against IR injury in cardiac cells. Similar studies were carried out using animal models. The expression of HSP72 and HSP27 were induced prior to insult by the creation of a MI in a rat animal model. These rats and appropriate wild type controls were exposed to IR injury. When compared, rats with prior HSP expression had reduced cardiac dysfunction upon IR injury (Pantos *et al*., 2007). This was demonstrated within the heart in other studies using rat animal models for HSP27, HSP20 and HSP32. The only difference was the method of HSP induction prior to injury (Yet *et al*., 2001; Fan *et al*., 2005; Pachori *et al*.. 2006; Kwon *et al*., 2007; Lu *et al*., 2008). The work herein looks at HSP expression as a result of IR injury in the myocardium. This methodology is different than used in the examples from the literature described above. Therefore it is difficult to compare them to the results in this thesis. We had hoped that if we

were able to increase expression of HSPs in ischemic human myocardium upon reperfusion we could then determine if this conferred protection to further stresses. No such differences were observed.

Other animal models studies have shown that knocking out the expression of HSP32 and HSP72 can cause more damage by IR injury when compared to wild type controls (Kim *et al*., 2006; Yoshida *et al*., 2001). This shows that lack of HSP expression is detrimental to the heart during IR injury. It would suggest that the presence of HSPs within the myocardium promotes cardiac cell survival. The results within this thesis demonstrated the presence of HSPs within the myocardium. It just didn't indicate any statistically significance differences in their expression upon varied O_2 exposure. Therefore their very presence within the myocardium may provide protection without being significantly induced. These studies assume that the lack of HSP expression is the cause of more damage by IR injury. They do not look at HSP expression as a result of IR injury. Therefore, as with the other animal model studies described above, the methodology used is different from the work within this thesis.

There are also examples of HSP expression during cardiac surgery within the literature. The expression of HSP27, HSP60, HSP70 and HSC70 was investigated in tissue from the RA from patients undergoing surgical repair of congenital heart defects. Tissue was removed before the ischemic period i.e. before the aortic-clamp was applied and during the ischemic period i.e. after aortic clamping. No tissue was removed during reperfusion i.e. removal of aortic-clamp. No significant differences of expression were found but the levels of HSP72 increased in 40% of patients and the other HSPs increased in 28% of patients after cardioplegic arrest. These patients were associated with a reduction in the level of biomarkers of cardiac injury (Giannessi *et al*., 2003). This study shows an increase (non significant) of HSP expression within the heart during surgery and that such expression is associated with better post-operative cardiac function. It does not however show the effect of re-oxygenation on HSP expression within the myocardium. The methodology used in the work herein was different. In this thesis, the affect of re-oxygenation on myocardial gene expression was performed in isolation. The lack of HSP expression due to 1.5 hours of 20% O_2 exposure during this experiment suggests such re-oxygenation is not enough to induce the expression of HSPs within myocardium. Such a finding is novel.

HSPs are well known for being intracellular molecular chaperones. They are also extracellular where they interact with aspects of the immune system (reviewed in Calderwood (i) *et al*, 2007). It was shown in adult patients undergoing CABG with CPB, that HSP72 was elevated in patient plasma samples taken after surgery was performed (Dybdahl *et al*., 2002). The same group demonstrated this in a later study but it was shown that the increase in HSP72 within the plasma was more pronounced after such surgery on bypass in comparison to surgery off bypass (Dybdahl *et al*., 2004). Another study showed HSP70 expression was significantly lower in plasma from adult patients that had undergone surgery without bypass when compared to surgery with bypass (Lin *et al*., 2010). A similar increase was seen for the expression of HSP27, HSP60, HSP70 and HSP90α in plasma from adult patients undergoing CABG on bypass when compared to surgery without bypass (Szerafin *et al*., 2008). These studies show an elevation of HSPs after cardiac surgery with CPB, but this was measured within the blood circulation, not the heart tissue. The work herein looked at HSP expression in isolated myocardium exposed to re-oxygenation injury in media only. No increase was seen after 1.5 hour exposure to 20% O_2. The studies described above demonstrate an increase in HSPs in the systemic circulation during surgery on bypass. Taken together, the lack of exposure of the infant myocardium to an active circulation in the work in this thesis could account for the lack of HSP induction within the myocardiym. HSPs are known DAMPs. They could be released into the plasma due to cellular injury and initiate inflammation. This work in this thesis has shown a lack of induced HSP expression with the myocardium and has indicated that it would be of value to measure circulating HSPs in future work. It has highlighted the next stage of investigation.

The expression of HSPs within the myocardium of children undergoing corrective surgery for congenital heart defects such as TOF has been previously demonstrated (Nakumura *et al*., 2000; Takeuchi *et al*., 2003). The presence of HSPs in infant myocardium during such procedures has again been shown herein. However, exposure of ischemic tissue to 20% O_2 reperfusion for 1.5 hours did not induce HSP expression.

Within the literature, prior expression of HSPs has been shown to be protective against insult such as IR injury in both cardiac cells and animal models. Such evidence used a different methodology than the work within this thesis. The studies that looked at HSP expression during cardiac surgery focused mostly on extracellular HSP expression within the

patient serum. Again this methodology is different than used herein, which should be taken into consideration when comparing such studies to the results in this thesis. The work herein demonstrated HSP expression within infant myocardium and that it was unaffected by normoxic re-oxygenation. It suggests that normoxic exposure for 1.5 hours, which is similar to the length of O_2 exposure during bypass after removal of the clamp, does not cause cellular stress within the myocardium. It also suggested combined with evidence within the literature described above, that the lack of exposure to an active blood circulation could be responsible for this. Therefore, the next stage in the investigation of cellular stress within the myocardium during surgery on bypass has been highlighted.

This experiment had a limited number of clinical samples. This was due to the fact they were rare samples that were difficult to obtain. Also, while waiting for the hyperoxic reperfusion system to be set up, this experiment allowed the reperfusion system to be optimised and properly established. Also, it helped produce pilot data that allowed the hyperoxic reperfusion experiment to be improved.

Surgical correction of congenital defects is carried out on bypass using hyperoxic levels of O_2 (usually 250 to 400 mmHg) (reviewed in Morita, 2012). This experiment only exposed the myocardial tissue to normoxic exposure for 1.5 hours. No differences in expression were observed for the inflammatory mediators or the stress proteins. The setup of the hyperoxic experimental model was problematic but once solved, was the basis of the next piece of work within this thesis described below.

4.4.2 Experiment 2: exposing myocardial tissue to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2

The aim of this experiment (see section 3.3.2.2) was to look at the differential expression of inflammatory mediators and stress proteins within ischemic infant myocardium and to determine if they are affected when exposed to 20% and 60% O_2 for 4 hours. This re-enacts re-oxygenation injury of the myocardium *in vitro* and is an extension of experiment 1 described above except it incorporated two different levels of O_2 exposure. Also, as no expression was observed in experiment 1 after only 1.5 hours of exposure, this time period was extended to 4 hours. Using RNA extracted from the ventricular myocardium and appropriate primers, mRNA gene expression was investigated using qPCR in all relevant

patients for all the genes of interest. The effect of chronic ischemic injury that results in cyanosis was also investigated in relation to the expression of the genes of interest.

Inflammatory mediators:

The expression of TLR4 (figure 3-17A), TLR2 (figure 3-17B) and MYD88 (figure 3-17C) were investigated (figure 3-17). Myocardial tissue was exposed to 2% O_2 exposure for 4 hours, which acted as an experimental control and no significant change in expression were observed. Upon exposure to 20% and 60% O_2 exposure for 4 hours, no significant changes in expression in the myocardial tissue were observed. When looking at the results there was no apparent trend of expression that could be expanded on with more patient samples.

This lack of differential expression of TLR4, TLR2 and MYD88 during the 4 hours exposure to normoxia (20% O_2) also occurred in experiment 1 except exposure was only for 1.5 hours. This could be because neither 1.5 hours nor 4 hours of normoxic exposure can cause inflammation within infant myocardium. Also, the fact no change in expression was seen after 4 hours as well as 1.5 hours makes it unlikely a change occurred at another time point. This supports that normoxic re-oxygenation of myocardium in isolation does not cause inflammation.

Evidence describing TLR4, TLR2 and MYD88 expression during IR injury within the literature has been described. Such studies involved animal models (Chong *et al*., 2004; Oyama *et al*., 2005; Favre *et al*., 2007; Arslan *et al*., 2010; Feng *et al*., 2008) where lack of functional forms of these genes reduced the size of MI. These studies make an assumption that the lack of these gene results in less IR injury. The work in this thesis looks at the effect of IR injury on gene expression therefore uses a different methodology. Also some animal studies brought up the question of whether it was the expression of inflammatory mediators within the heart or in the blood circulation that were responsible for cardiac damage/ dysfunction (Tavener *et al*., 2004; Fallach *et al*., 2010; Baumgarten *et al*., 2006; Binck *et al*., 2005). No clear resolutions were drawn. It suggests that perhaps circulating inflammatory mediators are responsible for myocardial inflammation during cardiac surgery. This is supported by the lack of their expression within the myocardium in isolation after 1.5 or 4 hours of normoxic reperfusion. Despite this, the evidence within these studies is hard to compare to work within this thesis (different methodology and studies involving animals rather than human tissue).

There was also evidence of TLR expression in the model of cardiac surgery (Dybdahl *et al*., 2002; Bennett-Guerrero *et al*., 2007). The question of whether it was circulating TLR expression causing inflammation was again drawn into question (Dybdahl *et al*., 2002). Also, a lack of TLR expression (TLR4 inhibitor) was shown to have no effect on inflammation or cardiac function after cardiac surgery on bypass (Bennett-Guerrero *et al*., 2007). Work herein looked at the effect of re-oxygenation in myocardium in isolation. It is again hard to compare this work to the work in this thesis, as they did not look at myocardial expression in isolation but at the model of cardiac surgery with CPB as a whole.

Normoxic re-oxygenation at both 1.5 hours and 4 hours did not cause an inflammatory response in the myocardium alone. The evidence in the literature was difficult to compare to the work in this thesis due to different methodologies. This thesis has highlighted that it is unlikely to be myocardial expression of inflammatory mediators that causes myocardial inflammation. It is more likely to be caused by circulating immunological factors activated by CPB, which would cause inflammation upon myocardial reperfusion.

Exposure to 60% O_2 for 4 hours also did not produce a significant change of expression of inflammatory mediators in infant myocardium. This was not looked at in experiment 1. In the literature, it was shown by some studies that hyperoxic exposure during cardiac surgery with CPB caused more oxidative stress and damage than normoxic O_2 exposure (Ihnken *et al*., 1998; Caputo *et al*., 2009). This would suggest that hyperoxic exposure for 4 hours would have caused an increase in the expression of inflammatory mediators within this experiment. This was not the case. Reasons for this will be similar to the lack of expression upon normoxic exposure that is described above.

TLR2 and TLR4 and have been shown to be induced by hyperoxic exposure. However, these studies mostly investigated TLR expression within the lungs and involved the use of animal models and human epithelial cells (Huang *et al*., 2011; Zhang *et al*., 2005; Ogawa *et al*., 2007). Therefore, the methodology used was different and the results therefore are hard to compare to work in this thesis. There is also evidence in the literature that hyperoxia can cause inflammation during cardiac surgery. It was shown that cardiac surgery with CPB caused an increase in pro-inflammatory cytokines within the lungs when using 100% O_2 when compared to using 50% O_2 (Pizov *et al*., 2000). This suggests that hyperoxia can cause an increase in TLR4 and TLR2 expression, as they are often responsible for the production of pro-

inflammatory cytokines. However, this study involved the lungs and therefore a direct comparison cannot be made to the work in this thesis. It does however suggest an avenue for future investigation.

Some studies, which used a pig animal model of cyanotic infant myocardium, suggested that chronic ischemic exposure resulting in pre-operative cyanosis increased the susceptibility of damage caused by hyperoxic exposure (Ihnken (i) *et al*., 1995; Ihnken (ii) *et al*., 1995; Morita (i) *et al*., 1995; Morita (ii) *et al*., 1995). This was also shown during paediatric corrective surgery for congenital heart defects (Modi *et al*., 2002; Bulutcu *et al*., 2002). This would suggest that the expression of inflammatory mediators within the myocardium from the cyanotic patient group would be higher than in the acyanotic patient group when exposed to high levels of O_2. In this experiment in this thesis, the expression of TLR4 (see figure 3-21A), TLR2 (see figure 3-21B) and MYD88 (see figure 3-21C) after exposure to 4 hours of 2%, 20% and 60% O_2 was compared between cyanotic and acyanotic patients and there was no significant differences seen. This indicated that cyanotic myocardium is not more vulnerable to normoxic or hyperoxic IR injury when compared to the acyanotic group, at least when assessed by these markers.

The studies within the literature described above indicate that hyperoxic exposure is known to cause injury, but there have been several studies that have shown the opposite both during cardiac surgery and cardiac arrest (Mariero *et al*., 2012; Foadoddini *et al*., 2011; Bellomo *et al*., 2011; Hoedemaekers and van der Hoeven, 2011). Therefore, no hard conclusions can be drawn. The lack of TLR4, TLR2 and MYD88 expression during the work of this thesis suggests that hyperoxic exposure for 4 hours does not cause inflammation within infant myocardium. This could support that the use of hyperoxic levels of O_2 to keep patients oxygenated during cardiac surgery with CPB does not have any additional detrimental effects. Although, this work only shows that hyperoxia does not cause myocardial inflammation after 4 hours in isolation. As there was no exposure of myocardial tissue to an active circulation, it gives no indication of the effect of circulating inflammatory meditators on myocardial inflammation. Further experimentation could investigate this.

Overall, the expression of inflammatory mediators was not initiated within infant myocardium undergoing normoxic or hyperoxic exposure for 4 hours. Evidence within the

literature, which includes animal models and models of cardiac surgery, all tended to use different methodology. Therefore a direct comparison to work in this thesis was not possible. This work therefore demonstrates that normoxic and hyperoxic re-oxygenation of the myocardium does not initiate a myocardial inflammatory response. Therefore, it suggests, along with the body of evidence in the literature that the myocardial inflammation during cardiac surgery with CPB is caused by other factors. This is likely the exposure of the myocardium to circulating blood factors. Therefore, the next step in this investigation has been shown.

Stress proteins:

The expression of HSP20 (figure 3-18A), HSP27 (figure 3-18B), HSP32, (figure 3-18C), HSP60 (figure 3-19A), HSP72 (figure 3-19B) and HSP90 (figure 3-19C) were investigated. There were no significant changes observed for any HSP upon exposure to hypoxia (2% O_2) for 4 hours (control). Exposure to normoxia and hyperoxia for 4 hours only produced significant changes for HSP32 and HSP72. HSP90 showed an increase when exposed to both normoxia and hyperoxia for 4 hours but none reached statistical significance.

HSP32 and HSP72 expression were significantly increased in infant myocardium after 4 hours of normoxic exposure. This increase in expression was not caused after 1.5 hours in experiment 1. It could be that 1.5 hours was not long enough exposure at 20% O_2 to cause cellular stress. 4 hours is an exaggerated exposure to such O_2 levels. The fact that a change in expression is seen indicates that the experimental model works. This helps validate the lack of expression of inflammatory mediators and stress proteins seen in experiment 1 and 2.

Examples within the literature of HSP expression during IR injury have already been discussed in section 4.4.1. These include studies involving cardiac cells (Mestril *et al*., 1984; Fan *et al*., 2004; Liu *et al*., 2007' Kwon *et al*., 2007; Lu *et al*., 2008), and animal models (Yet *et al*., 2001; Fan *et al*., 2005; Pachori *et al*., 2006; Kwon *et al*., 2007; Lu *et al*., 2008). These studies mostly involved the overexpression of HSPs prior to insult, which resulted in protection against subsequent IR injury. HSP32 and HSP72 were both among the examples of HSPs shown to give such protection. Knockout mice were also used, which indicated lack of functional HSPs increased the damage done by IR injury (Kim *et al*., 2006; Yoshida *et al*., 2001). It is hard to compare such studies to the evidence in this thesis, as the methods used

were different and they involved cardiac cells and animal models. Although they demonstrate the protective nature of HSP32 and HSP72, they do not show their response to IR injury within myocardium as is demonstrated in the work herein. Therefore, the fact HSP32 and HSP72 were shown be induced by normoxic exposure for 4 hours in this thesis is a new finding. Also, based on these studies, such expression could be protective against subsequent insult.

Several studies involving HSP expression during cardiac surgery were also discussed previously (see section 4.4.1). Many showed an increase of extracellular HSP expression i.e. within the patient's plasma (Dybdahl *et al*., 2002; Dybdahl *et al*., 2004; Szerafin *et al*., 2008; Lin *et al*., 2010). Such studies did not look at expression of HSPs within the myocardium. They suggested extracellular HSP expression could be increased within the systemic circulation in the patients studied herein, which could cause localised myocardial inflammation within the myocardium i.e. HSPs acting as DAMPS. The work in this thesis only investigated the expression in myocardium exposed to differential levels of O_2 in isolation. Therefore, other factors must be involved in myocardial insult during cardiac surgery. This could be a future investigation.

HSP32 and HSP72 expression were significantly increased in infant myocardium after 4 hours of hyperoxic exposure. HSP expression and exposure to hyperoxia has been investigated in the literature but mostly in studies that involve the lungs. Human respiratory epithelial cells that overexpressed HSP72 were shown to have better survival against hyperoxic exposure (Wong *et al*., 1998). A lack of HSP27 expression in lung epithelial cells resulted in reduced protection against hyperoxic exposure (Shao *et al*., 2009). Overexpression of HSP32 provided protection against such exposure in human pulmonary epithelial cells (Lee *et al*., 1996). These studies show that HSP expression is important in protecting against hyperoxic exposure but not how it affects their expression. Also, these studies do not look at expression within the myocardium. Therefore it is not entirely appropriate to compare these experiments to the evidence in the work of this thesis. The fact they show that HSPs are protective against IR insult suggests that the induced expression of HSP32 and HSP72 shown in his thesis during hyperoxic insult could have a protective affect during surgery on bypass.

Hyperoxic exposure during cardiac surgery with CPB has been shown to cause more damage when compared to normoxic exposure during the same procedure (Ihnken *et al*., 1998; Caputo *et al*., 2009). Despite this, it has been suggested that the level of O_2 used does not matter (Mariero *et al*., 2012). The increase in HSP32 and HSP72 expression in myocardium seen herein was seen after both normoxic and hyperoxic exposure for 4 hours. As HSP expression occurs in relation to stress, this suggests that the level of O_2 tension used during cardiac surgery does not affect the degree of cellular stress. This was supported by the fact HSP32 expression during the 20% and 60% O_2 4 hour exposure was not significantly different (figure 3-20A). However, it was demonstrated that the level of HSP72 expression during 20% O_2 4 hour exposure was significantly higher than the expression seen during the 60% O_2 4 hour exposure (figure 3-20B). This would indicate that the level of O_2 used does make a difference in realtion to myocardial IR injury.

In this thesis, the myocardium was exposed to normoxia and hyperoxia for 4 hours (reoxygenation of ischemic myocardial tissue), which is longer than would occur during cardiac surgery with CPB. Therefore this increase in the expression of HSP32 and HSP72 within the infant myocardium may be caused by an exaggerated O_2 exposure. The fact this increase was not seen at 2% O_2 for 4 hours (control) helps validate it was the O_2 exposure that resulted in such cellular stress but whether using 20% or 60% O_2 makes a difference still remains unclear. In the case of hyperoxic exposure for 4 hours, as only one time point was looked at, the increase in HSP32 and HSP72 expression may have been present before the 4 hour time point. Also, patients are usually exposed to 100% O_2 not 60% O_2 during surgery and this is a more extreme exposure. Therefore, using higher levels of O_2 during surgery with CPB may still be of some concern.

HSP32 and HSP72 expression was significantly increased within the myocardium upon exposure to normoxia and hyperoxia for 4 hours. Such insult could cause HSP release (DAMPs) from cardiac cells that were damaged beyond repair. Extracellular HSPs can interact with aspects of the immune system resulting in pro-inflammatory cytokines release (Asea *et al*., 2000; Asea *et al*., 2002; Basu *et al*., 2002). They can also cause an anti-inflammatory response, with HSP32 being a prime example (Zabalgoitia *et al*., 2008; Yeh *et al*., 2009; Wang *et al*., 2009). In the work within this thesis, such release would have been seen within the

media the tissue was incubated in. This was not measured for the work in this thesis but could be investigated in the future.

HSP32 and HSP72 expression have been demonstrated in RV cyanotic myocardium of children undergoing corrective surgery for congenital heart defects (Nakamura *et al*., 2000; Takeuchi *et al*., 2003). Cyanotic myocardium has been shown to be especially vulnerable to hyperoxic exposure (Modi *et al*., 2002; Bulutcu *et al*., 2002). As with the inflammatory mediators, the expression of HSPs in myocardium exposed to 4 hours of 2%, 20% and 60% O_2 was compared between cyanotic and acyanotic patients. No significant differences in expression were seen (figure 3-22 and 3-23). This suggests that cellular stress in myocardium from cyanotic patients during re-oxygenation injury is not more exaggerated than within myocardium from acyanotic patients but this would require further investigation.

Overall, HSP32 and HSP72 mRNA expression was induced by normoxic or hyperoxic exposure for 4 hours. Prior induction of HSPs has been shown to protect against IR injury in studies involving both cardiac cells and animal model. Such work provides evidence of the protective nature of HSPs. It could be that the increase of HSP32 and HSP72 within the infant myocardium shown herein may be an innate mechanism the body employs to provide protection against oxidative insult.

The majority of studies that show HSP expression during cardiac surgery focus on extracellular HSP expression within patient plasma. They do not investigate myocardial HSP expression. They suggest circulating factors may contribute to myocardial cellular stress, likely contributing to the HSP induction already caused by high level O_2 exposure shown herein. This is shown by the fact that the work in this thesis investigated the expression within isolated myocardium, which lacks contact with an active circulation, and also by the evidence within the literature.

4.5 The differential mRNA expression of GRP78

GRP78 is a stress protein located within the ER. Upon cellular stress, such as nutrient deprivation, misfolded proteins can accumulate in the ER, which can cause the expression of

proteins like GRP78 to accommodate the problem (Shiu *et al*., 1977; Li *et al*., 1993; Miles *et al*., 1994; reviewed in Lee, 2001). It has been shown that the expression of GRP78 is up regulated in the heart during ischemic insult (Sun *et al*., 2008; Hardy and Raiter, 2010; Xin *et al*., 2011). It is also increased during IR injury, where it was shown to be cardio-protective (Martindale *et al*., 2006).

4.5.1 Experiment 1: Screening of differential gene expression in ventricular myocardium excised from cyanotic and control (acyanotic) patient groups.

The aim of this experiment was to look at the differential mRNA expression of GRP78 (see section 3.4) and to determine if there was any difference in expression seen between the acyanotic and cyanotic patient groups. Using RNA extracted from the ventricular myocardium and appropriate primers, qPCR was used to investigate changes in mRNA expression and any differences seen in gene expression were calculated using the Comparative Ct method (see section 2.9.2) to give RQ of gene expression.

There was no significant change in the expression of GRP78 within the myocardium when comparing the cyanotic patient group with the acyanotic patient group (see figure 3-24). GRP78 is induced during ischemic insult (Sun *et al*., 2008; Hardy and Raiter, 2010; Xin *et al*., 2011). Cyanotic patients, who include patients with TOF, also have such damage because their heart is chronically exposed to low levels of O_2 (Apitz *et al*., 2009). Therefore, it may have been expected that the cyanotic group would have a higher expression than the acyanotic group. When looking at the results (figure 3-24) it does appear that the level of GRP78 is higher in the cyanotic group but this did not reach statistical significance.

4.5.2 Experiment 2: Reperfusion Experiment - exposing myocardial tissue to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2

The aim of this experiment was to look at the differential expression of GRP78 in ischemic infant myocardium when exposed to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2 for 4 hours. Therefore, re-oxygenation injury of the myocardium was re-enacted *in vitro*. Using RNA extracted from the ventricular myocardium and appropriate primers, mRNA gene expression was investigated using qPCR (see section 2.9). RQ of gene expression was calculated using the comparative C_t method (see section 2.9.2). The affect of

chronic ischemic injury, that results in cyanosis, on the expression of GRP78 during re-oxygenation injury was also investigated.

Myocardial tissue was exposed to 2% O_2 exposure for 4 hours, which acted as an experimental control and no significant change in GRP78 expression were observed. There were no significant differences seen in GRP78 when infant myocardium was exposed 20% (normoxia) and 60% (hyperoxia) O_2 for 4 hours (see figure 3-25). It has been shown in a mouse animal model that GRP78 expression was increased when exposed to IR injury within the heart (Martindale *et al*., 2006). Therefore it may have been expected to be increased upon exposure to either 20% (normoxia) or 60% (hyperoxia) O_2 for 4 hours within this thesis. Despite this, the study was not performed in the infant myocardium. It was done using an animal model and therefore it is hard to compare it to the work herein.

The expression of GRP78 was found to be upregulated in cultured human endothelial cells during hyperoxic exposure and conferred protection against the insult. This increase in GRP78 expression was due to the knockdown of the ER protein ERp57. When ERp57 knockdown was removed, expression of GRP78 was reduced and apoptotic cell death was increased (Xu *et al*., 2009). Therefore, upregulation of GRP78 expression seemed to protect against hyperoxic exposure. The results presented in this thesis looked at the response of GRP78 upon re-oxygenation injury within infant myocardium. It might have been expected for GRP78 expression to be increased upon hyperoxic exposure but this was not the case. The study described above involved the lungs and the use of a mouse animal model. Therefore despite GRP78 providing protection against hyperoxic insult, the results of this study cannot be easily compared to the work herein. In another study, the response of GRP78 to cigarette smoking was investigated. Smoking cigarettes exposes the lungs to a high level of ROS, which is a consequence of hyperoxic exposure. The expression of GRP78 was upregulated in lung tissue taken from smokers when compared to non-smokers and ex-smokers. Therefore GRP78 is increased upon hyperoxic exposure within the lungs (Kelsen *et al*., 2008). The work herein looked at the effect of hyperoxic exposure within the myocardium not the lungs. Also, smoking exposes tissue to other damaging factors. Therefore, this study cannot easily be compared to the results in this thesis despite it involving human subjects. This is because there will be other factors contributing to ER instability other than the ROS.

A comparison of GRP78 expression was performed with the tissue exposed to 2% O_2 for 4 hours, 20% O_2 for 4 hours and 60% O_2 for 4 hours between the cyanotic and acyanotic groups. No statistical differences were observed (figure 3-26). Cyanotic patient myocardium had been suggested as being more vulnerable than acyanotic patient myocardium but this experiment indicates that this is not the case.

In the literature GRP78 was induced by ischemic insult. Patients with congenital heart defects such as TOF are chronically exposed to low O_2 tension in their circulation resulting in cyanosis. There was no difference seen in GRP78 expression in the acyanotic and cyanotic patient groups in experiment 1. In experiment 2, GRP78 expression was unaffected by 4 hour exposure to 20% or 60% O_2. It has been demonstrated that GRP78 can be induced by hyperoxic exposure but this has not been shown within infant myocardium. The results of the present work suggest that normoxic and hyperoxic exposure does not cause ER stress and that cyanotic patient myocardium in not more vulnerable to IR injury than acyanotic patient myocardium.

4.6 The differential mRNA expression of APJ and its endogenous ligand Apelin

The Apelin receptor (APJ) and its cognate ligand Apelin have been implicated as having either a role as a vasodilator (Lee *et al*., 2000; Tatemoto *et al*., 2001; Cheng *et al*., 2003) or as having a hypothalamic role (De Mota *et al*., 2000; Reaux *et al*., 2001; Taheri *et al*., 2002; De Mota *et al*., 2004; Reaux-Le Goazigo *et al*., 2004). They are expressed in the heart where they improve cardiac contractile function (Szokodi *et al*., 2002; Berry *et al*., 2004; Ashley *et al*., 2005) and protects against cellular insult (Jia *et al*., 2006; Atluri *et al*., 2007).

4.6.1 Experiment 1: Screening of differential gene expression in ventricular myocardium excised from cyanotic and control (acyanotic) patient groups

The aim of this experiment was to look at the differential expression of APJ (see section 3-5) and to determine if there was any difference in expression seen between the acyanotic and cyanotic patient groups. Using RNA extracted from the ventricular myocardium and appropriate primers, qPCR was used to investigate changes in mRNA expression and any differences seen in gene expression were calculated using the Comparative Ct method (see section 2.9.2) to give RQ of gene expression.

There was no significant change in the mRNA expression of APJ when comparing the cyanotic patient group with the acyanotic patient group (figure 3-27). There was a study that looked at the mRNA and protein level of Apelin and APJ in a rat animal model of myocardial injury caused by the administration of isoproterenol. In such rats, the mRNA levels of Apelin and APJ in the myocardium were noticeably reduced. However the protein level of APJ in the myocardium was increased (Jia *et al*., 2006). Cyanotic patients, who include individuals with TOF, are chronically exposed to low O_2 tension in the blood. This is a form of myocardial injury. Therefore, it might have been expected that the mRNA level of APJ would have been reduced in the cyanotic group when compared to the acyanotic group. The mRNA expression of Apelin, the ligand of APJ, was found to be decreased in cultured neonatal rat ventricular myocytes exposed to mechanical stretch and in two models of chronic ventricular pressure overload. These are forms of cellular stress. As the mRNA expression of Apelin was reduced, one may have expected that APJ expression would also be lowered (Szokodi *et al*., 2002). Therefore, it may have been expected that APJ mRNA expression would be decreased in the cyanotic patient group, which have pre-operative ischemic damage. It was also shown using a mouse animal model that in heart and skeletal muscle the expression of Apelin and APJ were increased after ischemic myocardial injury. Also, in cultured endothelial cells exposed to hypoxia, Apelin mRNA and protein levels were increased by hypoxia (Sheikh *et al*., 2008). This would suggest that APJ mRNA expression would have been expected to be higher in the cyanotic patient group. This increase in Apelin and APJ expression after ischemic insult was again seen in another study but this was shown at the protein level (Atluri *et al*., 2007). There is therefore contradictory data in the literature as to how the expression of APJ and Apelin are affected by cardiac cellular insult. These studies are performed using both cardiac cells and animal models and do not look at myocardial APJ expression. As such, comparison to the work herein is problematic.

4.6.2 Experiment 2: Reperfusion Experiment - exposing myocardial tissue to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2

The aim of this experiment was to look at the differential expression of APJ in ischemic infant myocardium when exposed to 2% (hypoxia), 20% (normoxia) and 60% (hyperoxia) O_2 for 4 hours. Therefore, re-oxygenation injury of the myocardium was re-enacted *in vitro*. Using RNA extracted from the ventricular myocardium and appropriate primers, mRNA gene expression was investigated using qPCR (see section 2.9). Relative

quantification of gene expression was calculated using the comparative C_t method (see section 2.9.2). The affect of chronic ischemic injury, that results in cyanosis, on the expression of APJ after re-oxygenation injury was also investigated.

Myocardial tissue was exposed to 2% O_2 exposure for 4 hours, which acted as an experimental control and no significant change in APJ mRNA expression was observed (figure 3-28). There were no significant differences seen in APJ when infant myocardium was exposed to 20% (normoxia) and 60% (hyperoxia) O_2 for 4 hours (figure 3-28). A study was performed that looked at the expression of Apelin and APJ during IR injury using a rat animal model and cultured myocardial cells from neonatal rats. It was shown that rats exposed to IR injury had increased mRNA and protein levels of APJ as well as having a decrease in cardiac function. The myocardial cells exposed to IR injury had an increase in the mRNA levels of APJ and Apelin as well as an increase in apoptosis (Zeng *et al*., 2009). This may have suggested that exposure to 20% or 60% O_2 should have increased the expression of APJ. The same study indicated that pre-administration of Apelin improved the resulting cardiac dysfunction in the rats and also reduced damage sustained by the myocardial cells when exposed to IR injury. Such action of Apelin has been seen in other studies (Jia *et al*., 2006; Atluri *et al*., 2007). There are numerous studies using animal models that show that administration of exogenous Apelin can improve cardiac contractibility function which again is an improvement in cardiac function (Szokodi *et al*., 2002; Berry *et al*., 2004; Ashley *et al*., 2005). Taken together, such evidence suggests cellular insult such as ischemic insult and IR injury causes an increase in Apelin and APJ expression, but the pre-administration of exogenous Apelin provides cardio-protection by improving cardiac function. There was one study that indicated myocardial insult caused a decrease in APJ and Apelin mRNA expression within rat myocardium, which would disagree with this. However, the APJ protein expression was increased in the rat myocardium during the insult, which would agree with this evidence (Jia *et al*., 2006). Also, APJ mRNA was decreased after mechanical cardiac insult in cultured neonatal rat ventricular myocytes (Szokodi *et al*., 2002).

The results of this experiment indicates that APJ was unaffected by the exposure to 20% and 60% O_2 for 4 hours. The evidence within the literature is confusing therefore the results herein are not contradicted. Also, the evidence in the literature was based on work involving animal models. This involved different methodology than used within this thesis.

Therefore a direct comparison with this work and the studies within the literature is difficult. There was only one timepoint used in this experiment therefore changes in expression may be seen at other time points.

A comparison of APJ mRNA expression was performed with the tissue exposed to 2% O_2 for 4 hours, 20% O_2 for 4 hours and 60% O_2 for 4 hours between the cyanotic and acyanotic groups. No statistical differences were observed (figure 3-29).

Overall, in experiment 1, APJ expression within the myocardium was not different in the cyanotic and acyanotic patient groups. There is evidence in the literature that suggests cardiac ischemic and myocardial insult results in both a decrease or increase in APJ and Apelin expression. Cyanotic patients have pre-operative ischemic damage, which may have indicated that a difference in APJ expression would have been expected. In experiment 2, it is indicated that IR injury and other types of myocardial insult result in mainly an increase in the expression of APJ and Apelin, though some indicate the opposite. This would have again suggested that exposure of the infant myocardium to 20% and 60% O_2 for 4 hours would have produced a change in APJ expression. For both experiments, the evidence in the literature is inconclusive. Despite this, most suggest some change in APJ expression upon cardiac insult, therefore the lack of change seen in APJ expression in both experiments may not be what was expected. Also, these studies use animal models therefore cannot be directly compared to evidence within this thesis. There was no apparent difference seen when comparing the expression of APJ during varying O_2 exposure in the cyanotic patient group compared to the acyanotic patient group. It has been shown by others that administration of exogenous Apelin has been shown to improve cardiac dysfunction. This would indicate an interesting avenue of investigation for the future.

4.7 Decay curve experiments

The aim of these experiments was to investigate how the expression patterns of genes of interest within the infant myocardium were affected by varying incubations times. This experiment acted as a control for all other experiments as it showed whether expression seen was due to the variable used during the experiment or due to the length of time in the incubator. This experiment was very important for any results that demonstrated significant

changes in expression. A screening of the literature showed that other investigators have not performed these control experiments.

Inflammatory mediators:

The expression of TLR4, TLR2 and MYD88 was investigated (figure 3-30). Looking at the expression at all timepoints for all 3 TOF patients, there was no obvious pattern in expression. This mean the expression seen for these genes in the other experiments are due to the experimental environment the tissue was exposed to.

Stress proteins:

HSP20 and HSP27 did not show any particular trends in expression over incubation time (figure 3-31). HSP32 (figure 3-31) appeared to have an increase in expression at about 4-6 hours of incubation. HSP72, HSP60 and HSP90 (figure 3-32) all appeared to have slight trend of increase as time of incubation increased. Such changes seen did not appear to be significant.

The 6 hour time point relates to the HSP induction experiment 1 when looking at mRNA gene expression. HSP32, HSP72 and HSP90 were all induced upon exposure to heavy metal for 6 hours. Their induction was also increased in media only for 6 hours (control) but the degree by which the heavy metals increased expression was significantly higher than the increase in expression caused by the control. HSP32, HSP72 and HSP90 all increase with incubation time in this experiment (figure 3-31 and figure 3-32). This would draw into question whether or not the change in expression seen was due to the heavy metal exposure. The degree by which HSPs are increased by heavy metals compared to the gradual increase seen in this experiment by itself suggests that the heavy metal exposure was critical in increasing the expression of these HSPs. HSP32 seems to have a more immediate jump in expression at 4-6 hours in this experiment. When one compares the difference in RQ values seen for heavy metal induction of HSP32 when compared to the increase in RQ value of HSP32 in this experiment at 4-6 hours, there is a very distinct difference. This again supports that the heavy metals genuinely induced HSP expression in the infant myocardium. Although, the incubation time the tissue was exposed to cannot be disregarded as a factor. This is supported by the fact that for HSP32, HSP72 and HSP90, the tissue in media only also showed

a significant increase in expression after 6 hours. There was no heavy metal exposure of these tissues.

Overall, this experiment was a very important control for all other experiments performed as it validated others results found in other experiments.

4.8 Association of HSP-27 expression with improved RV function suggests a protective effect in cyanotic TOF

Although TOF (see section 1.1.3) can present with a wide spectrum of clinical and anatomical variations, the tetrad of anatomical defects that are prominent in the condition are RV outflow tract obstruction, VSD, biventricular origin of the aortic valve and RV hypertrophy. Early and late morbidity is related to the function of the right side of the heart which is exposed to increased pressure pre- and post-operatively. A larger VSD and outflow tract obstruction subjects the RV to chronic pressure overloading prior to surgery resulting in compromised function and slower recovery after repair (Wernovsky *et al*., 1995). Abnormal post-operative load within the ventricle, which can be due to residual lesions or pulmonary regurgitation, can lead to RV dilation, right heart failure, conductance disturbance, dysrhythmias and sudden death. Therefore, despite surgical repair, the patients may not be fully cured. This had led, over the last 50 years, to an expanding adult population with compromised right heart function (Davlouros *et al*., 2006).

This study is important since it is one of few that have investigated HSP levels in human infant myocardium. The study was powered based on previous studies from our group (Peng *et al*., 2011). The major finding was that cyanotic TOF myocardium baseline HSP27 levels (which close as possible represents pre-surgery status) was significantly increased compared to the non-cyanotic group and was also associated with better cardiac output/ contraction. Thus HSP27 may be important in myocardial protection. The cyanotic group have lower oxygen saturations and are more vulnerable than non-cyanotic patients. Interestingly this increase in HSP27 was not apparent in the tissue collected and combined after 15 minutes until the end of surgery (typically 15- 45 minutes). Whether this relates to protein stability, the surgical procedure or some other factor remains to be elucidated. Other possibilities are that as surgery time advances other protective mechanisms such as other protective HSPs are

increased to maintain protection. Also prior to surgery the myocardium from cyanotic and non-cyanotic groups were expose to different conditions (<15 minute group) whereas the >15 minute group are all now exposed to the same O_2 environment. Thus, even though after 15 minutes the HSP27 levels are reduced, it may be that post-operative function is improved because of other protective mechanisms being switched on during the cross clamp time. These would include other protective HSPs or it may be that repair mechanisms have been set in place by the increase in HSP27 (to repair protein folding) and once this happens the HSP27 declines.

An association of HSP27 was found with one out of the three echo ventricular function parameters (septal region) but not in the RV or LV free wall. This may be linked to the fact that the majority of muscle is removed from the septal area.

Despite a similar reduction in RV function in both groups the cyanotic group had higher troponin levels. Possible explanations are the longer cross-clamp and bypass time, more muscle resected, reduced myocardial protection or susceptibility to injury in the cyanotic group. Pediatric patients undergoing cardiac surgery have been shown to demonstrate greater susceptibility to IR injury and worse clinical outcome when cyanotic.

HSP27 (see section 1.6) is one of the most widely distributed of the small HSPs (Ghayour-Mobarhan *et al*., 2012). Animal and cell culture models have shown that the roles of HSP27 include protein chaperone function, regulation of glutathione levels and apoptosis (Arrigo *et al*., 2007) and heart tube formation (Brown *et al*., 2007). Intracellular HSP27 protects cells against external stress such as heat shock, mechanical and oxidative stress (Carper *et al*., 1997). HSP27 is also thought to be involved in an autoimmune response (Ghayour-Mobarhan *et al*., 2012). Like other small HSPs, HSP27 is regulated at both the transcriptional and post-translational level. HSP27 is phosphorylated by MAPKAP kinase 2 as a result of the activation of the p38 MAP kinase pathway (Arrigo *et al*., 2007). Since phosphorylated HSP27 is linked to actin-myosin interactions, it would be of interest in future studies to assess phosphorylated HSP27 and sarcomere structure in TOF.

Little is known about the role of HSP27 in the infant human heart. Most of the current knowledge has come from animal models or limited studies on adult cardiac disease. HSP27

expression has been shown to protect against myocardial infarction (Willis and Patterson, 2010; Efthymiou *et al*., 2004) and is increased in rat LV myocardium after coronary ligation and in human patients with dilated cardiomyopathy (Knowlton *et al*., 1998). Studies on HSP27 knockout mice have shown that, at least in this species, HSP27 is not essential for cardiac development but plays a role in the antioxidative response during IR injury (Willis and Patterson, 2010). HSP27 delivered with a virus vector into neonatal rat cardiomyoctes was shown to protect against apoptosis as well as against hypoxic stress (Brar *et al*., 1999) and modest increases in HSP27 have also been shown to protect against IR injury in dogs (Willis and Patterson 2010). These studies suggest that HSP27 plays a role in antioxidant mechanisms during IR injury.

Studies on the cardio-protective roles of other HSPs in right heart function are limited but suggest HSP32 and HSP72 may be cardio-protective in the right heart (Willis and Patterson, 2010; Peng *et al*., 2011; Nakamura *et al*., 2000). As well as HSPs it may be that other mediators of delayed ischemic pre-conditioning may occur in the myocardium and these will be the focus of future investigation. These may include investigation of anti-oxidant enzymes such as superoxide dismustase, nitric oxide synthase and cyclooxygenase (reviewed in Eisen *et al*., 2004).

Drugs are now used to increase cellular HSPs as therapy for neurodegenerative diseases, which are caused by misfolded proteins (Nakamura *et al*., 2000). Such drugs, gene therapy procedures as well as remote pre-conditioning to increase HSPs prior to surgery may one day be used in congenital heart disease in children. Before this can happen more studies such as the one herein are required to fully understand the role of HSPs in TOF.

4.9 Association of APJ expression with improved right ventricle function suggests a protective effect in cyanotic TOF

This is the first study to have shown evidence of APJ receptor expression in the early, infant myocardium. There has been extensive study into the expression and function of Apelin-APJ in the adult heart and its role in heart failure (Berry *et al*., 2004; Japp and Newby, 2008; Chandrasekaran *et al*., 2010; Falcão-Pires *et al*., 2010; Japp *et al*., 2010; Tycinska *et al*., 2012), but the characteristics of Apelin-APJ expression and function has never been established in the context of paediatric cardiac surgery for congenital heart defect repair.

Western blot analysis of the APJ receptor produced evidence that the APJ receptor is expressed in the RV of the infant myocardium. This analysis showed two band widths of sizes 50 and 60kD. These bands correspond to the findings of Atluri *et al* showing the isolated APJ receptor at 50kD and the glycosylated receptor represented at 60kD (Atluri *et al*., 2007).

This study has shown not only that the APJ receptor is expressed in the infant myocardium but also that expression of the phosphorylated APJ receptor increases as surgery progresses and, therefore, as time of cardioplegia and ischemia increases (figure- 3-40). These findings tie in with the hypothesis that ischemic conditions upregulate expression of APJ in the myocardium as part of a cardio-protective mechanism. Apelin-APJ expression being upregulated in this work could be considered part of a cardio-protective mechanism due to the physiological effects of Apelin (intropy, peripheral vasodilation and diuresis) taking strain off the heart and increasing cardiac output, as well as the known property of Apelin that it prevents myocardial damage through ischemia by reducing ER induced apoptosis (Tao *et al*., 2011). It has been hypothesized in other studies of Apelin in surgery that it may function to restore the epithelium of arteries after cessation of ischemia (Du *et al*., 2010). This provides further evidence that expression of Apelin-APJ is induced by ischemic conditions and provides information on the stress the heart is subjected to when undergoing CPB. In future studies we will expose myocardium to ischemic stress *in vitro* and monitor changes on APJ expression and Apelin release.

The rise in APJ expression as time in surgery progresses was not seen when each group, cyanotic and non-cyanotic, were looked at individually but was only seen when the entire patient pool was analysed together. There may be two reasons for this observation. Firstly, the sample size and power of the study may not have been sufficient to detect any differences in expression; as when the groups were separated the number of patients used in the calculation was smaller. Secondly, it could be hypothesized that the expression of APJ and changing expression of APJ is not related to pre-operative cyanotic status and is primarily a result of the ischemic stress and myocardial damage sustained by the myocardium during the procedure.

4.9.1 SvO_2

APJ expression in the period of surgery after 10 minutes was correlated to an increased SvO_2 (figure 3-41). SvO_2 is affected by O_2 uptake in the periphery, initial oxygenation of

blood and cardiac output. It can therefore be hypothesised that the increase in APJ expression after 10 minutes of surgery increased post-operative day 1 SvO_2 by increasing cardiac output in the post-operative recovery. This conclusion can be drawn given that Apelin-APJ function increases cardiac contractility and reduces ischemia-induced myocardiocyte damage.

4.9.2 Limitations

One of the main limitations of this study is the sample size as it was a pilot study. The samples in this study were from the same patient pool that was used in a previous MD study (Peng, 2010). The samples of myocardium resected for this project were all from the RV or RVOT. It is not known whether the expression of APJ receptor in these areas is representative for the rest of the infant heart. It is however the RV which is most at risk of post-operative damage in this case and is therefore the most appropriate place of the myocardium to measure APJ expression as affected by the surgery.

4.9.3 Conclusions

The known physiological functions of Apelin-APJ have made the system a focus for exogenous treatment in the context of heart failure since its discovery (Berry *et al*., 2004; Japp and Newby, 2008; Chandrasekaran *et al*., 2010; Falcão-Pires *et al*., 2010; Japp *et al*., 2010; Tycinska *et al*., 2012). Successful clinical trials (Tatemoto *et al*., 2001) and a new non-peptide APJ receptor agonist being described (Iturroiz *et al*., 2010) suggest that exogenous Apelin treatment is a possibility in heart failure. This project, however, presents the new hypothesis that, pending further study, Apelin administration could be considered in the context of congenital heart defects and prophylactic cardioprotection for surgical repair involving CPB.

Apelin-13 administration increases cardiac performance and protects the heart from damage in severe, end stage heart failure in mice (Koguchi *et al*., 2012), a time of ischemia and strain on the myocardium. CPB during correction of TOF induces comparable, if not similar, levels of cardiac stress (Allen *et al*., 1997). An animal model capable of reproducing the conditions of a CPB could further our knowledge of the potential benefit of Apelin-APJ function in a procedure of this nature.

For the first time, this thesis shows evidence that the APJ receptor is expressed in the infant human heart. The role of Apelin-APJ function is not fully understood in the context of TOF and surgery involving CPB. However, this study has shown that the stress and ischemic

conditions of such surgery can increase the expression of phosphorylated APJ receptor in the cells of the RV. There is also a correlation between increased levels of phosphorylated APJ receptor and a measure of improved clinical post-op recovery (SvO_2). There is much study needed in the area of Apelin-APJ function, however this project indicates a role for Apelin and APJ as a marker of ischemia-induced cardiac damage, as well as a potential therapeutic target for improving post-operative cardiac function. This warrants further investigation.

4.10 Concluding remarks

The primary aim of this project was to investigate the molecular changes that occur within the infant myocardium in the context of congenital heart disease by comparing cyanotic and acyanotic myocardium during the ischemic phase. The myocardium of children with cyanotic heart defects is exposed to chronic hypoxia whereas those with acyanotic defects are not. The study of these molecular mechanisms was also examined *in vitro* by attempting to mimic the re-oxygenation of the myocardium which occurs when the aortic-clamp is removed during surgery. In these procedures, the child is often exposed to high levels of O_2. The effect of these high O_2 concentrations on the myocardial molecular mechanisms in both the cyanotic and acyanotic patients groups were also looked at. This thesis focused on the expression of inflammatory mediators and stress proteins i.e. TLRs (see section 1.4) and HSPs (see section 1.6). Additional genes were also investigated, including the ER stress protein GRP78 (see section 3.4) and APJ (see section 3.5 and 3.6).

Initially the expression of these genes during the ischemic phase of corrective cardiac surgery was examined. This was performed to reflect as near to pre-operative gene expression levels as possible. The expression of these genes within myocardium in the two patient groups was compared. Expression of TLR4 ($p=0.0303$), TLR2 ($p=0.0177$) and HSP27 (0.0303) was significantly higher in cyanotic patient myocardium. This could indicate a consequence of the hypoxic environment to which the myocardium was exposed.

With regards to re-oxygenation injury of ischemic myocardium, exposure to 20% and 60% O_2 for 4 hours significantly induced mRNA expression of HSP32 ($p=0.0391$ and $p=0.0078$ respectively) and HSP72 ($p=0.0078$ and $p=0.0078$ respectively). This could indicate cellular stress during re-oxygenation of infant myocardium during surgical correction. These

changes were seen in both groups and no difference was found between the cyanotic and acyanotic patient group.

This project is mainly a scientific investigation. The changes seen are not related to any clinical data that describes cardiac function or contraction. Therefore, the molecular findings can only be looked at in the context in which the experiments were carried out. This is not the case for the investigation of HSP27 and APJ protein expression in TOF myocardium over ischemic time (see sections 3.7 and 3.8 respectively), which did relate to findings of clinical outcome.

The experiments, within this thesis examined myocardial gene and protein expression only. Whether factors within the systemic circulation are altered requires further investigation. Therefore, the myocardial inflammation that is seen during cardiac surgery on bypass may be caused by exposure to other circulating factors as re-oxygenation *in vitro* only affected some HSPs but not the inflammatory mediators studied.

Therefore it was important to do the work within this thesis in the controlled *in vitro* environment as the results obtained give initial insight into inflammatory changes and highlighted the need for future studies to look at the effect of circulating factors on myocardial inflammation. Incubating the myocardium with blood from the CPB unit could potentially address many of these issues, as this would contain biological factors that could influence the activation of certain genes of interest. Also, a comparison between clinical data about cardiac function and contraction and the differential expression of our genes of interest would allow the scientific findings more clinically relevant.

This project is novel because it looked at the expression of inflammatory mediators and stress proteins within the infant myocardium and how these genes were influenced by re-oxygenation injury. It also examined how such expression was affected by cyanosis. Despite the lack of correlation to cardiac functional data, this project allowed a better understanding about how these genes are expressed in the myocardium alone and how re-oxygenation can affect them. It has also highlighted the best ways in which such investigations could be extended. It has contributed important molecular data to the area of research and could

potentially help lead to an improvement in myocardial protection during cardiac surgery with CPB.

4.11 Plans for the future

Overall, some general potential future plans for this project include the following:

- Increase the patient numbers to increase the accuracy of results and look for more subtle changes.
- Include different and more time points of tissue incubation to enhance the data already recorded.
- Include more investigation of differential expression at the protein level to enhance the molecular data collected.
- Using different and more complex media including blood particularly taken from the infant during surgery at differing time points.

Reoxygenation at hyperoxic levels was the main focus and the project. Given more time, a bigger sample base would allow a more in depth investigation into the differences in expression between cyanotic and acyanotic myocardium than was possible during this project. Also, during these experiments, the ischemic tissue was exposed to high levels of O_2 in media only and therefore the tissue was not exposed to an active circulation. Therefore it would have been interesting to expose tissue to media supplemrnted with blood cardioplegia to try and determine if this would represent a more physiological state. This project highlighted the likelihood of such myocardial exposure as a possible source of inflammation. Therefore investigation into measuring circulating inflammaorty factors within the systemic circulation of patients (such as pro-inflammatory cytokines and circulating HSPs) that have tissue excised would allow the scientific data obtained to be compared to clinical information.

Appendices

1. Information sheets

1.1 Information sheet for TOF patient group:

PARENT/GUARDIAN INFORMATION SHEET – VERSION 3 (08/10/09)

PART ONE

1. Study title:

Myocardial protein expression in children undergoing cardiac surgery *(Full title: Myocardial cellular adaption to ischemia−reperfusion in children undergoing cardiac surgery: an integrated clinical and scientific approach to understand the molecular mechanisms involved).*

2. Invitation paragraph

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.

- **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.

Ask us if there is anything that is **not clear** or if you would like **more information**. Take time to decide whether or not you wish to take part.

3. What is the purpose of the research project?

To investigate how the heart muscle in children with congenital heart disorders functions caused during corrective heart surgery by looking at the expression of certain cellular proteins. By understanding the possible underlying mechanisms occurring in such children, we hope in the future to improve the protective methods employed during surgery and improve post-operative patient outcome.

4. Why has my child been chosen?

Your child has a heart condition called Tetralogy of Fallot (TOF), which is characterised by an obstruction to blood flow from the right side of the heart to the lungs (right ventricular outflow tract obstruction) and by blood flow between the two main pumping chambers of the heart, which is usually absent (ventricular septal defect). Patients with TOF have high pressure and low oxygen levels in the blood within the right ventricle of their heart, which can cause an impairment of heart contraction. Investigating the underlying mechanisms that occur in TOF could help improve our understanding and management of such conditions.

5. Does my child have to take part?

NO. It is up to you and your child (wherever possible) to decide whether or not to take part. You are both free to withdraw from the research at any time and without giving a reason. Your decision about this will not affect the standard of care your child will receive. 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.

6. What does my child have to do if we agree to take part?
This study will not alter the routine care 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 assessment will be more detailed to include a special mode to analyse the heart wall movement. This may take approximately 5-10 minutes longer than the usual examination. Also routine heart tracing will be done, where an electrical recording of the heart beat is taken to ensure that the heart is working properly.
During your child's surgery, removal of heart muscle tissue is routinely undertaken as part of the standard procedure to relieve obstruction in the right heart chamber to restore normal blood flow to the lungs. Such tissues are not normally retained for laboratory investigation, but will be for use in this study.
Apart from routine blood sampling, additional tests will be performed for cardiac hormone analysis. The child will not need an additional needle prick as the samples will be obtained during routine blood sampling. At the times when blood will be routinely be taken from your child for normal clinical purposes, 2 extra teaspoons of blood will be taken at 4 different times over the 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.

7. What are the possible benefits of taking part?
A more detailed and more frequent echocardiography assessment of the heart function will be performed for every participant. As this study does not alter the surgical or clinical care of your child, we do not anticipate any potential complications beyond that expected of the normal surgical treatment of your child's condition.

8. 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.

9. Who can I contact for further information?
If you require any further information on this study or will like to clarify any issues, please do not hesitate to contact us. Our contacts are as follows:

- Mr Mark Danton, Consultant Cardiac Surgeon, Yorkhill (01412010251)
- Miss Susan Walker, PhD student, University of Glasgow (01412010367)
- Dr W. B. Knight, Consultant Cardiac Surgeon, Yorkhill (brodie.knight@yorkhill.scot.nhs.uk)

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
1. 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.

2. 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.

3. 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, phone number: 201 0251 or 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.
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.

4. 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 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.

5. 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 injury caused during heart surgery.

6. Will any genetic tests be done? No.

7. 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. 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.

8. 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 in the University of Glasgow.

9. Who has reviewed the study?
This study was given formal ethical approval by the West of Scotland Research Ethics Committee 2 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.

1.2 Information sheet for control patient group:

PARENT/GUARDIAN INFORMATION SHEET – VERSION 4 (08/10/09)

PART ONE

1. Study title:

Myocardial protein expression in children undergoing cardiac surgery *(Full title: Myocardial cellular adaption to ischemia−reperfusion in children undergoing cardiac surgery: an integrated clinical and scientific approach to understand the molecular mechanisms involved).*

2. Invitation paragraph

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.

- **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.

Ask us if there is anything that is **not clear** or if you would like **more information**. Take time to decide whether or not you wish to take part.

3. What is the purpose of the research project?

To investigate how the heart muscle in children with congenital heart disorders functions during corrective heart surgery by looking at the expression of certain cellular proteins. By understanding the possible underlying mechanisms occurring in such children, we hope in the future to improve the protective methods employed during surgery and improve post-operative patient outcome.

4. Why has my child been chosen?

Your child has a heart condition called subaortic stenosis (narrowing of the left ventricular outflow tract, which obstructs blood from leaving the heart). As part of the standard surgical operation, a piece of muscle is removed to minimise the recurrence of this condition. This muscle tissue is not usually kept but we would like to retain this muscle specimen for protein analysis as part of a wider clinical investigation. Your child will be part of a control group for this study and any tissue you allow to be used will be a gift to others, with no direct benefits to your child.

5. Does my child have to take part?

NO. It is up to you and your child (wherever possible) to decide whether or not to take part. You are both free to withdraw from the research at any time and without giving a reason. Your decision about this will not affect the standard of care your child will receive. 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.

6. What does my child have to do if we agree to take part?

This study will not alter the routine care 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 assessment will be more detailed to include a special mode to analyse the heart wall movement. This may take approximately 5-10 minutes longer than the usual examination. Also routine heart tracing will be done, where an electrical recording of the heart beat is taken to ensure that the heart is working properly.
During your child's surgery, removal of heart muscle tissue is routinely undertaken as part of the standard procedure to relieve obstruction in the left heart chamber to restore normal blood flow to the rest of the body. Such tissues are not normally retained for laboratory investigation, but will be for use in this study.
Apart from routine blood sampling, additional tests will be performed for cardiac hormone analysis. The child will not need an additional needle prick as the samples will be obtained during routine blood sampling. At the times when blood will be routinely be taken from your child for normal clinical purposes, 2 extra teaspoons of blood will be taken at 4 different times over the 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.

7. What are the possible benefits of taking part?
A more detailed and more frequent echocardiography assessment of the heart function will be performed for every participant. As this study does not alter the surgical or clinical care of your child, we do not anticipate any potential complications beyond that expected of the normal surgical treatment of your child's condition.

8. 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.

9. Who can I contact for further information?
If you require any further information on this study or will like to clarify any issues, please do not hesitate to contact us. Our contacts are as follows:

- Mr Mark Danton, Consultant Cardiac Surgeon, Yorkhill (01412010251)
- Miss Susan Walker, PhD student, University of Glasgow (01412010367)
- Dr W. B. Knight, Consultant Cardiac Surgeon, Yorkhill (brodie.knight@yorkhill.scot.nhs.uk)

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
1. 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.

2. 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.

3. 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, phone number: 80251/80090). 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.
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.

4. 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 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.

5. 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 injury caused during heart surgery.

6. Will any genetic tests be done? No.

7. 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. 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.

8. 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 in the University of Glasgow.

9. Who has reviewed the study?
This study was given formal ethical approval by the West of Scotland Research Ethics Committee 2 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 your for taking time to read this information sheet.

2. Consent form:

Consent form – Version 2 (08/10/09)

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

CONSENT FORM

Title of Project: Myocardial protein expression in children undergoing cardiac surgery

(Full title: Myocardial cellular adaption to ischemia−reperfusion in children undergoing cardiac surgery: an integrated clinical and scientific approach to understand the molecular mechanisms involved).

Name of Researchers: Dr. Mark Danton (CI) and Miss Susan Walker (PI)

Please initial box

1. I confirm that I have read and understand the information sheet dated 08-10-09 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 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 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. ☐

_____	_____	_____
Name of Parent	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

3. Ethical approval

3.1 Approval from R and D:

NHS
Greater Glasgow and Clyde

Coordinator/administrator: Kirsty Theron
Telephone Number: 0141 211 6372
Fax Number: 0141 211 2811
E-Mail: Kirsty.theron2@ggc.ascot.nhs.uk

R&D Management Office
Western Infirmary
Tennent Institute
1st Floor, 38 Church Street
Glasgow, G11 6NT

16 November 2009

Dr Mark Danton
Consultant Cardiac Surgeon
Department of Cardiac Surgery
Royal Hospital for Sick Children
Yorkhill, Dalnair Street,
Glasgow G3 8SJ

R&D Management Approval

Dear Dr Danton,

Project Title: Myocardial cellular adaption to ischemia-reperfusion in children undergoing cardiac surgery: an integrated clinical and scientific approach to understand the molecular mechanisms involved
Chief Investigator: Dr Mark Danton
R&D Reference: GN09CA420
Protocol no (including version and date): Version 2 (12/10/09)

I am pleased to confirm that Greater Glasgow & Clyde Health Board is now able to grant **Management Approval** for the above study.
As a condition of this approval the following information is required during the lifespan of the project:

1. SAES/SUSARS – If the study is a **Clinical Trial** as defined by the Medicines for Human Use Clinical Trial Regulations, 2004 (CTIMP only)
2. Recruitment Numbers on a quarterly basis (not required for commercial trials)
3. Any change of Staff working on the project named on the ethics form
4. Change of CI
5. Amendments – Protocol/CRF etc
6. Notification of when the Trial / study has ended
7. Final Report
8. Copies of Publications & Abstracts

Please add this approval to your study file as this letter may be subject to audit and monitoring.

Yours sincerely

Dr Michael Barber
Research Co-ordinator

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3.2 REC approval:

West of Scotland REC 2
Western Infirmary
Ground floor, Tennent Institute
38 Church Street
Glasgow G11 6NT

Telephone: 0141 211 2123
Facsimile: 0141 211 1847
27 October 2009

Dr Mark Danton
Consultant Cardiac Surgeon
Royal Sick Children Hospital
Dept Cardiac Surgery
Dalnair Street
Glasgow G3 8SJ

Dear Dr Danton

Full title of study: **Myocardial cellular adaption to ischemia-reperfusion in children undergoing cardiac surgery: an integrated clinical and scientific approach to understand the molecular mechanisms involved.**
REC reference number: **09/S0709/61**
Protocol number: **2**

Thank you for the email from Susie Walker 12th October. I can confirm the REC has received the documents listed below as evidence of compliance with the approval conditions detailed in our letter dated 15 September 2009. Please note these documents are for information only and have not been reviewed by the committee.

Documents received

The documents received were as follows:

Document	*Version*	*Date*	
Protocol	Version 2	12 October 2009	
Covering Letter – by email		12 October 2009	
Participant Information Sheet: Subaortic Stenosis	Version 4	08 October 2009	
Participant Information Sheet: Tetralogy of Fallot (TOF)	Version 3	08 October 2009	
Participant Consent Form	Version 2	08 October 2009	

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

09/S0709/61	Please quote this number on all correspondence

Yours sincerely

Liz Jamieson
Committee Co-ordinator
E-mail: Liz.Jamieson@ggc.scot.nhs.uk
Copy to: *R&D office for NHS care organisation at lead site]*

4. List of reagents

Supplemented media for tissue culture (see section 2.2, 2.3 and 2.4):

- Medium 199 (With Earle′s salts, L-glutamine and sodium bicarbonate, liquid, sterile-filtered, cell culture tested) (Sigma, cat. no. M4530-100ML
- 5% fetal bovine serum (heat inactivated) (Sigma-Aldrich, cat. no. F9665)
- 1% antibiotic antimycotic solution. (Sigma-Aldrich, cat. no. A5955)

Supplemented media for HSP induction tissue culture (see section 2.4:

- Medium 199 (With Earle′s salts, L-glutamine and sodium bicarbonate, liquid, sterile-filtered, cell culture tested) (Sigma, cat. no. M4530-100ML
- 5% fetal bovine serum (heat inactivated) (Sigma-Aldrich, cat. no. F9665)
- 1% antibiotic antimycotic solution. (Sigma-Aldrich, cat. no. A5955)
- 100μM/l of sodium arsenite ($NaAsO_2$) (Sigma, cat. no 35000-1L-R) or 100μM/l of cadmium chloride ($CdCl_2$) (Sigma, cat. no. 28811-1ML-F)

Reagents for homogenization of tissue (see section 2.11.1):

- Homogenising buffer plus protease inhibitor cocktail in 1 litre:
    - + 500ml H_20
    - + 3.02g Tris (25mM) (121.14g/mol) (Sigma-Aldrich, cat. no. 93352)
    - + 0.4g EDTA (1mM) (372.24g/mol) (Sigma-Alrich, cat. no. E6758)
    - 85.6g Sucrose (250mM) (342.3g/mol) (Sigma-Aldrich, cat. no. S9378)
    - Make up 1 litre with dH_2O
    - Store at 4° C
  - Add 20ml (or 10ml if less samples) of buffer to a sterile universal container.
  - To this add a 250μl (or 125ul) aliquot of protease inhibitor cocktail immediately prior to use(Sigma-Aldrich, cat. no. P8340-5ML)

Reagents for Bradford Assay (see section 2.11.2):

- Bovine Serum Albumin (BSA) stock standard solution of for Bradford Assay
  - 1mg per ml of BSA (Sigma cat. no. A7906-50G).
  - 10mg BSA in 10ml H_2O.

Reagents for Western blotting (see section 2.12):

- Sample loading buffer (2x):
  - 1.2ml (1M) Tris (Sigma-Aldrich, cat. no. 93352)
  - 2ml glycerol (Sigma-Aldrich, cat. no. G2025)

- 4ml (10%) sodium dodecyl sulfate (SDS) (Sigma-Aldrich, cat. no. 35000)
- 2ml (1M) dithiothreitol (Sigma-Aldrich, cat. no. 646563)
- 0.8ml dH_20
- Bromophenol blue (solid) – add a small amount, add more if needed (Sigma-Aldrich, cat. no. B0126-25G)

- Buffer for resolving gel (pH 8.8) (in 1 litre):
  - In a 1litre bottle with stirring bar add 500ml dH_20
  - Add 181.65g of Tris (Sigma-Aldrich, cat. no. 93352), shake to dissolve (will increase volume)
  - Add 40 ml of 10% SDS (Sigma-Aldrich, cat. no. 35000) (final conc. of 0.4% in 1litre) slowly, allowing it to dissolve
  - pH buffer (5M HCl)
  - Add dH_20 to create a final vol. of 1l (black line)

- Buffer for stacking gel (pH 6.8) (in 1 litre):
  - In a 1litre bottle with stirring bar add 500ml dH_20
  - Add 60.55g of Tris, shake to dissolve (will increase volume)
  - Add 40 ml of 10% SDS solution (final conc. of 0.4% in 1litre) slowly, allowing it to dissolve
  - pH buffer (5M HCl)
  - Add dH_20 to create a final vol. of 1l (black line)

- Composition of resolving and stacking gels:

Reagent	**Resolving Gel (2 gels) (10%)**	**Stacking gel (2 gels) (4%)**
	Volume 2/ 1 gel	Volume
30% acrylamide/ bis- acrylamide	24ml or 12ml	2.7ml
50% glycerol	4.8ml or 2.4ml	n/a
Buffer A	18ml or 9ml	n/a
Buffer B	n/a	5ml
Distilled water	24.8ml or 12.4ml	12.2ml
10% APS	360µl or180ul	100µl
TEMED	36µl or 18ul	20µl
Total	36x2ml	20ml

- 10% ammonium persulfate (APS) solution (in table above):
  - Measure 1ml of dH_20
  - Add 0.1g of ammonium persulpahte (Sigma-Aldrich, cat. no. A3678)
  - Allow to dissolve

- Running buffer (5x) (pH 8.3) (in 4 litre):
  - Get 3litre of dH_2O
  - Add 60.4g of Tris
  - Add 288g of glycine
  - Add 200ml of 10% SDS solution
  - pH buffer (5M HCl)
  - Make up final vol. of 4 litres with dH_2O
  - Dilute 500ml pf running buffer (5x) in 2 litres of dH_2O when using for assay

Reagents for transfer of proteins to nitrocellulose (see section 2.12.2)

- Transfer buffer in 1 litre (pH 7.5):
    - 800ml H_20
    - 3g (25mM) Tris
    - 14.4g (190 mM) glycine
    - 200ml (20%) methanol then shake till all has dissolved in solution

- 0.1% NaOH:
    - 0.1g NaOH (Sigma-Aldrich, cat. no. 655104) in 100ml dH20

Reagents for immuno-detection of proteins (see section 2.12.3):

- TBSTB (in 1 litre) (pH 7.5):
    - Retrieve 800ml of dH_20.
    - Add 2.4g Tris (20 mM) then shake.
    - pH buffer (Plus 3ml 5M HCl).
    - Add 29.2g NaCl (0.5 M) then shake.
    - Add 4ml Tween20 (Sigma-Aldrich, cat. no. P1379)
    - Add 2.5g BSA (0.25%) (Sigma-Aldrich, cat. no. A7906) then shake.
    - Add dH_2O to make up the final 1 litre volume.

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List of publications, awards and conferences attended.

Walker, S., Danton, M., Peng, E. W. K., Lyall, F. (2012) Heat shock protein 27 is increased in Cyanotic Tetralogy of Fallot myocardium and is associated with improved cardiac output and contraction, Cell Stress and Chaperones, In press.

Vassalos, A., Peng, E., Young, D., **Walker, S**., Pollock, J., MacArthur, K., Lyall, F. Danton, M. H. (2011). Pre-operative sildenafil and pulmonary endothelial-related complications following cardiopulmonary bypass: a randomised trial in children undergoing cardiac surgery. Anaesthesia, 66, 472-80.

Walker, S., Danton, M., Peng, E. W. K., Lyall, F. HSP27 expression is associated with improved right ventricle function, suggesting a protective effect in cyanotic TOF. Yorkhill Research Day, Friday 18th November, 2011, Glasgow, Scotland (Presenter – oral session, winner of Long Communication presentations)

Walker, S., Danton, M., Peng, E. W. K., Lyall, F. HSP27 expression is increased in congenital heart disease in children and associated with improved right ventricle function: links to placental insufficiency Annual Scientific Meeting. Improving Women's Health through Personalized Medicine. March 21-24, 2012. *San Diego*, California, USA (Poster presentation).

Walker, S., Danton, M., Peng, E. W. K., Lyall, F. HSP27 expression is associated with improved right ventricle function, suggesting a protective effect in cyanotic TOF. TENOVUS-SCOTLAND: 30th Anniversary Symposium "Molecular Mechanisms of Disease", Wednesday 6 – Thursday 7 June 2012, Glasgow, Scotland (Poster presentation).