METABOLIC SUPPORT IN LEFT VENTRICULAR HYPERTROPHY

by

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To Amy - for all her patience

Abstract

Aims: To examine the metabolic changes associated with LVH secondary to AS, to examine the role of GIK as an adjunct to myocardial protection during AVR, and to examine the mechanism of its action.

Methods: Patients undergoing AVR for AS with evidence of LVH were randomly assigned to GIK or placebo. The trial was double blind and conducted at a single centre. The primary outcome was the incidence of low cardiac output syndrome. Left ventricular biopsies were analysed to assess changes in 5' adenosine monophosphate-activated protein kinase (AMPK) and Akt phosphorylation; and protein O-linked beta N-acetylglucosamination (O-GlcNAcylation). 217 patients were randomised over a four year period (107 control; 110 GIK).

Results: GIK treatment was associated with a significant reduction in the incidence of low cardiac output state (OR 0.22, p= 0.0001), and a significant reduction in inotrope use 6 to 12 hours postoperatively, (OR 0.30, p=0.0007). These changes were associated with a substantial increase in AMPK and Akt phosphorylation and a significant increase in the *O*-GlcNAcylation of selected protein bands.

Conclusions: Peri-operative treatment with GIK was associated with a significant reduction in the incidence of low cardiac output state and the need for inotropic support. This benefit was associated with increased signalling protein phosphorylation and O-GlcNAcylation. Multi-centre studies and late follow up will determine if routine use of GIK improves patient prognosis

Acknowledgments

The running of the HINGE trial and the laboratory analysis of the biopsies obtained would not have been possible without the help of a wide range of people and organisations for which I would like to express my gratitude.

I would like to thank the British Heart Foundation for supporting me during this research period and funding all the work and in particular I would like to thank Mr Pagano my supervisor for his continuing support and encouragement and for fixing all manner of problems encountered along the way.

The running of such a trial would not have been possible without the continued confidence of the consultant surgeons and I would therefore like to thank Messer's Bonser, Mascaro, Graham, Rooney and Wilson for allowing me to recruit their patients and manage them along the lines of the trial protocol. I would also like to thank all the anaesthetists and ODA who assisted me during the trial period and all the nursing staff on the intensive care unit who took the trouble to keep management of the patients within the trial specifications.

Clinical research in UHB is now well established and I would therefore like to thank all my predecessors for setting up this format of research especially Mr Quinn and Mr Ranasinghe who had previously trialled GIK in the context of coronary artery surgery.

The novel metabolomic research carried out was performed with significant input from Dr Viant's group in the Life Science department of the University of Birmingham.

I would like to thank them all for the huge patience they demonstrated in helping me to develop and validate the methodology and results that I present in this thesis.
The genomic and proteomic work was all planned after long discussions with Professor Frenneaux and Dr Ashrafian. I would also like to thank Dr Steeples and Dr Contractor for helping me produce the Western blots shown in this thesis. The biopsies were particularly challenging due to their small size and the known difficulty of immunoblotting the proposed targets and the final chapter in this thesis represents the end product of a very significant amount of preliminary work. The genomic work was carried out in Professor Stewart's laboratory in the IBR in Birmingham and I would like to thank Dr Hammer for all his help.

Finally, I would like to thank Mr Drury for assisting in the trial recruitment. Without his help this trial would not have been finished.

Extent of Personal Contribution

This study was conducted at University Hospital Birmingham between October 2005 and 2008 where I was employed as a British Heart Foundation Clinical Research Fellow, and Honorary Clinical Lecturer at the University of Birmingham following my appointment as an Academic National Trainee in Cardiothoracic Surgery in the West Midlands Deanery. The study was designed by Mr Pagano following completion of the MESSAGE trials. Ethical approval was obtained by Mr Quinn. I set up the trial and was responsible for the recruitment, logistics and financial management of the grant and I have completed a course in good clinical practice which deals with issues such as consent, ethics and investigator responsibilities.

I recruited all the patients from the start of the trial until my scheduled return to clinical practice in June of 2008. After this trial recruitment was assisted by Mr Drury. I was responsible for the pre- and post-operative management of the patients along pre-specified treatment algorithms. I collected the data and entered it into a MS Access database that I had written. I collected all the blood samples and centrifuged them prior to storage. I collected all the biopsies and stored them in line with the Human Tissue Act 2007.

I prepared all the clinical data for analysis. I underwent training in trial methodology and statistical analysis by Sheffield University and I underwent training in database management and statistical programming with SPSS and SAS at the University of Birmingham. I designed the statistical models for the primary and secondary endpoints in conjunction with Professor Freemantle, University of Birmingham.

These predefined primary and secondary endpoints were subsequently analysed by Dr Calvert, University of Birmingham. I performed the remainder of the statistical analysis presented in this thesis.

I designed the experiments to examine the metabolism in left ventricular hypertrophy following extensive discussion with Professor Frenneaux and Dr Ashrafian. The RNA work was carried out in Professor Stewart's lab at the University of Birmingham. I extracted the RNA from all the biopsies and performed all the reverse transcription and real-time polymerase chain reaction with assistance from Dr Hammer. Master transcription targets were chosen after discussion with Dr Ashrafian, Thomas Henry Wellcome Centre for Human Genetics, Oxford University. The metabolomic work was carried out by Dr Viant's team in the department of Life Sciences, University of Birmingham. He and I developed the idea for this. I was involved in extensive preparatory work in his laboratory; I performed all the extractions in his lab and assisted Dr Hill in performing the mass spectroscopy. The spectra obtained were analysed by Dr James.

The proteomic analysis was all carried out at the Thomas Henry Wellcome Centre for Human Genetics, Oxford University in Professor Watkins laboratory under the direct supervision of Dr Ashrafian. I travelled to Oxford with the biopsies and spent many weeks extracting protein and performing numerous westerns before the final results were obtained. Throughout this period I had extensive help from Dr Steples and Dr Contractor. The combined clinical and molecular biology results from this work have now been accepted for publication in *Circulation*. This paper is included in Appendix J.

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List of Abbreviations

Adenosine diphosphate / triphosphate	ADP / ATP
Adenosine monophosphate kinase	AMPK
Aortic cross clamp	AXC
Aortic stenosis	AS
Aortic valve replacement	AVR
Cardiac index	CI
Cardiopulmonary bypass	CPB
Coronary artery bypass graft	CABG
Electrocardiogram	ECG
Free fatty acids	FFA
Fourier transform ion cyclotron resonance	FT-ICR
Glucose insulin potassium	GIK
Glucose transporter protein	GLUT
Intensive care unit	ITU
Ischaemia-reperfusion	IR
Low cardiac output episode	LCOE
Left ventricle	LV
Left ventricular hypertrophy	LVH
Mass spectroscopy	MS
Mitochondrial permeability pore	mPTP
Myocardial infarction	MI
Nicotinamide adenine dinucleotide (reduced)	NAD^+ (NADH)
Polymerase chain reaction	PCR
Phosphocreatine	PCr
Pyruvate dehydrogenase	PDH
Phosphofructokinase	PFK
Reactive oxygen species	ROS
Ribonucleic Acid	RNA
Systemic Vascular Resistance	SVR
Trans thoracic echocardiography	TTE
Troponin T	TnT
Tricarboxcylic acid	TCA

Chapter 1. Introduction

1. Aortic Stenosis

1.1 Background

Obstruction to the left ventricular (LV) outflow tract is localised most commonly at the aortic valve. In the developed world the most common acquired cause is degenerative calcific aortic stenosis (AS). The cusps of the aortic valve become immobilised by deposits of calcium at their bases as a consequence of years of normal mechanical stress on the valve. This is an active disease process with lipid accumulation, inflammation and calcification [1-8]. Degenerative calcification may also extend in the direction of the cusps resulting in commissural fusion.

1.2 Pathophysiology

In degenerative calcific AS there is slow and progressive LV outflow tract obstruction. Left ventricular output is maintained by the development of left ventricular hypertrophy which may sustain a large pressure gradient across the aortic valve for many years without a reduction in cardiac output, left ventricular dilatation or the development of symptoms. The increased systolic wall stress induced by AS leads to a parallel replication of sarcomeres and concentric hypertrophy and the increase in left ventricular wall thickness is often sufficient to counterbalance the increased pressure so that peak systolic wall tension returns to normal [1-3]. An inverse correlation between wall stress and ejection fraction exists suggesting that the depressed ejection fraction found in some patients is a consequence of inadequate wall thickening [4] resulting in afterload mismatch [5]. Patients with AS and

compensated pressure overload may have normal interventricular stress (dσ/dt) and pressure (dP/dt) development indicative of normal contractility [6].

Although LV hypertrophy is a key adaptive mechanism to the pressure load imposed by AS, it has an adverse pathophysiological consequence by increasing diastolic stiffness. This leads to an increased intracavity filling pressure [7, 8]. In some this increase in stiffness is directly related to muscle mass but with no alteration in diastolic properties of each unit of myocardium, in others its is due to increases in both chamber and muscle stiffness which contribute toward the elevation of ventricular diastolic filling pressure at any level of ventricular diastolic volume [9, 10]. A raised left ventricular end diastolic pressure is therefore reflective of diastolic dysfunction rather than systolic dysfunction. Forceful atrial contraction that contributes to an elevated end-diastolic pressure plays an important role in ventricular filling without increasing mean left atrial or pulmonary venous pressure [11] and therefore loss of atrial transport with atrial fibrillation often results in significant clinical deterioration.

Left ventricular hypertrophy in severe AS is associated with a variety of changes in the myocardial ultrastructure including large nuclei, loss of myofibrils, accumulation of mitochondria, large cytoplasmic areas devoid of contractile material and proliferation of fibroblasts and collagen fibres in the interstitial space [12]. The depression of cardiac function that occurs late in the course of aortic stenosis may well be related to these morphological alterations.

Although coronary flow in LVH is elevated in absolute terms, it is normal when corrected for myocardial mass [13], myocardial oxygenation in severe AS may be inadequate, even in the absence of coronary artery disease. The hypertrophied left ventricular muscle mass, the increased systolic pressure and the prolongation of ejection all elevate myocardial oxygen consumption [14]. The abnormally high diastolic pressure compressing the intramuscular coronary arteries exceeds the coronary perfusion pressure thereby interfering with coronary blood flow [15] thus leading to a potential imbalance between myocardial oxygen supply and demand. Myocardial perfusion is also impaired by the relative decrease in myocardial capillary density and the elevation of left ventricular end-diastolic pressure which lowers the aortic-left ventricular end-diastolic pressure gradient in diastole. The subendocardium in severe AS in particular is susceptible to ischaemia and this malperfusion may be responsible in part for the development of subendocardial ischaemia [15]. Recent work combining cardiac magnetic resonance to measure LV mass and PET to quantify resting and hyperaemic (dipyridamole) myocardial blood flow and coronary vasodilator reserve (CVR) in patients with severe LVH secondary to AS with angiographically normal coronary arteries and non-hypertrophied controls demonstrated that patients with severe LVH manifested significantly lower subendocardial CVR (1.43 ± 0.17) than subepicardial CVR (1.78 ± 0.35; p=0.01), with a further reduction in subendocardial CVR with hyperaemia (0.92 ± 0.17; p=0.05) [16]. Quantitative analysis of high energy phosphate levels in patients with severe LVH secondary to aortic stenosis with angiographically normal coronary arteries has also demonstrated a significant reduction in ATP concentration in the subendocardium (25.9 ± 1.7 μmoles.g protein^{-1}) compared to the subepicardium (31.5 ± 1.6; p=0.01)[17] . Hypertrophied hearts also exhibit an increased sensitivity to

ischaemic injury with larger infarcts and higher mortality rates than are seen in the absence of hypertrophy [16-18] . Elderly patients, particularly women, often develop excessive concentric hypertrophy resulting in a reduced end systolic volume and increased ejection fraction a combination associated with increased peri-operative morbidity and mortality [18, 19].

1.3 Grading the degree of stenosis

The American Heart Association guidelines grade the severity of stenosis on the basis of a number of haemodynamic and natural history data [20].

Indicator	Mild	Moderate	Severe
Jet Velocity ms^{-1}	<3.0	3.0-4.0	>4.0
Mean Gradient (mmHg)			6o
Valve area (cm^2)	>1.5	1.0-1.5	<1.0

Table 1. The grading of severity of aortic stenosis [20].

1.4 Natural History

Aortic stenosis is associated with a prolonged latent period during which there is no increased risk of cardiac morbidity or mortality. Once the stenosis is moderate the average rate of progression is an increase in jet velocity of 0.3 ms^{-1} per year, an increase in mean gradient of 7mmHg per year and a decrease in valve area of 0.1cm^2 per year [19-25] but individuals may manifest marked variability in haemodynamic progression.

Following the long latent period, symptoms of angina, syncope or heart failure develop and the clinical prognosis changes significantly. After the onset of symptoms mean survival is 2-3 years [19-23] with a significantly higher risk of sudden death (Fig 1.2). The onset of symptoms is therefore the key step in considering surgical intervention for aortic stenosis.

1.5 Echocardiographic assessment

Echocardiographic assessment is indicated when there is a systolic murmur that is grade 3/6 or greater, a single S2, or symptoms that might be due to AS. The 2D echocardiogram is valuable for interrogation of valve anatomy and function and determining the LV response to pressure overload [20].

1.6 Indications for Surgery

In adults with severe symptomatic calcific AS, AVR is the only effective treatment improving both symptoms and survival [19-23]. The outcome is similar in patients with preserved and with moderately impaired LV contractility[21]. The ejection fraction in patients with severely impaired function caused by excessive afterload improves after AVR but if the ventricular dysfunction is not caused by afterload mismatch then improvement in ventricular function and performance status is reduced. In the absence of severe co-morbidity AVR is therefore indicated in virtually all symptomatic patients. In 1998, a joint task force of the American College of Cardiology (ACC) and the American Heart Association (AHA) developed evidence-based consensus guidelines for management of valvular heart disease which were updated in 2006 [20]. These indications for AVR are listed in Appendix A.

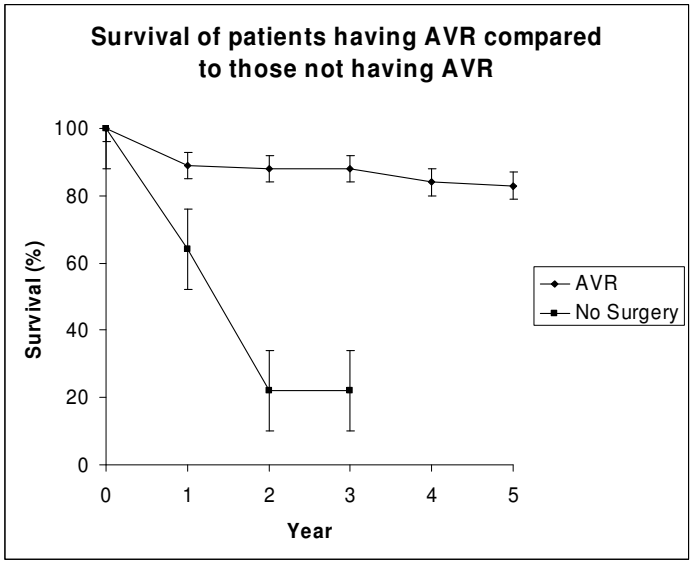

Figure 1 Survival in untreated patient vs surgically treated patients with severe Aortic Stenosis

2. Myocardial Metabolism

2.1 Overview

Cardiomyocytes oxidise fatty acids derived from both the plasma and the breakdown of intracellular triacylglycerol stores, while pyruvate is derived from either lactate dehydrogenase or glycolysis. The rates of these metabolic pathways are tightly coupled to the rate of contractile work, and conversely, contractile work is coupled to the supply of oxygen and the rate of oxidative phosphorylation.

The fasted heart extracts free fatty acids (FFAs), lactate and glucose from the blood, and assuming complete oxidation of extracted substrates, fatty acid oxidation accounts for 60-80% of the oxygen consumption with a lesser contribution from lactate and glucose, each accounting for 10-20%.

2.2 Glycolysis and Glycogen metabolism

The uptake of extracellular glucose is regulated by the transmembrane glucose gradient and the concentration and activity of glucose transporters. The two isoforms GLUT 1 and GLUT 4 [22, 23] are located in the sarcolemmal membrane and in intracellular microsomal vesicles [22-24]. Insulin and ischaemia result in translocation from the intracellular site into the plasma membrane resulting in an increase in the capacitance for glucose transport [24-26]. On entering the cell glucose is rapidly phosphorylated by hexokinase rendering it impermeable to the cell membrane[27]. Insulin activates hexokinase, and cell culture data suggest that glucose phosphorylation by hexokinase is the rate limiting step in insulin stimulated glucose utilisation [28]. Glucose 6-phosphate (G 6-P) can be used for either glycogen synthesis or it can proceed down the glycolytic pathway to form pyruvate [27] (Fig 1). The glycolytic pathway produces two molecules of ATP and two molecules of pyruvate per molecule of glucose. Under aerobic conditions pyruvate enters the mitochondria and is decarboxylated to actetyl coenzyme a (CoA) whereas during anaerobic conditions pyruvate is reduced in the cytosol to lactate[27]. If the concentration of G 6-P is constant the primary regulators of glycolytic rate are the activity of phosphofructokinase (PFK) and the ability to form reduced NADH [29-32]. The activity of PFK is inhibited by H^+ , citrate and ATP and is stimulated by ADP, Ca^{2+}, and fructose 2,6-biphosphate from F 6-P. NAD^+ is reduced to NADH and cytosolic NADH can be converted back to NAD^+ through the conversion of pyruvate to lactate by lactate dehydrogenase or the reducing equivalents can be shuttled into the mitochondria via the malate-aspartate shuttle[27]. Glyceraldehyde 3-phosphate dehydrogenase would appear to be a rate limiting step for glycolysis with either high rate of contractile work [32] or during ischaemia [33].

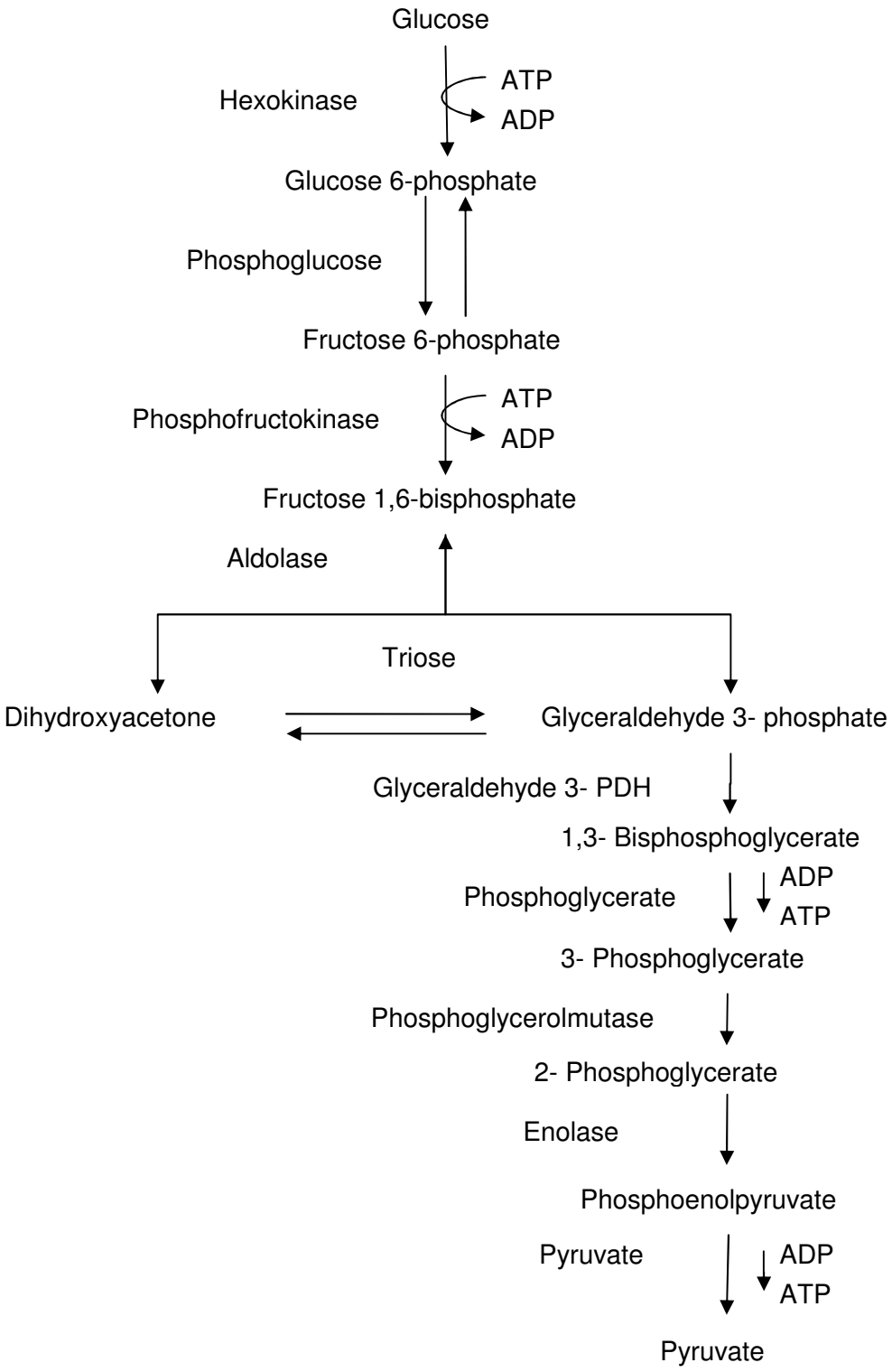

Figure 2 **The Glycolytic pathway.**

It has been hypothesised that glycolytically derived ATP is preferentially used for Ca^{2+} re-uptake into the sarcoplasmic reticulum and that it is essential for optimal diastolic relaxation [34-36]. This evidence comes from studies in isolated tissue that demonstrated that inhibition of glycolysis resulted in impaired relaxation especially in ischaemic or reperfused myocardium [35]. It has also been demonstrated that key glycolytic enzymes are associated with the cardiac ATP-sensitive K+ channels and that glycolytically derived ATP preferentially inhibits these channels [37]. Glycolysis is also important for optimal function of the Na^+ /K^+ ATPase and prevention of intracellular Na^+ accumulation during ischaemia [38].

The rate of glycogen synthesis is regulated by the concentration of G 6-P and the activity of glycogen synthase. Glycogen synthase activity and glycogen storage are both increased by insulin [39-41]. Glycogenolysis results in the formation of G 6-P and is regulated by the activity of glycogen phosphorylase. Glycogen phosphorylase is activated when phosphorylated by phosphorylase kinase which is activated by Ca^{2+}. This is regulated by protein kinase a, and thus activated by cAMP [39]. Glycogen phosphorylase activity is therefore controlled by both hormonal stimulation including β-adrenergic receptor stimulation resulting in increased cAMP and Ca^{2+}, and metabolic feedback from AMP, P_i and Ca^{2+}. In vivo there is simultaneous synthetase and phosphorylase activity [41-44].

The overall rates of glucose uptake, glycogen synthesis and breakdown and the rate of glycolysis are controlled by multiple steps distributed along these pathways and are not subject to control at discrete steps [29].

2.3 Pyruvate and Lactate Oxidation

Under well perfused conditions in vivo lactate is a major source of pyruvate formation, and under some conditions lactate uptake can exceed glycolysis as a source of pyruvate [45, 46]. Studies with carbon labelled lactate tracers in humans have shown that 80-100% of the lactate taken up by the heart in health is immediately released as labelled CO_2 into the coronary effluent suggesting that extracted lactate is rapidly oxidised by lactate dehydrogenase, decarboxylated by pyruvate dehydrogenase (PDH) and oxidised to CO_2 in the TCA cycle [46].

Pyruvate decarboxylation is the key irreversible step in carbohydrate oxidation and is catalysed by PDH [47]. The activity of PDH is regulated by a variety of mechanisms. It is inactivated by phosphorylation by PDH kinase and is activated by dephosphorylation by PDH phosphatase.[48-50]. The rate of pyruvate oxidation is strongly dependent on the degree of phosphorylation of PDH and on the concentrations of its substrates and products in the mitochondria. The activity of PDH phosphatase is increased by Ca^{2+} and Mg^{2+}, while PDH kinase is inhibited by pyruvate and ADP and activated by increases in AcylCoA /CoA and NADH / NAD^+ [47, 51].

Pyruvate oxidation and the activity of PDH in the heart are decreased by elevated rates of fatty acid oxidation and caused by increased plasma levels of FFA and are enhanced by suppression of FFA oxidation induced by a decrease in plasma FFA levels [47]. Positive inotropic agents also increase the amount of active enzyme by a Ca^{2+} dependent activation of PDH phosphatase [52].

2.4 Fatty acid metabolism

Although the energy yield from complete oxidation of fatty acids is more than that of carbohydrates, providing 130 molecules ATP per mole FFA versus 38 molecules ATP per mole of glucose, it is more oxygen dependent. The ATP yield using FFA per mole of oxygen is 2.83 compared to 3.17 for glucose [27].

Fatty acids are stored as triacylglycerols (TAGs). Oxidation of free fatty acids in the mitochondria can only occur after a series of essential steps. It has been hypothesised that during ischaemia accumulation of these metabolites due to inhibition of mitochondrial metabolism contributes towards cellular dysfunction [53].

Hydrolysis of TAGs by hormone sensitive lipases generates glycerol and FFAs. Glycerol is then converted to glyceraldehyde-3-phosphate which is then converted to either pyruvate or glucose by the liver. The mitochondrial membrane is impermeable to fatty acids therefore long chain acyl groups from fatty acids are transported into the mitochondrial matrix by carnitine so that they can undergo β-oxidation [27]. On the outer mitochondrial membrane fatty acids are attached with a thioester bond to coenzyme A (CoA), a reaction catalysed by fatty-acyl-CoA synthetase [27]. Acyl CoA is conjugated to carnitine by carnitine palmitoyltransferase I (CPT-I) on the outer mitochondrial membrane and it is then shuttled inside by carnitine-acylcarnitine translocase. Acyl-carnitine is then converted to acyl-CoA by CPT-II on the inner mitochondrial membrane where it can then undergo β-oxidation [27].

CPT-I undergoes allosteric inhibition as a result of malonyl-CoA an intermediate in fatty acid biosynthesis, in order to prevent futile cycling between β-oxidation and fatty acid synthesis [47].

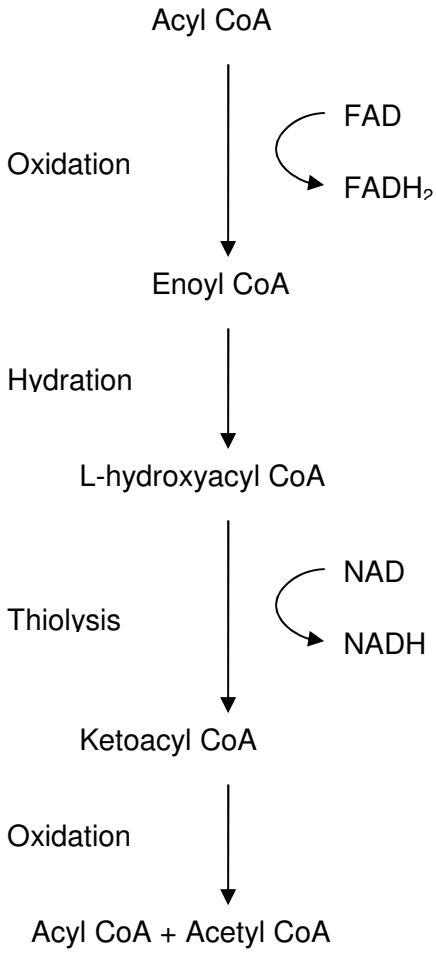

Figure 3 Acyl CoA degradation pathway

2.5 TCA Cycle and Oxidative phosphorylation

The tricarboxylic acid cycle (TCA) operates only under aerobic conditions requiring a constant supply of NAD^+ and FAD. The enzymes involved are located in the mitochondrial matrix or attached to the inner surface of the mitochondrial membrane. This facilitates the transfer of reducing equivalents to the enzymes of the electron transport chain. There are four oxidation-reduction reactions that occur in the cycle and these donate four pairs of electrons, three to NAD+ and one to FAD [27]. These reduced electron carriers are subsequently oxidised by the electron transport chain leading to the generation of 11 molecules of ATP, an additional high energy phosphate bond in the formation of succinate means a total of 12 molecules of ATP are produced for each Acyl CoA that is completely oxidised [27].

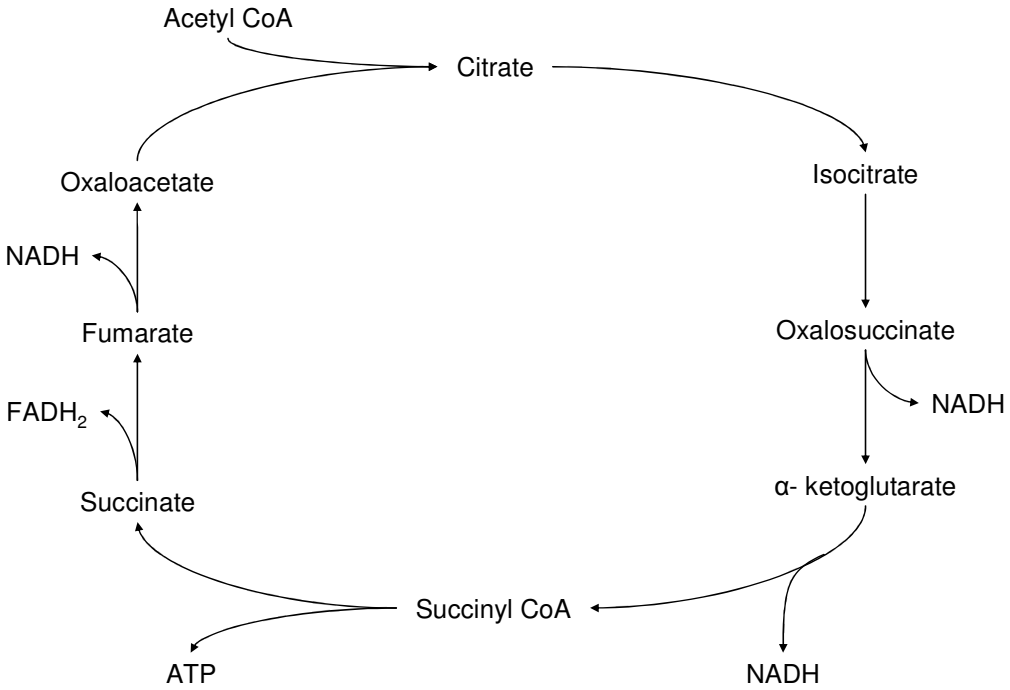

Figure 4 The TCA Cycle [27]

The NADH and $FADH_2$ formed by glycolysis and the TCA cycle are energy rich molecules due to a pair of electrons with a high transfer potential. The transfer of electrons from NADH and $FADH_2$ to oxygen leads to protons being pumped out of the mitochondrial matrix. This process generates a pH and transmembrane electric potential gradient. Flow of protons back to the mitochondrial matrix via ATP synthase leads to ATP synthesis. This process of oxidative phosphorylation is highly efficient generating 32 of the 36 molecules of ATP generated from complete oxidation of glucose to carbon dioxide and water [27]. The ATP yield is shown below:

Glycolysis: Glucose into pyruvate (in the cytosol)	**ATP Yield per glucose**
Phosphorylation of glucose	**-1**
Phosphorylation of fructose-6-phosphate	**-1**
Dephosphorylation of 2 molecules of 1,3 BPG	**+2**
Dephosphorylation 2 molecules phosphoenolpyruvtae	**+2**

Conversion of pyruvate into acetyl CoA (inside mitochondria)

2 NAD are formed

Citric Acid cycle

2 molecules of guanine triphosphate formed from 2 of succinyl CoA

6 NADH from oxidation of 2 each of isocitrate, ketoglutarate, malate **+2**

2 FADH formed in oxidation of 2 molecules succinate

Oxidative Phosphorylation

2 NADH formed in glycolysis each yield 2 ATP	**+4**
2 NADH from oxidative decarboxylation of pyruvate	**+6**
2 $FADH_2$ formed incitric acid cycle each yields 2 ATP	**+4**
6 NADH formed in citric acid cycle each yields 3 ATP	**+18**
Net yield of Glucose	**+36**

ATP yield from the complete oxidation of glucose

The rate of oxidative phosphorylation is closely related to the work of the heart. Electrons do not flow through the electron transport chain unless ADP is simultaneously phosphorylated to ATP. This regulation of oxidative phosphorylation by ADP is termed respiratory control. The level of ADP also affects the rate of the TCA cycle because of its need for NAD^+ and FAD therefore as ATP is consumed ADP levels increase therefore oxidative phosphorylation is coupled to ATP utilisation.

2.6 Phosphocreatine and Hypertrophy

Myocardial energetic demands are met primarily through mitochondrial ATP production via oxidative phosphorylation. Phosphocreatine (PCr) is an important short term energy source which maintains a high phosphorylation potential under conditions of increased energy demand. The transfer of a phosphoryl group from PCr to ADP by the enzyme creatine kinase (CK) rapidly and reversibly generates

ATP and creatine, approximately 10 times faster than the maximum rate of ATP generation by oxidative phosphorylation [54].

The PCr/ATP ratio is an indicator of the energetic state of the myocardium. Studies in animals in both hypertrophy and failure have demonstrated a progressive reduction in the creatine pool of 60% largely due to a reduced number of creatine transporters [55, 56]. In heart failure in man studies with ^{31}P NMR spectroscopy have demonstrated a reduced PCr/ATP with the magnitude of this reduction being predictive of late mortality [57 58] . This reduction is partly due to a reduced creatine pool and partly to reduced CK isoenzyme expression, particularly mitochondrial CK.

2.7 Control of Metabolism

Central to the role of regulating energy homeostasis is the trimeric enzyme AMP-activated protein kinase (AMPK) [59]. AMPK induces a cascade of events within the cell in response to the ever changing energy charge of the cell [60, 61]. Once activated, AMPK-mediated phosphorylation events switch cells from net active ATP consumption to activate ATP production [59]. These events are rapidly initiated and referred to as short-term regulatory processes. AMPK is activated by phosphorylation by one or more upstream AMPK kinases (AMPKKs), and in the absence of phosphorylation, there is no detectable activity of AMPK towards any substrates [59, 62].

AMPK is also regulated by AMP. The effects of AMP are twofold. Direct allosteric activation makes AMPK a poorer substrate for dephosphorylation, and because AMP affects both the rate of AMPK phosphorylation in the positive direction, and

dephosphorylation in the negative direction, the cascade is ultrasensitive[59]. This means that a very small rise in AMP levels can induce a dramatic rise in the activity of AMPK. Negative allosteric regulation of AMPK also occurs and this effect is exerted by phosphocreatine with a high glycogen content suppressing AMPK activity [60].

The signalling cascade initiated by the activation of AMPK exerts effects on glucose and lipid metabolism, gene expression and protein synthesis[61]. During myocardial ischaemia, AMPK activation leads to the phosphorylation and activation of phosphofructo-kinase 2, one of the most important regulators of the rate of flux through glyolysis and gluconeogenesis[63]. AMPK activation also leads to phosphorylation and inhibition of acetyl-CoA carboxylase (ACC)[61]. This inhibition results in a decrease in the level of malonyl-CoA which itself is an inhibitor of carnitine palmitoyltransferase 1 (CPT-1)[61]. In addition, AMPK has been demonstrated to phosphorylate and thus regulate the activity of HMG-CoA reductase, malonyl CoA decarboxylase, glycogen synthase and creatine kinase thus regulating the spectrum of fatty acid, cholesterol, glucose, glycogen, and phosphocreatine metabolism [60, 64, 65]

2.8 Metabolism in hypertrophy

The hypertrophic response of the heart enables it to meet the demands of chronically elevated haemodynamic workload. Whilst initially compensatory, LVH is ultimately a maladaptive response, associated with markedly increased cardiovascular mortality and a substantial increase in the development of heart failure [66] initiated through two mechanisms. Firstly, pathological LVH is associated with activation of the circulating and tissue renin-angiotensin-aldosterone systems coupled with local up-regulation of

NADPH oxidase which both lead to progressive cardiac fibrosis and impaired function[67]. Secondly, there is increasing evidence that the hypertrophied heart is relatively energy starved and that energy deficiency may play a key role in the transition from LVH to HF [68-70].

Animal models of hypertrophy and failure suggest that the loss of ATP in the myocardium is slow and progressive ~0.35% of the ATP pool per day. This low rate, whilst not necessarily immediately translatable to man, means that a decrease in ATP content would be difficult to detect until the heart was in severe failure thus explaining the conflicting literature on this topic [71, 72]. The loss of ATP in the failing heart is caused by loss in the total adenine nucleotide (TAN) pool [73]. The combination of a fall in ATP and TAN had previously been observed in the context of ischaemia [54]. Whether the fall in this case is due to a failure of de novo purine synthesis or failure to increase synthesis to support the hypertrophied myocyte mass or because reactions converting adenine nucleotides to diffuse purine nucleosides and bases are accelerated remains unknown.

Analysis of biopsies in patients with LVH secondary to severe AS showed that before the decrease in ATP level becomes significant the total creatine pool falls by over 60% [74]. This finding has been confirmed more recently non-invasively using MR spectroscopy, and further extended by relating the magnitude of the depletion to the severity of heart failure [56]. The observation that the PCr to ATP ratio and the PCr concentration are lower in both compensated LVH as well as failing hearts suggests that the loss of creatine cannot be a specific marker of the failing heart. Instead it has been hypothesised that loss of PCr is a more general marker of a mismatch in the

integration of the pathways maintaining sufficient ATP supply to meet the demand for ATP utilisation [74].

The mechanisms responsible for energy starvation in LVH are controversial and include both tissue ischaemia as a consequence of increased myocardial oxygen demand, increased LVEDP resulting in a reduced trans-coronary perfusion gradient and increased capillary-myocyte diffusion distance [67] and altered metabolism and substrate utilisation. FFA account for the majority (~70%) of energy production in the healthy adult heart, with the remainder accounted for by carbohydrates, ketone bodies and amino acids via anaplerosis [75]. Under certain circumstances this balance may change, for example during ischaemia a shift to relatively greater carbohydrate utilisation occurs [70]. Teleologically this shift results from the lower oxygen requirement for ATP production when using carbohydrates vs FFA [65, 69]. This shift away from FFA recapitulates the metabolism of the fetal heart which is relatively oxygen starved [76].

On the basis of stoichiometry, a shift from oleate (FFA) to carbohydrate metabolism would render the heart only ~12% more efficient with respect to oxygen [65, 69] but in animal models where substrate utilisation is acutely shifted from FFA to glucose, there is an ~40% increase in oxygen efficiency [77-80] . Two principal explanations have been proposed for this apparent discrepancy between stoichiometry and empirical observations. Firstly, FFA may undergo wasteful “futile” intracellular cycles [65] and secondly, mitochondrial uncoupling may be increased with dominant FFA utilisation [81]. Uncoupling proteins (UCPs) are present in the mitochondrial membrane of several tissues including cardiac and skeletal muscle [82]. The principal role of UCPs

is to export lipid peroxides out of the mitochondrion as they accumulate, protecting the mitochondrial DNA from damage [83]. However, the concomitant dissipation of mitochondrial electrochemical gradient results in a reduced efficiency of ATP production. Uncoupling protein expression is increased by FFA excess within the cytosol, acting via the PPARα receptor [83].

A large number of studies have examined substrate utilisation in animal models of LVH. Although a source of persistent controversy, these studies broadly suggest that in LVH, there is a shift away from FFA utilisation [65]. Furthermore, gene expression studies have shown a shift towards a foetal pattern of myocardial gene expression with down regulation of genes involved in FFA metabolism [75]. There has also been considerable debate as to whether this shift in substrate utilisation is an adaptive response (to reduced tissue oxygen) or a maladaptive response, predisposing to the development of HF [65].

Recent studies in other animal models of LVH have shown that while glucose uptake and glycolysis are maintained or even increased, glucose oxidation is reduced [84] . This uncoupling of glycolysis from glucose oxidation was not due to reduced activity of the pyruvate dehydrogenase complex [84, 85] but it is unknown whether the reduced pyruvate oxidation described in animal models of LVH occurs in human LVH. In patients with LVH due to aortic stenosis cardiac insulin resistance has also been demonstrated with normal basal glucose uptake but a marked reduction in insulin stimulated uptake [86]. In summary, the data in animal models of LVH suggests a down regulation of all metabolic pathways (i.e. FFA and carbohydrate oxidation). This maladaptive down regulation of all metabolic pathways would render the heart

energetically vulnerable. Furthermore if down regulation of FA β-oxidation is greater than down regulation of FA uptake, accumulation of LCFA's might increase uncoupling of the mitochondrial electron transport chain further aggravating energetic impairment.

2.9 Genomic control of metabolism

Analysis of gene expression during the induction of cardiac hypertrophy has identified a specific transcription pattern [87]. Early mediators of the hypertrophic pattern include the immediate early genes (e.g., *c-fos, c-myc, c-jun* and *Egr1*), followed by a cascade of mitogen activated protein kinases. These changes result in substantial alterations to the expression and organisation of sarcomeric and structural proteins [87].

Although many of the differentially expressed genes identified in initial studies are involved in cytoskeletal and matrix remodelling, myosin isoform switching (MHCα to MHCβ) and TGFβ signalling, downregulation of components of the fatty acid oxidation pathway has also been note. This distinct change in transcription has been described as a general reactivation of the fetal gene programme [88]

From a metabolic point of view, further genome wide expression profiling has however contradicted this initial work. Rather than an isolated downregulation of fatty acid and a switch to carbohydrate metabolism, LV hypertrophy has additionally been demonstrated to manifest a downregulation of the genes for oxidative phosphorylation. Over 40 genes associated with complexes I-V of the mitochondrial

oxidative phosphorylation and respiratory chain machinery have been identified as downregulated, with an additional seven TCA genes also downregulated [89].

In addition to the individual genes of any specific pathway, it is also now accepted that a number of master regulators control the regulation of such pathways. In controlling metabolism 2 master regulators have been identified Peroxisome proliferator activated receptor alpha (PPAR α), peroxisome proliferator activated γ coactivator alpha (PGC1α) and Oestrogen recepetor alpha (EERα).

PPARs regulate a wide variety of target genes involved in cellular lipid catabolism and storage. In the heart, activation of PPARα increases the expression of genes involved in three major steps in the cellular fatty acid utilization pathway: (i) fatty acid transport and esterification (via FATP / FAT/CD36, FABP and ACS), (ii) fatty acid mitochondrial import (via M-CPY I/CPT1β) and (iii) mitochondrial (MCAD, LCAD) and peroxisomal (ACO) β-oxidation, and recent evidence has linked PPARα to the known alterations in cardiac energy substrate utilisation during pathological LVH including reduced FAO rates and increase glycolysis. PGC-1α is a transcriptional co-activator, i.e., it increases the probability of a gene being transcribed by interacting with transcription factors, but it does not itself bind to DNA in a sequence specific manner. PGC-1α is involved in a wide variety of biological responses including mitochondrial biogenesis, glucose / fatty acid metabolism and heart development. When the heart undergoes hypertrophy PGC-1α expression is markedly decreased, and this has been associated with a conversion of fatty acid oxidation to glycolytic metabolism.

2.10 Effects of ischaemia

Myocardial substrate metabolism during ischaemia is highly dependent on the severity of ischaemia [22, 33, 90, 91]. At normothermia and at a normal workload complete elimination of coronary flow quickly results in depletion of high energy phosphates, lactate accumulation and contractile akinesis which with time evolves into tissue necrosis and myocardial infarction.

In animal models mild to moderate ischaemia results in a rapid decrease in mechanical work associated with a decrease in ATP and creatine phosphate concentrations and a transient net output of lactate by the myocardium. With time there is partial restoration of ATP [92] and lactate output decreases but contractile function does not return to normal. Following reperfusion there is a return to normal contractile function. More severe ischaemia results in greater rates of lactate accumulation and glycogen breakdown, and more severe contractile dysfunction or if the ischaemia is of sufficient duration, myocardial necrosis and infarction.

Complete cessation of coronary flow differs from partial reductions in flow in that there is no residual re-synthesis of ATP by oxidative phosphorylation and there is total dependence upon anaerobic metabolism with endogenous substrates. The sole source of glycolytic substrate under these conditions becomes glycogen since there is no blood flow to deliver glucose to the tissue [93]. There is also no washout of lactate and therefore intracellular pH decreases, and eventually a reduction in the rate of glycolysis occurs due to H^+ inhibition of phosphofructokinase. Additionally, the flux through glyceraldehyde 3-phosphate dehydrogenase may also become rate limiting due to low cytosolic NAD^+/NADH ratio [33].

Myocardial ischaemia results in a decrease in flux through pyruvate dehydrogenase (PDH) as seen by a decreased rate of glucose oxidation and a switch from net lactate uptake to net production. In vivo studies have demonstrated no inhibition of PDH with a 60% reduction in coronary flow despite increased lactate production [94] and therefore it has hypothesised that the decreased flux through PDH is due to a build up of NADH and acetyl CoA in the mitochondria independent of the phosphorylation state [93]

Reductions in coronary flow result in non-uniform distribution of blood flow across the left ventricular wall [95] with a relatively modest decrease in subepicardial flow but a more profound decrease in subendocardial flow [90, 96] . Therefore during ischaemia there are lower ATP, creatine phosphate and glycogen levels and higher lactate content in the subendocardium than in the subepicardium [95]. Some reports have suggested a small but significant increase in the capacity for glycolysis in the subendocardium relative to the subepicardium [97], and this has led to the hypothesis that the subendocardium may be inherently better suited to withstand ischaemia because of higher glycogen levels, and a greater activity of key glycolytic enzymes. However there is not a consistent transmural gradient in these parameters and therefore any transmural difference in metabolism would appear to be due to differences in blood flow and contractile work and not to the inherent capacity for glycolysis [95, 97, 98].

2.11 Effects of reperfusion

During ischaemia anaerobic glycolysis becomes an important source of ATP production but this leads to H^+ production which is a major contributor to the acidosis that occurs in the myocardium [99]. Whilst the sodium-hydrogen exchanger NHE1 provides an important mechanism whereby myocardial intracellular pH is restored, this in itself can contribute towards further cellular damage and dysfunction. Although intracellular pH is restored, the intracellular concentration of Na^+ increases, and is further exacerbated by ischaemia-induced depression of sodium-potassium pump [100]. This results in reduced calcium efflux and even calcium influx [101] leading to intra-cellular calcium overload.

Continued production of H^+ during the critical early period of reperfusion has the potential to further exacerbate myocardial injury [102]. Animal studies have demonstrated that during reperfusion there is an increase in the rate of fatty acid oxidation [103-108], impaired pyruvate oxidation and accelerated anaerobic glycolysis [109]. High rates of fatty acid β-oxidation dramatically inhibit glucose oxidation resulting in an imbalance between glycolysis and glucose oxidation [108, 110]. This uncoupling is a major source of H^+ production. When glycolysis is uncoupled from glucose oxidation and pyruvate derived from glycolysis is converted to lactate there is a net production of two H^+ from each glucose molecule which results from the hydrolysis of glycolytically derived ATP [111]. Pyruvate oxidation is also inhibited by the high plasma FFAs which result in low tissue malonyl CoA levels and subsequently less inhibition of CPT-1 and fatty acid oxidation [111].

2.12 Cell signalling in myocardial ischaemia reperfusion

Myocardial ischaemia results in a dramatic activation of AMPK [61, 112]. This activation restores myocyte ATP levels, primarily by turning on ATP generating pathways through both glucose and fatty acid metabolism in the ischaemic heart.

AMPK increases cardiac glucose utilisation by a number of different mechanisms. AMPK activation promotes the translocation of GLUT-4 to the sarcolemma of the myocytes promoting glucose uptake [112]. AMPK also phosphorylates and activates phosphofructokinase 2 (PFK-2) producing fructose-2,6-bisphosphate, a potent promoter of glycolysis. AMPK activation during ischaemia may therefore be beneficial to the heart in terms of increasing glucose utilisation and subsequent anaerobic ATP production.

A second major metabolic consequence of AMPK activation during ischaemia and reperfusion, is a stimulation of fatty acid oxidation [61] via phosphorylated and therefore inhibition of Acetyl-CoA Carboxylase (ACC), the enzyme that converts acetyl CoA to malonyl-CoA, an inhibitor of CPT-1 [62]. Inactivation therefore of ACC results in increase in fatty acid transport into the mitochondria and subsequent oxidation.

Fatty acid oxidation can be dramatically accelerated during ischaemia reperfusion such that 95% of acetyl CoA derived ATP originates from fatty acids [61]. These high rates of fatty acid oxidation are in part due to an increase in the circulating levels of free fatty acids that occur during ischaemia. The combination of AMPK activation and high circulating levels of free fatty acids can lead to high rates of fatty acid oxidation via the Randle cycle [47]. Fatty acid derived acetyl CoA decreases the

production of glucose derived acetyl CoA via inhibition of pyruvate dehydrogenase (PDH), resulting in a continued low rate of glucose oxidation, an uncoupling of glucose oxidation and a decrease in cardiac efficiency during this critical period of reperfusion.

The question therefore of whether or not AMPK activation is beneficial during reperfusion remains to be fully elucidated. The majority of studies examining AMPK activation in the ischaemic heart suggest that AMPK activation is an adaptive response that allows the heart to generate ATP and thereby protect cardiac tissue in the presence of oxygen deprivation [113] but there remains a paucity of data on the effect of AMPK activation on cardiac function.

To analyse the effect of AMPK activation during reperfusion, initial work was carried out using AMPK knock-out mice. These experiments were conducted using isolated hearts perfused with low levels of fatty acids (0.14mM oleate). AMPK activity, glucose uptake and lactate production were all increased in wild-type mice but not in knock-out mice. AMPK knock-out mice also demonstrated worse function during and after ischaemia, an increase in creatine kinase and lactate dehydrogenase production, and an increase in the pro-apoptotic caspase-3 and TUNEL positive cells [112]. These hearts also demonstrated significant increases in myocardial necrosis after ischaemia suggesting that AMPK activation is cardioprotective in isolated hearts undergoing ischaemia reperfusion and important in allowing myocytes to function following a period of ischaemia [112]. It was also noted that during ischaemia and reperfusion, both rates of fatty acid oxidation and glucose oxidation were similar between wild-type and AMPK knock-out mice[112].

Recently it has also been shown that activation of the survival pathways associated with ischaemic preconditioning is also associated with significant activation of AMPK [114]. This activation is associated with up-regulation of GLUT-4 expression, which occurs in a protein kinase C-dependent manner [115]. The activation of H11 kinase that occurs in preconditioned hearts is also associated with an activation of AMPK to the nucleus, therefore resulting in an increase in the transcription of metabolic enzymes such as GLUT-4. Although the activation of AMPK and increases in glucose uptake has the potential to benefit the heart, it has yet to be directly determined whether this activation of AMPK is associated with cardioprotection in the setting of ischaemic preconditioning [115].

AMPK activation itself has also been shown to induce preconditioning in isolated cardiomyocytes and to prevent hypoxic injury [116], although the degree to which AMPK activation is either required or sufficient to induce preconditioning is uncertain. The molecular mechanism behind which AMPK activation might induce preconditioning are complex but may include activation of ATP-sensitive potassium channels [116]. Activation stimulates the movement of these channels from storage membranes to cell surface membranes where they are physiologically active and can shorten the action potential and therefore potentially reduce calcium overload during reperfusion [116].

3. Myocardial Protection

3.1 Damage from Myocardial ischaemia

Immediately after the onset of ischaemia contractile force and pH rapidly decline as lactate and protons accumulate in the myocardial cytoplasm. High energy phosphates and glycogen reserves diminish whilst adenosine, inosine and other nucleotide products of ATP catabolism leave the cell. The ultrastructural changes in early ischaemia are limited to loss of glycogen granules and a degree of intracellular and organelle swelling.

If the period of ischaemia continues, further accumulation of fatty acids within the cell leads to diastolic arrest. Within fifteen minutes of ischaemia the control of sarcolemmal permeability is lost [117] resulting in further loss of adenosine, lactate, cytoplasmic proteins and enzymes. Macromolecules in the cell are converted to smaller more osmotically active molecules further exacerbating cell swelling. Cellular metabolism and ATP production cease and glycogen stores are eventually depleted. As glycolysis and mitochondrial function are totally lost, cellular autolysis begins and cell contents leak more extensively into the interstitial space. Myocardial contracture begins when ATP levels reach critically low levels developing firstly in the subendocardium and more rapidly with myocardial hypertrophy [118].

3.2 Damage from Myocardial Reperfusion

The response of myocardial cells to reperfusion depends upon the damage the cell has sustained during ischaemia. The critical point in the pathway to cell death at

which reperfusion results in explosive cellular response remains unknown. The spectrum of reperfusion injury ranges from myocardial stunning to more severe injury including cell death and necrosis. The histological features of some of these more advanced forms of reperfusion injury include myofibrillar disruption and contraction bands [119].

Oxygen derived free radicals generated during reperfusion are the fundamental cause of reperfusion injury and are characterised by the presence of unpaired electrons, including superoxide, hydrogen peroxide and hydroxyl radicals. In normal physiological states myocardial cells are exposed to low levels of oxygen free radicals which are well controlled. Superoxide dismutase catalyzes the reduction of superoxide anions to hydrogen peroxide and water. Hydrogen peroxide is then further reduced to water and oxygen by catalase and glutathione peroxidise. ischaemia progressively reduces the level of superoxide dismutase and increases the metabolic end-products of ATP catabolism such as hypoxanthine and xanthine which can then supply oxygen free radicals to xanthine oxidase. Reperfusion leads to a chain reaction producing overwhelming amounts of free radicals. Iron converts the superoxide radical into hydroxyl radicals which results in peroxidation of membrane lipids leading to an increase in membrane permeability, decreased calcium transport into the sarcoplasmic reticulum and altered mitochondrial function [120] leading to myocardial stunning or necrosis.

Reperfusion-induced elevation in cytotoxic reactive oxygen species (ROS), can trigger opening of the mitochondrial permeability pore (mPTP) resulting in immediate dissipation of the mitochondrial membrane potential [121], and it is ROS rather than

calcium overload that would appear to be the more important trigger for mPTP opening [122]. It has further been demonstrated in isolated cell culture that ROS-induced mPTP opening is followed by an additional ROS burst that occurs simultaneously with mitochondrial membrane potential dissipation [122-124]. This ROS-induced ROS release (RIRR) would appear to be responsible for a large fraction of the oxidative damage detected in cells after reperfusion [124]. Additionally, RIRR-induced mitochondrial depolarisation can propagate to neighbouring cardiomyocytes resulting in regional pathological disturbance in excitability [122, 124-126]. .

The most significant consequence of mPTP opening is loss of the mitochondrial membrane electrochemical gradient, critical to the synthesis of ATP. This can result in further membrane instability as ATP levels become inadequate to fuel activity of the Na^+/K^+ ion channels [127]. The mPTP can also allow leakage of components of the electron transport chain, notably cytochrome c which may lead to further production of free radicals. The mPTP also causes the mitochondria to become permeable to small osmotically active molecules resulting swelling and membrane rupture further releasing cytochrome c. Cytochrome c can cause further cellular injury by activating the pro-apoptotic caspase cascade [127]. Unless pore closure occurs, these changes will cause irreversible cell damage resulting in necrosis. Even if closure occurs, the mitochondrial swelling and outer membrane rupture may be sufficient to set the apoptotic cascade in motion

3.3 Cardioprotection from ischaemia-Reperfusion Injury

3.3.1 Cardioprotective Signalling Pathways and the mPTP

Cardiomyocyte survival after reperfusion has been negatively correlated to the fraction of cellular mitochondria that undergo mPTP induction [122]. Activation of a number of complex sets of cell signalling pathways which increase the mPTP-ROS threshold can provide the cardiomyocyte with increased protection. These signalling pathways have been employed clinically through pre- and post- ischaemic conditioning and by various pharmacological agents [128-131]. Activation at a variety of points along the various signalling pathways will result in comparable levels of protection of cardiomyocytes from reperfusion injury and it has therefore been hypothesised that there exists a common final mechanism to integrate these diverse signals to the end effecter, the mPTP [122].

3.3.2 GSK -3β mediated cardioprotection

Recently it has been proposed that GSK-3β is the most likely candidate for this point of convergence [122]. GSK-3 is a serine/threonine kinase that phosphorylates and down regulates glycogen synthase, the rate limiting step of glycogen synthase [132]. It has also been implicated in many other critical regulatory roles; it regulates multiple transcription factors, apoptosis, cell cycle progression, cell migration and cell survival. Unlike most kinases, GSK-3 is highly active in the basal state and exerts a tonic-negative inhibitory effect on down-stream pathways. Phosphorylation results in enzyme inactivation and therefore relief of its tonic inhibition and activation of downstream targets [131]. Two isoforms exist, α and β. They have similar biochemical properties and substrate recognition, but they are often but not always functionally identical and interchangeable [122] and it is GSK-3β not GSK-3α that is involved in cardioprotection [122].

Extensive evidence has now accumulated that GSK-3β is critical to the role of cardioprotection [133-136]. It has been demonstrated that both pre-conditioning [133, 134] and post-conditioning[136] involve inhibition of GSK-3β. Moreover, substantial evidence implicates GSK-3β as the critical mediator of pharmacological conditioning triggered by numerous agents including opioid antagonists [137, 138], erythropoietin [135], anaesthetics such as propofol and isoflurane[139]. It has also been demonstrated that a number of agents thought to function specifically by free radical scavenging compounds also activate prosurvival kinases acting through GSK-3β inhibition by its phosphorylation [140] .

It has been suggested that protection signalling should be separated into two main mechanistic categories; memory-associated signalling, which results in preconditioning that persists for several hours, and memory-lacking signalling, which results in relatively short-lived cell protection once the triggering agent, and therefore the phosphorylation state of one or more key proteins, is removed from the system. It has been suggested that both memory-associated and memory-lacking protection requires GSK-3β and that this enzyme is located proximally to the mPTP complex [122]. Furthermore it has been hypothesised that it acts as a master switch to convey multiplicity of protective signals to their final point the mPTP [122]. Whether or not other kinase signalling pathways can bypass this intersection remains unknown.

Memory-lacking signalling can be induced by insulin, insulin-like growth factor, glucagon like peptide-1 and, β2-adrenoreceptor activation, resulting in activation of one or more of the multiple signalling transduction pathways including P13K, PKA, PKB/AKT, PKG, PKC and their down-stream targets including GSK-3β [122].

Recent evidence supports a direct functional role of the Bcl-2 protein family in GSK-3β related cardioprotection against mPTP induction by ROS. A direct link between the induction of pre-conditioning to protect against ischaemia-reperfusion injury and GSK-3β phosphorylation-dependent modulation of mitochondrial Bcl-2 protein levels has been demonstrated [141]. In addition to a direct modulation of the mitochondrial voltage-dependent anion channels (VDAC), activity may be regulated by binding of Bcl-2 proteins to VDAC; and in a model of isolated cardiomyocytes, infusion of a peptide comprised of the BH4 domain of BcL-$X_{L,}$ increased the ROS threshold for mPTP induction [122]. Recently it has also been demonstrated that cardioprotection

induced by GSK-3β inhibition results in a significant decrease in VDAC phosphorylation with increased levels of Bcl-2 in mitochondria and specifically, increased Bcl-2 affinity for VDAC [141].

In summary, it has been proposed that in the basal (active) state a GSK-3β subdomain pool relevant to mitochondrial signalling is active and binds ANT forming a dynamic complex with phosphorylated VDAC. In this basal state the Bcl-2 BH4 domain-containing proteins would be inhibited by the BH3 proteins. Cardioprotective signalling deactivates GSK-3β by phosphorylation and leads either directly or indirectly by involving de-phosphorylation of VDAC and the release of VDAC from the GSK-3β-bound pool of ANT, to a shift in balance within the Bcl-2 family of proteins. The resultant de-phosphorylation of VDAC may then increase the ROS threshold for mPTP induction [141] thus improving myocardial protection.

3.3.4 Protein O-GlcNAcylation and Cardiac Protection

Post-translational modification (PTM) of proteins is a common mechanism for the modulation of protein function, location and turnover. Protein phosphorylation is the most widely studied form, but there are many other forms of PTM including acylation, methylation and glycoslylation. Protein O-GlcNAcylation refers to the PTM of serine and threonine residues of nuclear and cytoplasmic proteins by the O-linked attachment of the monosaccharide β-N-acetylglucosamine (O-GlcNAc). However in contrast to the hundreds of kinases and phosphatases that regulate protein phosphorylation, to date, only one enzyme has been identified that catalyses the formation of O-GlcNAc and one enzyme that catalyses its removal. It has been

estimated that approximately 5% of total glucose entering the cell is metabolised via the hexosamine biosynthesis pathway (HBP) [142].

Glucose entry into the HBP is controlled by GFAT (L-glutamate-D-fructose 6-phosphate amidotransferase) which is glutamine dependent. Two isoforms exist: GFAT-1 and GFAT-2. GFAT-1 is subject to phosphorylation by cAMP dependent protein kinase and AMPK phosphorylates GFAT-1. AMPK activity is stimulated by increased HBP flux and therefore GFAT may represent a key regulatory point between HBP and AMPK signalling [103].

The attachment of a single β-N-acetylglucosamine molecule via a O-linkage to specific serine-threonine residues of nuclear and cytoplasmic proteins is catalysed by O-GlcNAc transferase (OGT). The rate of protein O- GlcNAcylation is strongly regulated by HBP flux since OGT catalytic activity is highly sensitive to changes in UDP-GlcNAc concentrations [143]. In addition, insulin has been shown to increase both tyrosine phosphorylation and O-GlcNAcylation of OGT leading to increased activity and transient translocation from the nucleus to the cytoplasm [144, 145].

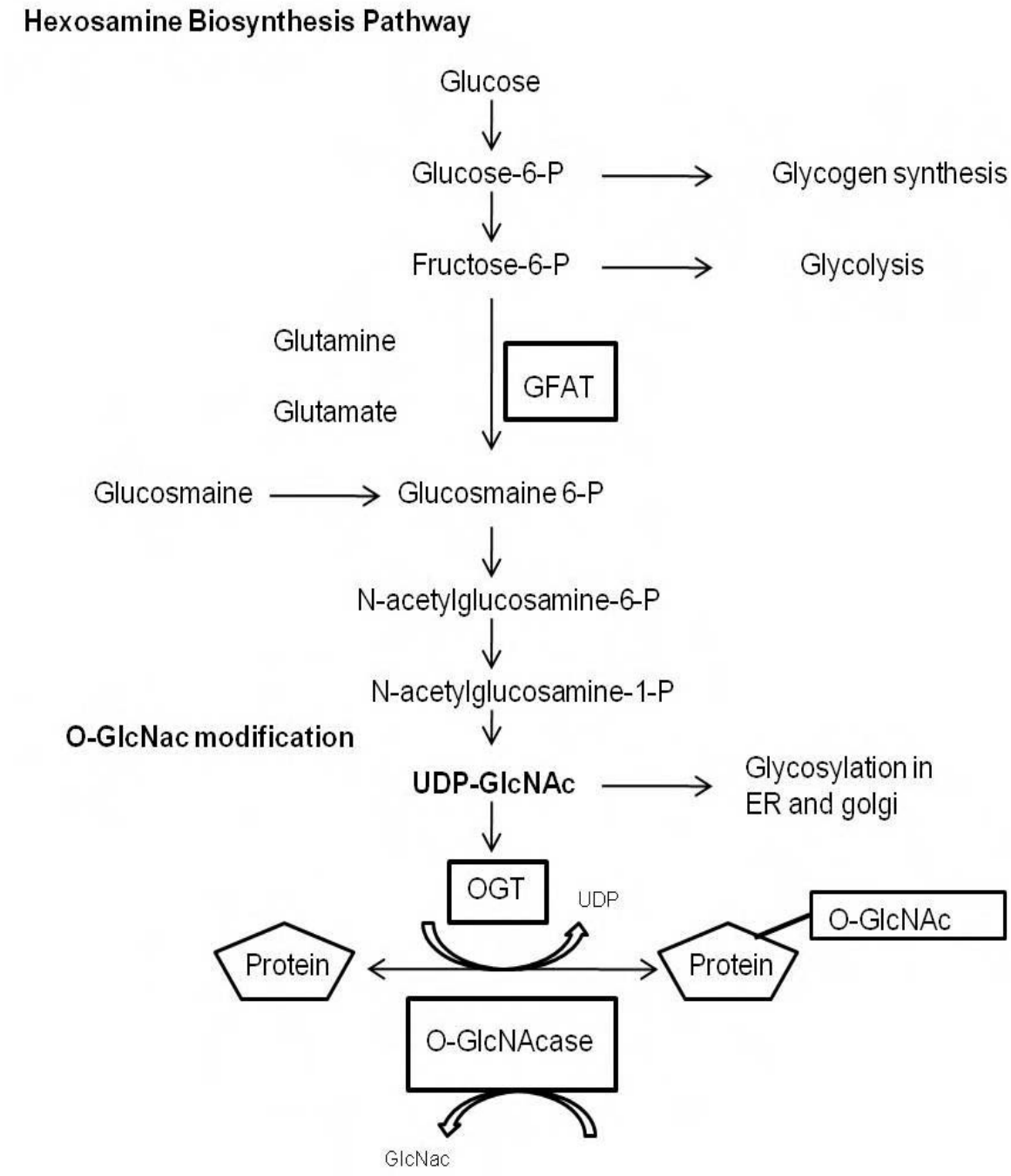

Figure 5. The Hexosamine biosynthesis pathway (HBP) and protein Protein O-GlcNAcylation [142].

Protein O-GlcNAcylation is emerging as a key regulator of a number of critical biological processes including nuclear transport, translation and transcription, signal transduction and apoptosis [146]. Although chronic activation of O-GlcNAc would appear to be deleterious in a variety of settings, acute activation appears to be an endogenous stress response designed to enhance cell survival [146]. Overall O-GlcNAc levels have been shown to be significantly increased in response to multiple experimental stress stimuli in several mammalian cell lines [147].

In the isolated perfused heart, ischaemia has been shown to increase both UDP-GlcNAc and overall O-GlcNAc levels [148] indicating that this endogenous stress-activated pathway is active in the heart. Activation of the HBP either by glucosamine or glutamine prior to the induction of ischaemia significantly increased O-GlcNAc levels [146], improved contractile function and decreased tissue injury after reperfusion [149]. Additionally, perfusion with the GFAT inhibitor Azaserine and the OGT inhibitor Alloxan prevented the increase in O-GlcNAc levels, decreased functional recovery, and exacerbated tissue injury after ischaemia-reperfusion. There was also significant correlation between increased O-GlcNAc levels and cTnI release [149].

Several putative mechanisms have been suggested to explain the increased tolerance to ischaemic stress associated with increased O-GlcNAc levels [146]. Increasing O-GlcNAc levels have been associated with increased transcription of heat shock proteins (HSP) including HSP40 and HSP70 [147]. In particular HSP70 has been identified as a target for GlcNAcylation [150]. Activation of O-GlcNAc formation has also been shown to attenuate mPTP opening, a critical step in oxygen free radical-induced apoptosis and necrosis [151, 152]. One of the O-GlcNAc modified

proteins identified as a potential O-GlcNAc target is VDAC, and it has been hypothesised that VDAC may preserve mitochondrial integrity by interfering with mPTP formation [151].

A number of other pathways may also contribute to O-GlcNAc-mediated cardioprotection. Increasing O-GlcNAc levels with glucosamine attenuated the ischaemia-induced p38 MAPK phosphorylation, decreased ischaemic contracture and reduced the incidence of reperfusion arrhythmias [146, 148]. Paradoxically, at the end of reperfusion, phosphorylated p38 levels were increased in response to glucosamine treatment which could lead to activation of pro-survival pathways through downstream effectors such as αβ-crystallin and HSP27, both of which have been shown to play a role in ischaemic protection and are also targets for GlcNAclyation [153]. It has been shown that ischaemia-reperfusion alters the level of O-GlcNAc modification of glycogen phosphorylase b, mitochondrial aconitase 2 and the cytoskeletal protein vinculin [154] all of which have been hypothesised to improve functional response to ischaemia-reperfusion injury [154]. Increased levels of O-GlcNAc have also been reported to inhibit protein degradation [147] most likely due to inhibition of the proteasome [147] and this could also contribute to improved cell survival. In addition there is also some experimental evidence that increasing O-GlcNAc levels inhibit Ca^{2+} overload during reperfusion [155].

Cellular stress leads to an increase in ROS production and one consequence of this is increased flux through HBP and an increase in O-GlcNAc synthesis [156] which in turn leads to increase in O-GlcNAcylation of mitochondrial proteins and increased tolerance of mitochondria to stress [151]. Furthermore, there is some experimental data

suggesting that inhibition of GADPH by mitochondrial superoxide is an important factor leading to increased O-GlcNAcylation [156] and taken together these studies suggest that ROS and mitochondria may contribute towards the regulation of O-GlcNAc synthesis which could in turn modulate the response to oxidative stress.

Nitric oxide is one of the most widely recognised reactive species in redox signalling and nitric oxide synthase (NOS) is subject to both phosphorylation and O-GlcNAcylation and therefore is a potent target for O-GlcNAc signalling [146]. eNOS is activated by Akt-mediated phosphorylation thereby increasing NO production. This Akt phosphorylation site is also subject to O-GlcNAcylation leading to lower activity and decreased NO production [156]. Therefore the modulation of NO production by O-GlcNAc demonstrates a convergence between redox signalling and O-GlcNAc protein modification [146].

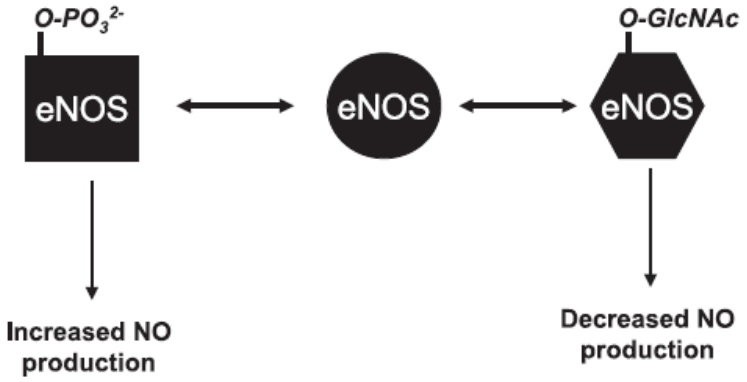

Figure 6. Phosphorylation of endothelial nitric oxide synthetase (eNOS) results in increased eNOS activity and NO production, whereas O-GlcNAcylation at the same site leads to decreased enzyme activity and NO production[146].

The intersection of redox and O-GlcNAc signalling may apply not only to specific proteins such as eNOS but may be involved in the regulation of entire signalling pathway[146]; the insulin pathway is such a proposed pathway. A number of components of insulin signalling include IR-β, IRS-1/2, PI3K, Akt and GSK-3β have all been reported to be O-GlcNAcylated and the result is lowered activity and an attenuated response to insulin [157]. Insulin signalling is also regulated by NO, the production of which may be regulated by direct O-GlcNAcylation of NOS. Thus increasing flux through OGT is envisaged to decrease NO production not only by direct modification of NOS but also by reducing NOS phosphorylation via O-GlcNAcylation of the IR complex and Akt.

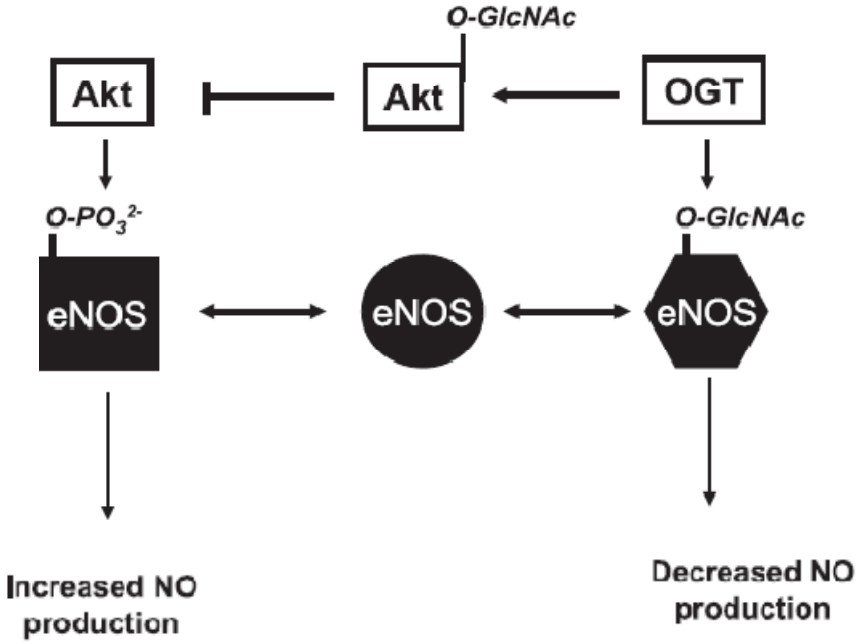

Figure 7 O-GlcNAc modification of eNOS decreases its activity and NO production. At the same site Akt-mediated phosphorylation and activation of eNOS decreases its activity and NO production. However, Akt is also subject to O-GlcNAcylation which reduces its activity thereby inhibiting eNOS phosphorylation and NO production [146].

3.4 Myocardial Protection for Aortic Valve Surgery

Protective strategies are employed during cardiac surgery to produce a motionless bloodless operating field enabling access to the aortic valve to facilitate precise surgical repair whilst preserving myocardial function by limiting ischaemia and controlling reperfusion.

Establishing cardiopulmonary bypass alone results in a significant reduction in cardiac work. It reduces ATP requirements from a resting level ~85µmol/g/min to 50µmol/g/min [158]. This requirement can be reduced to less than 10µmol/g/min by preventing myocardial depolarisation [158]. The resting membrane potential is defined by the ion concentration differences between the intracellular and extracellular areas, and by the permeability of the membrane to each type of ion. The interactions between ions that generate the resting potential are modelled by the Goldman-Hodgkin-Katz voltage equation which in the ventricular myocardium is between -85mV to -95mV. The maintenance of this gradient is due to the $Na^{+/}K^{+}$ ion exchange pump as well as the Na^{+}/Ca^{2+} exchanger current and the inwardly rectifying K^{+} current. Excitation in the resting phase (Phase 4) results in opening of fast Na^{+} channels resulting in a rapid influx of Na^{+} ions

This can be achieved with the use of cardioplegia, a solution containing a high concentration of potassium.

Initial work by Melrose [159] and then by Sealy[160] demonstrated proof of concept in the 1950s but clinical application was limited by the development of myocardial necrosis. Further refinements to the composition of crystalloid cardioplegia solutions by Gay and Ebert [161] led to the development of the first clinically accepted St Thomas' cardioplegia solutions by Hearse in 1973 [162]. This concept was taken further by Buckberg who reported use of blood based cardioplegia in 1978 with the theoretical advantages of improved buffering, endogenous oxygen free radical scavenging and superior oxygen transport [163, 164].

3.5 Special considerations with LVH

The hypertrophied left ventricle is particularly vulnerable to ischaemia-reperfusion injury [165-167]. Hypertrophy is accompanied by distinct quantitative and qualitative changes including a switch of contractile proteins to a foetal isoform and a reintroduction of fetal isoforms for numerous enzymes including lactate dehydrogenase and creatine kinase [111, 168], changes in calcium handling[34], impaired insulin signalling [169] as well as the metabolic changes discussed earlier in in this chapter [65, 170, 171].

These adaptive changes should render the heart less susceptible to ischaemic injury as anaerobic glycolysis becomes the main source of energy production but paradoxically glucose uptake and metabolism is impaired during ischaemia and early reperfusion. Hypertrophied hearts use both exogenous carbohydrates such as glucose and lactate, and endogenous stores of glucose from glycogen [110]. During myocardial ischaemia hypertrophied hearts exhibit accelerated loss of high energy

phosphates [102], greater accumulation of tissue lactate and hydrogen ions, earlier onset of ischaemic contracture and accelerated calcium overloading during early reperfusion [110]. This increased vulnerability is due to a combination of morphological, metabolic and physiological changes that occur in the hypertrophied myocardium.

4. Glucose-Insulin-Potassium Therapy

4.1 History

The use of Glucose-insulin-potassium (GIK) as a metabolic intervention to treat myocardial ischaemia was first reported by Sodi-Pallares in 1962 [172]. He demonstrated in animals and then patients with acute myocardial infarction that GIK reduced electrocardiographic signs of ischaemia, reduced infarct size and improved survival. However, a trial by the Medical Research Council, UK found that patients with acute myocardial infarction treated with GIK had no positive survival benefits. The combination of this and the introduction of thrombolysis for the primary treatment of acute myocardial infarction tempered its further use.

The potential benefit of this therapy to improve contractile function in patients with myocardial stunning following cardiac surgery was demonstrated by Baimbridge in 1969, and in the early days of cardiac surgery GIK was used for both cardioprotection and weaning from cardiopulmonary bypass. Its use was then abandoned due to the introduction of St Thomas' cardioplegia, the development of hypothermic cardiopulmonary bypass and the introduction of positive inotropic agents

such as dopamine and dobutamine alongside less cardiodepressant anaesthetic agents.

In the 1980s there was an increase in the number of patients with unstable coronary artery syndromes presenting for emergency surgical revascularisation. In addition elderly patients and patients with a history of severe coronary artery disease, ventricular hypertrophy and chronic heart failure were referred for surgery. These patients required more complex surgery and frequently developed significant myocardial stunning. Poor outcomes in these patients using contemporary myocardial protection strategies led to further interest in protective adjuncts such as GIK.

4.2 Proposed Mechanisms of Action

The potential beneficial mechanisms of action of GIK therapy can be broken down by the timing of ischaemia. Prior to ischaemia GIK may increase myocardial glycogen stores resulting in improved ischaemic tolerance [173]. During ischaemia when oxidative metabolism is reduced, GIK may augment anaerobic glycolysis, the predominant source of ATP production [174]. In reperfusion GIK can limit the high plasma concentrations of fatty acids high levels which result in an increase in the rate of myocardial fatty acid uptake and oxidation [104, 107, 175], and therefore lead to a reduction in glucose utilisation by the myocardium and suppression of glucose oxidation to a greater extent than glycolysis resulting in an uncoupling of glycolysis from glucose oxidation [102].

4.2.1 Glucose uptake & glycogenesis prior to ischaemia

Insulin and ischaemia both stimulate the translocation of GLUT-1 and GLUT-4 to the sarcolemma [24] and therefore increase the capacity of the myocardium to take up glucose. Importantly, these effects have been shown to be additive in terms of glucose transporter translocation and glucose uptake [25]. Insulin also increases glycogen synthetase activity and promotes glycogen storage [40]. Studies have demonstrated a 50-70% increase in cardiac glycogen concentration with overnight treatment with GIK prior to surgery with associated improved clinical outcomes [173, 176]. However it is unknown whether the improved outcome was related to a greater rate of carbohydrate oxidation due to lower FFA levels and higher rates of glycolysis and pyruvate oxidation or due to glycolytically derived ATP.

4.2.2 Glucose uptake and glycolysis during ischaemia

Myocardial substrate metabolism during ischaemia is highly dependent on the severity of ischaemia [70]. During ischaemia mitochondrial oxidative metabolism is suppressed and anaerobic glycolysis becomes an important source of ATP production [174]. Some of the beneficial effects of GIK may be due to an increase in glycolytically derived ATP, a decrease in PDH activity due to decreased plasma FFA concentration and increased insulin levels.

Animal studies of GIK during periods of low-flow ischaemia have demonstrated an increase anaerobic glycolytic flux and therefore a decrease in ATP depletion [93] resulting in improvements in systolic and diastolic function. Preservation of high energy phosphates during ischaemia is beneficial as it may help to maintain critical membrane function including sodium and calcium ion homeostasis and ultimately, the enhanced production of energy may delay or reduce the extent of ischaemic necrosis.

4.2.3 Glucose and fatty acid oxidation during reperfusion

The recovery of myocardial oxidative metabolism is rapid following reperfusion [104]. During this period fatty acid metabolism dominates as a source of oxygen consumption for a number of reasons [104]. Circulating fatty acid levels rise as a result of heparin and of elevated endogenous catecholamines [102] and high plasma FFA levels result in an increase in the rate of myocardial fatty acid uptake. High cytosolic concentrations of FFA result in both stimulation of fatty acid oxidation and an accumulation of fatty acid intermediates such as acyl-carnitine which contributes

towards cell membrane damage [61]. Incomplete oxidation of FFA during periods of ischaemia also leads to peroxide formation which impairs cellular function. ischaemia reperfusion also results in phosphorylation of AMPK which reduces malonyl coenzyme (CoA) to inactive ACC therefore reducing inhibition of CPT-1 mediated fatty acid uptake into the mitochondria [175].

The dominance of fatty acid oxidation during reperfusion leads to a reduction in glucose utilisation by the myocardium. Importantly glucose oxidation is suppressed to a much greater extent than glycolysis. This results in a far greater uncoupling of glycolysis from glucose oxidation than normal. This increased uncoupling leads to accelerated proton production that is an important contributor to the myocardial dysfunction and decreased contractile efficiency observed during reperfusion [98, 177]. Thus high levels of fatty acid oxidation contribute towards myocardial dysfunction and contractile inefficiency during oxidation by decreasing rates of glucose oxidation and increasing proton production from glucose metabolism [53, 104, 175].

GIK therapy during reperfusion results in high insulin levels which inhibit hormone sensitive lipase in adipose tissue. This leads to a significant reduction in circulating fatty acid levels resulting in reduced myocardial fatty acid uptake and oxidation [93]. This concept has been supported by the observation that the greatest clinical benefit of GIK therapy occurs with therapeutic regimens that maximally suppress circulating fatty acid levels [178]. Fatty acid oxidation may also be directly reduced by the action of insulin on AMPK [179].

GIK infusion during the reperfusion period may also directly stimulate glucose uptake, again via GLUT translocation [25, 26], and this combined with the reduction in fatty acid oxidation results in an increase in myocardial glucose oxidation [104]. This may lead to improved coupling of glycolysis to glucose oxidation [103], decreased proton production [99] and therefore an increase in myocardial contractile efficiency.

4.2.4 Anapleurosis via pyruvate during reperfusion

Depletion of Krebs cycle intermediates during reperfusion has been hypothesised to be a significant cause of impaired energy production during reperfusion [90]. Anapleurosis refers to the pathways which are involved in the replenishing of intermediate substrates of metabolic cycles. There are 4 major anapleurotic reactions including carboxylation of pyruvate to oxaloacetate; transamination of aspartate to oxaloacetate; glutamate-dehydrogenase of glutamate to α-ketoglutarate and β-oxidation of fatty acids to succinyl-CoA. Of these reactions, carboxylation of pyruvate is the major mechanism of aneuplorosis in the heart [178]. GIK has been proposed to increase pyruvate production by increasing glucose uptake and glycolytic flux. Therefore an additional benefit of GIK therapy may be rapid replenishment of the depleted citric acid cycle intermediate pool.

4.2.5 Direct Insulin activation of AKT signalling

Recent work by Yellon has demonstrated that insulin administered at the onset of reperfusion attenuated infarct size by more than 45% in an isolated perfused rat heart subjected to 35 minutes of regional ischaemia and 2 hours of perfusion [180]. Perfusing the heart with either glucose or pyruvate showed no significant differences in the degree of cardioprotection suggesting that the effects of GIK are predominantly due to insulin. It was also found that administration of insulin directly at the onset of

reperfusion was crucial in mediating cardioprotection. A delay of 15 minutes after reperfusion negated any cardioprotective effect of insulin administration.

Insulin is known to activate the prosurvival kinase Akt. Akt is activated in the proximity of the cell membrane with subsequent translocation to the cytosol. ischaemia alone induces Akt phosphorylation >4 fold in the absence of insulin. On the basis of the cardioprotective effect of insulin being independent of glucose administration it was proposed that it was achieved via Akt-mediated cell survival signalling. The administration of insulin at the onset of reperfusion maintained activated/phosphorylated Akt in both the cytosolic and membrane fraction of the myocardium compared to placebo treated hearts. Addition of an Akt inhibitor such as the PI3-kinase inhibitor wortmannin, completely abolished the cardioprotective effect of insulin[180].

The conclusion of this paper was that the classic tyrosine kinase and Akt-mediated cell survival programmes are activated by insulin when administered at the moment of reperfusion.

4.3 Studies of GIK in Acute Myocardial ischaemia

Interpreting the results of GIK as a therapeutic strategy in the treatment of acute myocardial infarction (AMI) have been complicated by the study designs which have differed significantly in terms of composition of the GIK solutions and in the timing and duration of therapy. A meta-analysis by Ordoubardi in 1997 identified 15 randomised control trials of GIK versus placebo therapy in the treatment of AMI [181].

Six trials were excluded leaving a total of 9 trials containing 1932 randomised patients. The hospital mortality in the GIK group was 16.1% versus 21% in the placebo group, a relative reduction of 23% resulting in a number needed to treat to prevent one death of 20 patients.

Since this meta-analysis there have been four further randomised controlled trials of GIK. The Diabetes Mellitus Insulin Glucose Infusion in Acute Myocardial Infarction (DIGAMI) trial [182], the Polish GIK trial (Pol-GIK) [183], the Dutch Glucose Insulin Potassium Study (GIPS) [184] and the Estudios Cardiolgicos Latinoamerica (ECLA) study [185].

The DIGAMI study was a multi-centre European study which examined the effects of in-hospital glucose-insulin infusions and subsequent tight glycaemic control with subcutaneous insulin in diabetic patients with AMI. 620 diabetic patients were randomised to either conventional treatment or an infusion of 5% glucose with 80 IU L^{-1} of actrapid insulin. Although they did not demonstrate a reduction in in-hospital mortality, late follow up demonstrated an absolute risk reduction in mortality of 11% [182].

A second DIAGMI 2 study recruited a further 1253 non-insulin dependent diabetic patients with AMI [186]. They were randomised to either glucose-insulin infusion followed by insulin, glucose-insulin followed by clinician led management or routine glucose management but this trial failed to detect a significant difference in mortality at 2 year follow up. In the original DIGAMI trial all patients recruited had a blood glucose of >11.0mmoll^{-1} [182] but in DIGAMI 2 blood glucose was not part of the

inclusion criteria [186]. DIGAMI patients therefore had a higher baseline level of blood glucose with a greater initial reduction than patients recruited in DIAGMI 2.

The Pol-GIK trial was a prospective randomised multi-centre trial examining the effects of low dose GIK (10% dextrose;20-32 IU Insulin l^{-1}, KCl 80mmoll^{-1}) versus placebo in non-diabetic patients presenting with AMI for a 24 hour period [183]. 954 patients were randomised but this trial was terminated early after the interim analysis found no difference in mortality or specific cardiac events. The lack of effect may have been due to a large number of low risk patients recruited with a lower than predicted overall mortality. In addition, the GIK regimen employed may not have been adequate to suppress FFA plasma levels and the investigators proposed further work using a higher dose of GIK [183].

The GIPS trial randomised 490 patients who were suitable for PCI to either GIK or placebo [187]. There was no statistical difference in 30 day survival overall but a sub-group analysis demonstrated a reduction in mortality for those without heart failure. This led to the design of GIPS-2 which examined the outcome of GIK as an adjunct in patients without heart failure undergoing primary PCI [184]. The trial was halted early after an interim analysis reported no difference between groups [188]. At one year follow up there was no difference in mortality, recurrent MI or need for repeat intervention between groups.

The multi-centre CREATE-ECLA study randomised 20,201 patients with AMI presenting within 12 hours to receive either GIK (25% glucose, 50IUl^{-1} Insulin, Potassium 80mmoll^{-1} at 1.5.ml/$kg^{-1}h^{-1}$) for 24 hours or standard care [185, 189]. 74.1% of

patients received thrombolytic therapy and 9.1% underwent primary PCI. The trial had a 99% and 95% power to detect a 20% and 15% relative risk reduction respectively with the use of GIK. The trial demonstrated no significant differences between groups on the primary outcome of 30 day all-cause mortality or any of the secondary endpoints which included a composite endpoint of death or non-fatal cardiac arrest, death or cardiogenic shock, death or re-infarction and each of these individually. There were also no differences in any of the pre-specified subgroup analysis which included diabetes status, heart failure, time to presentation, reperfusion strategy.

4.4 Studies of GIK in Cardiac Surgery

Interpreting the trials of GIK in cardiac surgery has been complicated by the same heterogeneity of dosage regimens, timing of treatment, length of treatment and route of administration as in the trials of GIK in AMI. The majority of these studies have been carried out in patient undergoing coronary bypass grafting (CABG) using cardiopulmonary bypass and cardioplegia based myocardial protection [190-195]. Although they have consistently demonstrated improvements in post operative haemodynamics, they have been less clear on the effect of GIK on reducing myocardial injury.

Bothe et al conducted a meta-analysis of the randomised trials examining the effects of GIK in cardiac surgery [190]. 35 trials were included, the majority performed in patients undergoing CABG with cardiopulmonary bypass. This meta-analysis demonstrated an 11% improvement in post-operative cardiac index. When trials

were split into peri-operative versus post-operative infusion strategies the calculated improvement was 6.1% versus 19.5% respectively. The meta-analysis also demonstrated a consistent reduction in inotrope usage.

4.4.1 The MESSAGE Trials

Between January 2000 and September 2004, two consecutive randomised double-blind prospective placebo controlled trials were performed at University Hospital Birmingham examining the effects of GIK versus placebo in non-diabetic patients undergoing isolated coronary artery bypass grafting [196-198]. This trial also contained a third arm examining the effect of thyroid hormone in addition to or separate from GIK. 160 patients were randomised to placebo and 157 to GIK[198].

The main clinical findings of these studies were that GIK improved haemodynamics in the post-operative period during the period of GIK infusion. In addition, the incidence of low cardiac output episode as defined by a cardiac index of $<2.2 l min^{-1} m^{-2}$ was lower in the GIK group although after post hoc testing this did not reach significance. In the GIK arm 33 / 157 patients (21%) required inotropic support for LCOE compared to 50 / 160 (31.3%) in the control arm [197]. However, GIK was associated with a consistent reduction in systemic vascular resistance and hence a parallel increase in vasopressor requirements. In the GIK arm 108 / 157 (69%) of patients required vasoconstrictor therapy compared with 73 / 160 (46%) in the control group, p=0.0001.

The MESSAGE studies also examined myocardial injury at the time of surgery by analysing serial Troponin I plasma levels and serial ECGS. There was no statistical difference in the incidence of new peri-operative myocardial infarction as defined by the presence of new Q-waves or new LBBB on serial ECGs and in the second no significant difference in Troponin release between the two treatment groups [197, 198].

4.4.2 Previous studies of GIK in ventricular hypertrophy

The majority of clinical trials in cardiac surgery have examined the effect of GIK in patients undergoing isolated coronary artery bypass grafting. Although some of these patients will have had significant left ventricular hypertrophy secondary to hypertension, this is not detailed in any of the trials nor in the meta-analysis discussed above.

A study from Finland by Koskenkari and colleagues randomised 40 patients undergoing combined AVR and CABG randomised 1:1 to GIK or control [199]. Although they did not examine the degree of hypotrophy in these patients, the trial demonstrated a reduction in LCOE from 17 / 20 (85%) in the control arm to 13 / 20 (65%) in the GIK arm with similar vasopressor requirements. One patient in the GIK arm had a peri-operative myocardial infarction defined by peak Troponin I >60$\mu g l^{-1}$ compared to two patients in the control arm (N/S). No other studies have examined the effects of peri-operative GIK administration on patients with demonstrable hypertrophy undergoing cardiac surgery.

5. Metabolomics

Whilst it has been consistently demonstrated that LVH is associated with changes in the PCr:ATP ratio there is an appreciation that this may well be the end point of a down regulation of multiple metabolic pathways. Such a down regulation may result in differing concentrations of multiple intermediary metabolites.

Metabolomics is a novel field of investigation that attempts to analyse the small changes in metabolites, that individually, may be insignificant, but when compared together may demonstrate significant changes in the metabolome. The metabolome represents the collection of all metabolites in a biological cell thus giving an instant snapshot of the physiology of that cell. It spans a variety of chemical compound classes, including those that are anionic versus cationic, and lipophilic versus lipophobic. Metabolites in tissue are present across a broad range of concentration and no individual analytical method is capable of analysing all metabolites.

5.1 Techniques for Metabolomic Analysis

Metabolites can be measures by several available analytical methods. Chromatographic procedures such as gas chromatography (GC), high performance liquid chromatography (HP-LC) and capillary electrophoresis have all been used to identify and quantify metabolite subsets e.g., purine metabolites but are best used for initial separation in combination with other detection techniques [200, 201]. Recently, two high-throughput techniques have been used for profiling large numbers of

metabolites simultaneously: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). NMR spectroscopy uses magnetic properties of nuclei to determine the number and type of molecular entities in a molecule whereas MS distinguishes metabolites on the basis of mass/charge ratio (m/z). It requires a separation of the metabolite components but offers far greater sensitivity than NMR and has been used to resolve compounds in the femtomole / litre range.

Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry ionizes compounds to generate charged molecules and then measures their mass-to-charge (m/z) ratio. FT-ICR measures mass by detecting the image current produced by ions cyclotroning in the presence of a magnetic field. Cyclotroning is the method of using a perpendicular magnetic field to accelerate charged particles to spiral almost in a circle so that they re-encounter the accelerating voltage many times. Instead of measuring the deflection of ions within a detector such as an electron multiplier, the ions are injected into a static electric/magnetic ion trap (Penning trap) where they effectively form part of a circuit. Detectors at fixed positions measure the electrical signal of ions which mass near them over time, producing a periodic signal. Since the frequency of an ion's cycling is determined by its mass to charge ratio, the m/z ratio can be calculated by a process of deconvoluting by performing a Fourier transform on the signal. Fourier transform is a mathematical algorithm which converts the wave form function into a secondary mathematical function [202].

5.2 Statistical Approaches to Metabolomic Data Reduction and Pathway Analysis

High-throughput metabolomics risks generating false-positive associations due to multiple testing and over-fitting of data. However, traditional statistical techniques to correct for such errors e.g. Bonferroni correction, result in an insurmountable statistical penalty, and even newer statistical techniques such as re-sampling methods, or control of false discovery rate do not really adequately address the fundamental problem of how to detect subtle but important changes in multiple variables identified in an 'omics' approach [203].

For metabolites participating in set biological pathways, a bioinformatics approach using pathway analysis can instead be employed. Although measurement error in the marker discovery phase often prevents high confidence in any individual metabolite's correlation, the observation that multiple metabolites in a particular biological pathway are moving in a related fashion allows the correlation of changes in the biomarkers of that pathway to be made.

There are a number of data reduction strategies that have been validated for processing high-throughput data including supervised learning multivariate analysis, cluster based discriminant analysis, partial least squares linear regression analysis and principal component analysis (PCA) [204].

6. Hypotheses

Symptomatic aortic stenosis is associated with a poor natural history with almost universal progression to heart failure and death if left untreated. Animal models suggest that pathological hypertrophy is associated with impaired cardiac energetics secondary to maladaptive changes in metabolism. Human data from patients in heart failure also suggests that impaired cardiac energetics as defined by a reduced PCr:ATP ratio is also highly predictive of survival. Patients with significant hypertrophy undergoing cardiac surgery are known to be at increased risk of inadequate myocardial protection due to a number of morphological and functional abnormalities. In addition significantly impaired energetics due to maladaptive changes in metabolism may further jeopardise their myocardial protection.

Glucose-insulin-potassium has the potential to improve myocardial protection in these patients via a number of mechanisms. High levels of glucose and insulin in the immediate pre-ischaemic period may improve myocardial glycogen storage. Switching substrate to more efficient glucose oxidation during reperfusion should improve aerobic ATP production and therefore also reduce the production of oxygen free radical species. Insulin itself has also been linked to “memory-lacking” cardioprotection through the conditioning pathways. This may therefore also decrease mPTP susceptibility to ROS through GSK-3β signalling pathway.

1. Myocardial protection in patients undergoing aortic valve replacement for aortic stenosis may be improved by metabolic manipulation in the immediate peri-operative period.

2. Left ventricular hypertrophy in man secondary to aortic stenosis is associated with significant impaired energetics due to down-regulation of cardiac metabolic pathways as demonstrated by an abnormal metabolome.
3. Glucose-insulin-potassium improves myocardial protection by suppressing plasma free fatty acid levels and up-regulating glucose transport in the early reperfusion period thus driving more efficient glucose metabolism.
4. Insulin also exerts a direct cardio-protective effect on the cardiomyocyte via Akt / AMPK signalling.
5. Insulin also increases protein O-GlcNAcylation and therefore decreasing the susceptibility of the mitochondria to oxidative stress and improving myocardial function following ischaemia reperfusion.

Chapter 2. Design and Methodology

7. Trial Design

7.1 Patient Selection

The aim of this study was to assess the impact of GIK on patients with with echocardiographic evidence of LVH as defined by a left ventricular mass index greater than 134 $g.m^{-2}$ for men, or 100 $g.m^{-2}$ for women [205, 206], undergoing AVR for AS ± CABG. It was decided to include patients undergoing concomitant coronary artery revascularisation as this effects over 30% of patients undergoing AVR for AS and indeed patients with a combination of LVH and coronary artery disease may be at further risk from significant ischaemia-reperfusion injury. To select a relatively homogenous patient group patients undergoing ascending aortic replacement were included but not those undergoing root or arch procedures. Aortic root procedures are uncommon in patients with AS, and patients with arch pathology represent a different disease pathology, and tend to require more extensive surgery including circulatory arrest.

Patients with isolated aortic regurgitation AVR were excluded. These patients demonstrate eccentric remodelling with compensatory larger stroke volumes and therefore haemodynamically behave very differently. Patients with other significant valvular lesions were also excluded due to the impact of this haemodynamic pathology on ventricular function.

Patients with atrial fibrillation, or those in sinus rhythm maintained with Amiodarone were also excluded. Patients with significant LVH have a greater dependence on

active atrial contractility to maintain cardiac output, and the presence of AF can result in difficulties in measuring cardiac output using a thermodilution technique.

7.2 Endpoint Selection and Power Calculation

In 2003 when this trial was being designed, the mortality for isolated AVR was ~3%, and for patients undergoing AVR + CABG ~6% [207]. The operative mortality has continued to improve and the mortality until the year ending 2008 in the UK was 2 and 4.5% respectively [208]. Powering a study designed to analyse survival in a single centre is therefore not possible. We therefore examined potential surrogate markers of outcome.

The incidence of LOCS following surgery in the UK however remains approximately 30%. This is thought to represent a type of myocardial stunning, and although most patients develop no permanent myocardial injury [209], some patients require prolonged or high dose inotropic support which may be associated with permanent injury resulting in further remodelling and fibrosis [210]. Additionally, of the patients that do not survive cardiac surgery, LCOE is implicated in 30% of deaths, and late outcome may be jeopardised [211, 212]

In previous trials of GIK in the department, the use of GIK was associated with a reduction in LCOE from 31% to 21% [198] The incidence of LCOE in patients undergoing AVR as recorded by the department PATS database is approximately 35%. Based upon the assumption that GIK might offer similar benefits in patients

undergoing AVR, 220 patients randomised 1:1 to GIK or control would give the trial a 90% power with 5% sensitivity.

On this basis it was decided that LCOE would be both a feasible and clinically relevant primary endpoint. To allow for comparison with previous work in the department, LCOE was defined as CI<2.2 $lmin^{-1}m^{-2}$.in the presence of adequate filling pressures (CVP 8-12mmHg or PAWP 12-16mmHg) and adequate heart rate, (80-110bpm) where systolic BP <90mmHg and/or inotropic support +- IABP for >60 minutes to maintain such a clinical picture within six hours of cross clamp removal. Any patient meeting any of these criteria was to be presented to a blinded end-points committee where a diagnosis of LCOE was to be adjudicated.

Secondary endpoints were pre-defined as need for inotrope therapy and total dose of inotrope therapy administered, post operative haemodynamics as defined by the cardiac index at 6 hours following AXC removal, stratified for surgeon and intent to perform CABG and new peri-operative myocardial infarction. There is a paucity of data on which to define myocardial infarction following aortic valve surgery. It was therefore decided to compare mean TnT at six hours post AXC removal, again stratified by surgeon and intent to perform CABG. Additionally, a significant Troponin rise for patients undergoing isolated AVR was defined >0.8 μl^{-1} and in patients undergoing AVR + CABG >1.3 μl^{-1}. Safety outcome measures including length of stay, glucose control and wound infection rates.

7.3 Clinical Protocols

To allow for appropriate comparison between groups pre-determined surgical and anaesthetic protocols were agreed by all trialists. The management of potential LCOE episodes was also standardised. Dopamine was selected as the first line inotrope as per standard clinical practice in our department. The first choice vasopressor however was changed from norepinephrine to phenylephrine. Although in clinical practice norepinepherine is considered primarily an α-agonist it also has significant β1 effects resulting in an increase in contractility. In contrast, phenylephrine is a pure α-agonist with no β effects.

7.4 Haemodynamic monitoring

Haemodynamic measurements were obtained in all cases with a Pulmonary artery flotation catheter (PAFC). The cardiac output was measured using thermodilution. Three successive injections of 10 ml cold (6–10°C) dextrose 5% in water at end-expiration were made. The recorded value was the mean of three individual measurements. Recorded values for pulmonary artery pressure, pulmonary artery wedge pressure (PWP) and CVP were obtained at end-expiration from graphic recordings.

The indices of LV function used in this trial as a surrogate of LV function are both load and afterload dependent. Therefore, in all patients, it was attempted to manage these parameters with appropriate filling to achieve a PAWP of 8-14mmHg and via the use of vasoactive drugs to achieve a SVR of 800-1200 dyne.cm^{-1}.sec^{-5}.

7.5 GIK Administration

Previous studies with GIK in our department had infused GIK from the induction of anaesthesia until six hours post aortic-cross clamp removal. The data from these previous studies had shown that this approach had resulted in a median infusion time of 85 minutes until the onset of global ischemia secondary to aortic cross-clamping. Animal data from GIK experiments had previously demonstrated maximal glycogen loading within one hour of administration. Following aortic cross clamp removal, the nadir of ventricular function has been demonstrated to occur within six hours of aortic cross clamp removal [213,214]. Unless the myocardium is subjected to further ischemia, systolic function starts to improve although diastolic function may continue to decline [213]. To allow comparison of our results with these previous trials which were designed with the above rationale, it was elected to continue to infuse GIK from induction to six hours post aortic cross clamping. Although overnight administration of GIK pre-operatively has been hypothesised to improve myocardial protection by increasing glycogen storage, due to staffing and logistical reasons it was agreed that this approach would not be appropriate in the current setting.

7.6 Data Collection

All data were prospectively collected and stored on a custom designed data sheet. Once data acquisition was complete for a patient the data were entered into a custom designed Microsoft™ Access© Database. All data was anonymised and stored securely in compliance with Caldicott principles and the Data Protection Act.

7.7 Statistical Analysis

Continuous data was assessed for normality and are presented as mean (standard error mean) or median (interquartile range). Normally distributed data was analysed by Student's t-test or by analysis of variance (ANOVA) with a post hoc Bonferroni test to allow for multiple comparisons. Skewed data were analysed with Mann-Whitney u-test. Categorical data was analysed by Fisher's exact test. Main outcome analyses were pre-specified and conducted according to the intention-to-treat principle with the use of SAS software (version 9.1, SAS Institute, Ca USA). p values other than for the primary end point are nominal. Dichotomous outcomes were analysed with the use of nonlinear mixed models, which included CABG as a patient-level covariate and Consultant as a random effects. Continuous data were analysed with the use of mixed models, which included CABG as a patient-level covariate and Consultant as random effects. [215]. The rates of adverse events were compared between groups by means of Fisher's exact test.

8. Experimental Design

8.1 Left Ventricular biopsies

To allow analysis of the cellular changes that occur in LVH, and the effect of GIK on ischemia/reperfusion it was elected to take biopsies of the anterior wall of the left ventricle between the LAD and its first diagonal branch. Ethical approval was obtained to take biopsies in patients under the age of 80 years. The upper age limit was chosen in light of surgical concerns in a potentially frail and high risk patient group. Additionally, in some frail patients, biopsies were not taken at Consultant

request. To preserve high energy phosphates, protein and RNA intact at the point of biopsy, samples were immediately snap-frozen in liquid nitrogen. With these caveats in place, complete biopsies were collected on 124 patients enrolled in the trial.

Full thickness left ventricular biopsies were taken with a tru-cut needle. This method allowed a minimum of tissue (sometimes as little as 8mg) that could be processed for analysis. It is accepted, particularly in patients with severe LVH, that cellular conditions may not be comparable between the epi- and endo-cardium. There are a number of reasons why it was decided that this was acceptable and no further manipulation was required. When comparing between LVH and non-LVH samples, whilst accepting the known differences in the sub-endocardium, it is not known to what level these differences reach, nor of how this is influenced by the degree of hypertrophy. It was therefore decided that crude estimates of sub-endocardium would have been unhelpful. When comparing between patients with LVH, because the echocardiographic markers of LVH were similar between groups, it was agreed that full thickness biopsy would therefore have been equally representative between groups.

To date a number of experiments have been completed on the biopsies and a further set are planned. A total of 26 patient samples were used in the metabolomics experiments, 42 in the western blotting of Akt, AMPK and Protein O-GlcNAcylation. Protein O-GlcNAcylation in particular is recognised as being very difficult to blot, and many samples were analysed before satisfactory blots could be obtained. 32 samples were used in other experiments that are beyond the scope of this thesis.

The remaining samples have undergone metabolomic extraction and further work is ongoing as detailed in the final chapter.

8.2 Metabolism in Left Ventricular Hypertrophy

To assess changes in genotype expression, mRNA of the major transcription regulators of carbohydrate, fatty acid metabolism were compared using Taqman PCR.

Following successful pilot experiments with mass spectroscopy, it was decided to develop this novel technique not previously reported in LV hypertrophy in man to analyse the many thousands of metabolites that construct the myocardial metabolome. Metabolomics is a powerful technique in terms of high-throughput sampling, processing and analysis, however, it has a number of limitations. There is a lack of systemised method for metabolite analysis, a bias to the only the most abundant or processable chemical metabolites, the lability of metabolites that require stringent extraction; and multiple confounding variables including age, gender and pharmacotherapy.

To compare the metabolome and the transcription of master regulators of metabolism a control group needed to be identified. This group must be of a similar age of the hypertrophy group because changes in the cellular phenotype with ageing have been demonstrated. The control group clearly must have no evidence of hypertrophy or infarction nor of diabetes, insulin resistance or the metabolic syndrome. More importantly, LV tissue samples needed to be obtained from this

group. After much discussion, it was elected to use LV samples from patients with stable coronary artery disease undergoing elective CABG with no evidence of previous infarction, or echocardiographic evidence of hypertrophy.

8.3 Cardioprotection and GIK

Cardioprotective conditioning signalling pathways have been proposed as an important mechanism of the myocyte protecting itself against ischemia-reperfusion. It has been hypothesised that the protective effects of GIK is via a direct memory lacking cardioprotective effect, and animal data has suggested it is mediated via Akt and GSK-3β signalling pathway. Ischemia is also known to up-regulate this pathways but not to the same extent. Therefore to assess whether GIK upregulates these signalling pathways, biopsies of the left ventricle taken prior to the onset of ischemia in patients treated with GIK were compared to matched patients in the control arm. Following protein extraction western blotting was used to quantify the total expression of both ACC and GSK-3β and the phosphorylated forms. The ratio between the total expression and phosphorylated expression were then compared to give a marker of activation.

8.4 GIK and Protein O-GlcNAcylation

Recently it has been demonstrated that increased O-GlcNAc levels are associated with an increased tolerance to ischaemic stress increased transcription of HSPs [146] [147], and activation of O-GlcNAc formation has also been shown to attenuate mPTP opening, a critical step in oxygen free radical-induced apoptosis and necrosis [151, 152].

To examine whether GIK therapy is associated with increased O-GlcNAc levels through both an increase in glucose substrate for the HBP and also increased flux via a direct action of insulin, left ventricular biopsies in patients treated with GIK were compared to matched patients in the control arm prior to the onset of ischaemia. Following protein extraction western blotting was used to quantify the total expression of protein O-GlcNAc.

8.5 Metabolism and GIK

To attempt to analyse the effect of GIK on metabolism, plasma glucose, free fatty acid and insulin levels were measured at pre-specified intervals during ischaemia and reperfusion. Left ventricular biopsies in patients treated with GIK were compared to matched patients in the control arm after 10 minutes of reperfusion. AMPK activity was analysed by comparing active phosphorylated-AMPK to total AMPK levels. In the presence of suppressed free fatty acid levels, a significant increase in AMPK phosphorylation has been hypothesised to imply increased glucose oxidation which may improve recovery of high energy phosphates and therefore reduce the severity of injury secondary to reactive oxygen species during reperfusion ultimately leading to improved contractile function.

9. Trial Protocol

9.1 Patient Selection

All patients undergoing elective or urgent first time aortic valve replacement for aortic stenosis in the context of echocardiographic or magnetic resonance quantification of LVH were considered for recruitment to this study. The patient inclusion and exclusion criteria used in this trial is detailed in Table 2.0.

Inclusion criteria	Exclusion criteria
Aortic valve replacement for aortic stenosis +- concomitant coronary artery bypass grafting	Other procedures required in addition to coronary artery bypass grafting
Left Ventricular Hypertrophy	Diabetes mellitus
	Atrial fibrillation or Amiodarone
	Renal failure (creatinine>200mmol.l^{-1})
	Liver failure
	Previous cardiac surgery
	Pregnancy
	Age <18 years

Table 2 Inclusion and exclusion criteria

9.2 Patient Recruitment

Suitable patients were approached prior to surgery and counselled as to the nature of the trial. A patient information sheet was then made available to the patients. After having given the patients and relatives time to read and discuss the information sheet

the patient was then approached again and if agreeable written informed consent was obtained for participation in the trial. The patient information leaflet and consent form please see appendix B and C.

9.3 Randomisation

The allocation of treatment or placebo is randomly determined in a blinded fashion and includes stratification need for concomitant CABG. The randomisation schedule for the allocation of treatment or placebo uses a blinded minimisation procedure designed by Professor Nick Freemantle, Health Care Evaluation Group, University of Birmingham.

9.4 Treatment Groups

The treatment arm received a central GIK infusion consisting of 500ml of 40% glucose, 35IU Actrapid® insulin (Novo Nordisk A/S, Bagsvaerd, Denmark) 50mmol of potassium chloride (KCl)) run at $0.75ml.kg^{-1}.h^{-1}$ rounded to the nearest $10ml.h^{-1}$ starting at sternotomy and finishing six hours following release of the AXC. The control group received 5% dextrose run at $0.75ml.kg^{-1}.h^{-1}$ rounded to the nearest $10ml.h^{-1}$ starting at sternotomy and finishing six hours following release of the aortic cross clamp (AXC). To ensure blinding of investigators, trial solution bags of placebo/GIK were wrapped in opaque tape to conceal the identity of the solution and simply labelled as “trial solution”.

9.5 Anaesthetic Protocols

9.5.1 Premedication, induction and maintenance of anaesthesia

Pre-medications were administered 90 minutes prior to surgery and consisted of temazepam (maximum dose of 30mg), ranitidine 150mg and metoclopramide 10mg. Anaesthetic induction was with fentanyl 10-15$\mu g.kg^{-1}$ or alfentanyl 15$\mu g.kg^{-1}$ (anaesthetist choice), etomidate 0.1-0.2$mg.kg^{-1}$ and pancuronium 0.1$mg.kg^{-1}$ Anaesthesia was maintained by a propofol based anaesthetic with either propofol (1-2% neat strength at the discretion of the anaesthetist) infused at 4-8$mg.kg^{-1}.h^{-1}$ or using target controlled rate at 2-4$\mu g.ml^{-1}$ and alfentanyl 25mg in 50ml (neat) infused at 50$\mu g.kg^{-1}.h^{-1}$. enflurane was used to supplement propofol-based anaesthesia but was not mandatory. Other volatile anaesthesia were not permitted. All patients received a quadruple lumen central venous (CVP) line and a pulmonary artery flotation catheter (PAFC) fully floated into the pulmonary artery prior to sternotomy.

9.5.2 Other medication

Mannitol (20%) as an osmotic diuretic (0.5$ml.kg^{-1}$) was administered via the CVP line prior to institution of CPB. Phenylephrine (0.1$mg.ml^{-1}$) was administered in aliquots to maintain a MAP 60-70mmHg. Aprotinin was administered at full-dose with a test dose of 1 000 000 units, followed by an iv bolus dose of 2 000 000 units with 2 000 000 units given into the CPB circuit followed by a continuous infusion of 500 000 $units.hr^{-1}$ in all cases. Heparin was administered prior to the institution of bypass and after the termination of bypass was reversed by Protamine at a dose of 1mg per 100iu of heparin.

9.6 Perfusion Protocol

All patients underwent surgery utilising CPB. For all cases a roller pump with a 2l Hartman's prime with 8000U heparin and a Capiox SX oxygenator were used. Intermittent antegrade cold blood cardioplegia was administered via the aortic root and directly into the coronary ostia following aortotomy using St Thomas A for induction at 12ml.kg^{-1} and St Thomas B for maintenance at 6ml.kg^{-1} diluted 4:1 with blood (see Table 5.2 for composition) at a rate of 300ml.min^{-1} or a pressure of 120-150mmHg. Maintenance doses of cardioplegia were administered after 20 minutes or at earlier intervals at the discretion of the surgeon. Supplemental retrograde cardioplegia and "hot shots" were not permitted. All patients were actively cooled to 32^{o}C. A target mean radial artery perfusion pressure of 50-60mmHg was maintained using phenylephrine (0.1mg.ml^{-1}). For all cooling and re-warming, blood/water temperature gradients were $\leq$ 7^{o}C. On re-warming once the nasopharyngeal temperature reached 35^{o}C, the heater/ chiller temperature was set to 37^{o}C.

St Thomas A Solution	St Thomas B Solution
898ml Ringers compound NaCl	966ml Ringer's compound NaCl
102ml Cardioplegia solution containing	34ml Cardioplegia containing
16.6g Magnesium Chloride	5.2g Magnesium chloride
6.1g Potassium Chloride	1.9g Potassium chloride
1.4g Procaine Hydrochloride	0.4g Procaine hydrochloride

Table 3 Composition of Cardioplegia

9.6 Surgical Protocol

9.7.1. Conduct of Operation

After transfer to the operating theatre the patient was prepped and draped as standard. If concomitant bypass grafting were to be performed then the internal mammary artery and saphenous vein/radial artery were harvested synchronously. After harvest of conduit CPB was instituted in a standard fashion with venous drainage from the right atrium using a two-stage cannula, with single arterial return to the ascending aorta. An AXC was applied proximal to the aortic cannula and cardioplegia solution delivered into the aortic root as previously detailed.

For patients undergoing concomitant revascularisation, the distal anastomoses of all free grafts were first performed. The aorta was then opened, the valve explanted, the annulus sized and the valve replaced in a manner of the surgeons choosing. Supplemental doses of cardioplegia were delivered by direct ostial cannulation. The heart was vented either through a direct left ventricular vent or left superior pulmonary vein vent. The heart was then de-aired and the aortotomy closed. If pedicled coronary artery grafts were to be placed then the distal anastomes were then performed. The aortic cross clamp was then removed and all proximal aorto-spahenous anastomses performed during a period of partial aortic occlusion.

In cases of severe ascending aortic calcification instead of a standard cross clamp, a 22 Fr TRIUMPH aortic occlusion arterial cannula was introduced directly into the ascending aorta. If aortic calcification made application of a side biting clamp perilous, proximal anastomses were performed using a period of single aortic cross

clamping. If the ascending aorta was dilated then it was replace an interposition graft (Gelweave, Vasctex Terumo, Scotland).

Right atrial and ventricular pacing wires were placed prior to separation from CPB. Patients were separated from CPB with a heart rate of 70-110bpm (native or paced) without inotropic support if possible. Drains were placed to the mediastinum and open pleural cavities and closure was performed in a standard fashion with sternal wires and layers of Vicryl.

9.7.2. Myocardial Biopsy

Biopsies were full thickness and taken from the left ventricular (LV) free wall. LV biopsies were obtained using a Trucut biopsy needle (Allegiance Healthcare, McGaw Park, IL). LV biopsies were taken prior to application of the AXC (pre-ischaemia), immediately before removal of the AXC (ischaemia) and approximately 10 minutes after removal of the AXC (reperfusion). Biopsies were immediately snap frozen in liquid nitrogen and subsequently stored at -80^{o}C. Biopsy sites were oversewn with 5'O' prolene to ensure haemostasis.

9.8 Assessment and management of hypotension

For the purposes of this study hypotension was defined as MAP< 60mmHg. Prior to institution of any inotrope or vasoconstrictor therapy the following were assessed: the accuracy and calibration of the arterial line; heart rate and rhythm and right and left sided filling pressures. If any therapy could be instituted at this stage e.g. pacing rate,

fluid challenge, then these were performed. Finally a set of cardiac output studies was performed. If the CI was less than 2.2l.min^{-1}.m^{-2} with a normal SVR 9800-1200dyne.cm^{-1}.sec^{-5}) and adequate filling and heart rate, the protocol for management of LCOE was followed (section 8.9). In the event of a CI greater than 3.0l.min^{-1}.m^{-2} with a low SVR (< 800dyne.cm^{-1}.sec^{-5}) vasoconstrictor therapy with phenylephrine (10mg in 50ml of dextrose 5% at 0-0.2μg.kg^{-1}.h^{-1}) titrated to the response of the MAP was instituted. If the ceiling dose of 0.2μg.kg^{-1}.h^{-1} of phenylephrine was reached without the MABP returning to the range 65-85mmHg then vasopressin (20U in 40 ml of 5% dextrose) at 5 U.h^{-1} was started. Further treatment above these levels was at the discretion of the patient's clinical team

9.9 Management of suspected low cardiac output episode

For the purpose of this study a LCOE was defined as a CI of less than 2.2l.min^{-1}.m^{-2} refractory to appropriate intra-vascular volume expansion following correction or attempted correction of any dysrhythmias. In the case of inotropic support being instituted due to a LCOE, all data were analysed by a blinded endpoints comprising at least two consultant surgeons. In order to qualify as a LCOE episode a unanimous verdict was sought but in the event of disagreement, contentious cases were discussed and a consensus opinion agreed.

9.9.1 Inotrope Therapy

In the event of a LCOE inotropic therapy was instituted. Dopamine was used as the first line inotropic agent in this study (200mg in 50ml 5% dextrose). and instituted at a

dose of 5-10 μg.kg.$^{-1}$.min^{-1} according to a prepared nomogram. Thereafter, all management considered necessary above and beyond dopamine 10μg.kg.$^{-1}$.min^{-1} was at the discretion of the clinical team.

9.9.2 Low cardiac output episode in theatres and anticipatory treatment

Whenever possible cardiac output studies were performed and recorded prior to institution of inotropic support. In the case of an anticipated LCOE based on subjective and objective features of cardiac performance before and during surgery, confirmatory cardiac output studies were performed if possible. Otherwise inotropic therapy was started at the discretion of the clinical team and charts underwent review by the endpoint committee.

9.10 Biochemical monitoring

After initiating the trial solutions in theatre serial arterial blood gases (ABGs) were performed every 30 minutes until institution of CPB, every 20 minutes during CPB and hourly for the first 12 hours following removal of the AXC. Thereafter ABGs were performed at the discretion of the clinical team.

During the trial, blood samples were taken from all patients at baseline, during reperfusion and then at 6 and 12 hours following cross clamp removal. Plasma glucose was measured in fluoride oxalate samples using a Yellow Springs analyzer (YSI2300; Yellow springs instruments Aldershot, UK), plasma insulin levels were measured by radioimmunossay (Pharmacia, Uppsala, Sweden) and FFA by a commercial enzymatic system (NEFA-C, Wako, Neuss, Germany). The area under

the curve for the mean plasma levels was calculated and compared between GIK and control using Mann-Whitney u-test.

9.11 Blood glucose management

The aim of glycaemic control in this study was to maintain a blood glucose level within a target range of 4.1-10.0mmol.l^{-1}. Levels of blood glucose greater than 10.0mmol.l^{-1} were treated with an insulin actrapid sliding scale (50 units actrapid in 50ml normal saline – see Appendix C for sliding scales). Supplemental boluses of insulin were permitted to obtain glycaemic control. Regardless of blood glucose readings actrapid infusions were terminated one hour prior to termination of trial solutions to prevent rebound hypoglycaemia.

Hypoglycaemia for the purposes of this study was defined as blood glucose less than 4.0mmol.l^{-1}. In the event of hypoglycaemia, treatment was given as a bolus of 25mls of 50% dextrose. Blood glucose measurement was then repeated after 30 minutes and supplemental boluses of 50% dextrose administered as required.

9.12 Potassium Management

Serum potassium levels were maintained in the range of 4.5-5.5mmol.l^{-1}. Prior to and during cardiopulmonary bypass supplemental potassium was not administered unless clinically indicated. After cardiopulmonary bypass intravenous potassium chloride was administered to maintain potassium levels within the range 4.5-5.5mmol.l^{-1} during the first 12 hours.

10. Laboratory Protocols

10.1 RNA and Protein Extraction

Biopsies were initially homogenised using Ultraturax homogeniser at 10,000 rpm for 30-60 seconds in the presence of 1ml Tri Reagent©. The samples were allowed to stand at room temperature for 5 minutes. 0.2ml chloroform per ml of TRI Reagent© was then added and thoroughly mixed. This was then allowed to stand for a further 10 minutes at room temperature.

Samples were centrifuged at 12000g for 15 minutes at 4 ^{o}C separating into an upper colourless aqueous phase containing RNA, a middle layer of protein and a lower layer of DNA.

10.1.2 Isolation of RNA

The RNA phase was transferred to a clean eppendorf tube adding 0.5ml of isopropanol per ml of Tri Reagent©. This was thoroughly mixed and left to stand at room temperature for 10 minutes.

Samples were centrifuged at 12000g for 10 minutes at 4^{o}C to precipitate a pellet of RNA. The supernatant was removed and discarded and the RNA pellet washed with 1ml 75% ethanol per ml TRI Reagent©. Samples were vortexed and then centrifuged at 12000g for 5 minutes at 4^{o}C.

Samples were air-dried to remove all residual ethanol. The RNA was then dissolved in 50 µl nuclease free water by repeat pipetting at room temperature.

10.1.3 Isolation of Protein

Following removal of the aqueous phase containing RNA, the DNA was precipitated with 0.3ml of 100% ethanol per 1ml TRI Reagent© used for the initial homogenisation. The samples were thoroughly mixed and incubated at room temperature for 2-3 minutes. The sample was centrifuged at 2000g for 5 minutes at 4 ^{o}C to sediment the DNA.

300 µl of the supernatant (phenol/ethanol phase) was transferred to a clean eppendorf and the DNA destroyed. Proteins were precipitated by adding three volumes of acetone. The sample was thoroughly mixed for 10-15 seconds to obtain a homogenous solution and stored at room temperature for 10 minutes at room temperature before being centrifuged at 2000g for 10 minutes at 4^{o} C to sediment the protein.

The supernatant was then discarded and the protein pellet re-suspended in 0.5mk of 0.3M guanidine HCl in 95% ethanol 2.5% glycerol (1:1). Following dispersion a further 0.5ml of guanidine HCL in 95% ethanol 2.5%glycerol (1:1) wash solution was added and the sample incubated at room temperature for a further 10 minutes. The protein was sedimented by centrifugation at 8000g for 5 minutes at room temperature.

The wash solution was then decanted and two further washes with incubations and spins in 1 ml of guanidine HCL in 95% ethanol 2.5%glycerol (1:1) were made. After each wash the sample was vortexed to disperse the pellet and remove residual phenol.

The final wash was made in 1ml ethanol containing 2.5% glycerol (v:v) and the sample was then incubated for 10 minutes at room temperature. The proteins were sedimented by centrifuging at 8000g for 5 minutes at room temperature. The alcohol was decanted and the samples air dried for 30 minutes at room temperature. The proteins were solubilised by adding 200µl 1% SDS and gently dispersed and supplemented with 0.025% Tween 80. The proteins were stored at -80^{o}C until required.

10.2 Reverse Transcription and Quantitative PCR

RNA was reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI) in a total reaction of 20 µl, with 1 µl of total RNA, 30pmol random hexamer primers, 4 µl of 5x AMV reverse transcriptase buffer 2 µl of deoxynucleotide triphosphate (dNTP) mix (200 µM each), 20U ribonuclease inhibitor (RNain; Promega) and 15U of AMV reverse transcriptase.

Expression of specific mRNA was determined using the ABI PRISM 7500 sequence detection system. RT-PCR was carried out in 25 µl volumes on 96 well plates in a reaction buffer containing 1x TaqMan Universal PCR Master Mix, 0.75 µl TaqMan probe per reaction and 4.5 µl forward and reverse primer per reaction. All reactions

were multiplexed with a pre-optimised control probe for 18s RNA (ABI, Warrington, UK). As per the manufacturer's guidelines, data were expressed as cycle threshold (Ct) values and used to determine the ΔCt value. This was calculated by the Ct of the housekeeping gene from the Ct of the target gene.

Statistical comparison was made between the ΔCt values to exclude potential bias caused by averaging data that had been transformed through the equation $2^{-\Delta\Delta Ct}$ to give the fold changes in gene expression.

Target genes were labelled with FAM and housekeeping genes with VIC. All measurements were carried out twice on each sample. Reactions were at 50 ^{o}C for 2 minutes and at 95 ^{o}C for 10 minutes followed by 40 cycles.

10.3 Western Blotting

Resolving gels of varying concentrations were prepared depending upon the intended target. Resolving gel preparations are shown in Appendix E. The gel was pipetted into clean gel plate and 1000 µl of isopropranol was added. The gel was allowed to set and then the isopropranol removed.

A stacking gel of 5% acylamide, 0.2M Tris-HCl pH 6.87, 7mM SDS, 0.2% v/v TEMED and 8.8mM APS was prepared and layered on top of the resolving gel. Protein samples were loaded alongside Bio-rad Precision Plus Protein Prestained Dual Colour standards. Gels were then run using the Mini-Protean III electrophoresis cell

(Bio-Rad) and electrophoresed at 200 V through the resolving gel in SDS running buffer (25mM Trizma base, 192 mM glycine, 3.5 mM SDS) for 50 minutes.

Two pieces of 3mm filter paper (Whatmann, Kent, UK), a 10 cm x 8 cm sheet of PVDF membrane (Hybond-P, Amersham Biosciences, Amersham, UK) pre-soaked in 100% methanol, and two fibre pads were equilibrated in transfer buffer (25mM Trizma base, 192mM glycine, 20% methanol), and the electrophoresed SDS-PAGE gel was sandwiched in a transfer cassette of the Mini Protean III transfer apparatus (Bio-Rad, Hemel Hempstead, UK).

Protein was transferred onto the PVDF membrane at 250 mAmp for ninety minutes. Following transfer, the membrane was blocked with 5% (w/v) Marvel dried skimmed milk in TBS-T (Tris-buffered saline containing 20mM Tris-HCl pH 7.6, 140mM NaCl and supplemental with 0.025% Tween 80) for at least one hour on a rocker bed at room temperature.

Membranes were probed overnight with the primary antibodies diluted in 5% (w/v) milk-TBS-T at 4 ^{o}C on a rocker bed. Primary antibodies are detailed in Appendix F. Membranes were then washed four times in TBS-T with fifteen minute incubations on a rocker bed between each wash.

The membrane was incubated with the appropriate secondary antibody diluted in 5% milk TBS-T for one hour on a rocker bed. The membranes were further washed for one hour with TBS-T at room temperature changing the wash solution every ten minutes. Excess wash buffer was then discarded and the membranes incubated with

ECL (Enhanced Chemiluminesence Plus (Amersham Biosciences) at 5 minutes at room temperature. The excess detection reagent was drained off, the blots covered and then placed protein side up in an X-Ray film cassette. A sheet of autoradiography film (Hyperfilm© ECL, Amersham Biosciences) was placed on top of the membrane, the cassette closed and exposed for 10 seconds and developed. On the basis of the appearance of this film, the exposure time for further films was adjusted to give optimal results.

The limitations of western blotting are widely accepted. It is a slow, complex and cumbersome technique which is very operator dependent. Only one protein can be analysed at time, and repeated stripping and re-probing may result in protein loss and incorrect results. It is however a robust and reproducible method of protein analysis and recent advances in digital image processing have made analysis of the final photographic results increasingly accurate.

10.4 Mass Spectroscopy

Metabolites were extracted from 10 non-LVH and 10 LVH cardiac tissue biopsies using a two-step methanol:water:chloroform protocol. Samples were initially homogenised using a 24 bead-based homogeniser (Stretton Scientific, Stretton, UK) 320µl methanol and 128µl water. The homogenate was removed into a 1.8ml glass vial and all of the remaining solvents were added (320µl chloroform 128µl water). Samples were then vortexed for 60 seconds and left on ice for 10 minutes to allow partition. The samples were centrifuged at 2000g at 4^{o}C for 5 minutes.

The polar fraction from each biphasic mixture was removed, split into two equal volumes, and then dried in a centrifugal concentrator. An “extract blank” was also prepared using identical methods except that no biological material was added to the solvents. These were then stored at -80°C until analysis.

Following resuspension of the dried polar extracts in 80:20 methanol:water, containing 0.25% formic acid or 20 mM ammonium acetate for positive and negative ion analysis respectively, Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry-based metabolomics was conducted using a hybrid 7-T LTQ FT (Thermo Scientific, Bremen, Germany) equipped with a chip-based direct infusion nanoelectrospray ion source (Triversa, Advion Biosciences, Ithaca, NY).

The nanoelectrospray conditions comprised of a 200nl/min flow rate, 0.3psi backing pressure and +1.7 or -1.7 kV electrospray voltage respectively for positive and negative ion analysis controlled by ChipSoft software (version 8.1.0 Advion Biosciences, UK).

Each sample was analysed in triplicate with Fourier transform ion cyclotron mass spectroscopy (FT-ICR MS) collecting multiple adjacent selected ion monitoring (SIM) windows which are stitched together from *m/z* 70 to 500 with each window overlapping by m/z 10 (3) with automatic gain control (AGC) target of 1×10^5 charges and a mass resolution of 100,000. Data was recorded for 5.5 minutes per replicate analysis using Xcalibur software version 2.0, (Thermo Scientific, USA).

Transient data from the FT-ICR detector were processed using averaging of transients, Hanning apodisation and application of a fast Fourier transformation all using custom written code in MATLAB Version 7.0 (The MathWorks, MA USA).

Next the SIM-stitching algorithm was applied (Custom written code, MATLAB, Version 7.0 The MathWorks) [216] which stitched together multiple SIM windows, rejected all peaks with a signal-to-noise ratio (SNR) <3.5, and then internally calibrated each mass spectrum using a pre-defined calibrant list of metabolites (3).

The data were normalised using probabilistic quotient normalisation (PQN) to minimise the effects of particularly high and low intensity peaks [96]. They were then subject to generalised log transformation [217] to stabilise the variance across the several thousands of detected peaks in order to avoid the highest intensity peaks dominating in the multivariate analysis. Principal components analysis (PCA) was used to assess the metabolic differences between the sample groups in an unbiased manner, using the PLS_Toolbox (version 3.53, Eigenvector Research, Manson, WA, USA) within Matlab (version 7.1; The MathsWorks, Natick, MA, USA), and confirmed using Student's *t*-tests (MS Excel, Seattle, USA). To determine empirical formulae and putative metabolite identities of key peaks in the mass spectra, the strategy of Taylor *et al.,* [218] was followed, and Student's *t*-tests with appropriate correction for multiple hypothesis testing were conducted to assess the significance of these metabolic changes between groups.

10.5 Principal component analysis

Principal component analysis (PCA) is a mathematical reduction strategy that has been validated for processing high-throughput data. It attempts to transform a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The aim is for the first principal component to account for as much variability in the data as possible, and each following component to account for as much of the remaining variability as possible. Although this is a highly complex mathematical technique, it is actually one of the simplest available tools for this type of data processing. In the multivariable metabolomic dataset produced by our FT-ICR MS analysis, the PCA plots each individual dataset as a set of co-ordinates.

This technique has accepted limitations including assumptions on linearity, and on the importance of mean and covariance, but a detailed description of this computational technique and its limitations is beyond the scope of this work.

Chapter 3. An integrative systems biology study of the metabolic profile in ventricular hypertrophy secondary to aortic stenosis in man.

11. Introduction

Integrative systems biology is the attempt to explain biological phenomenon not only by the delineation of genes and molecules but by reconstructing biochemical supramolecular networks that represent various cellular functions. The process leading to integrative systems biology consists of combining elements of the genome, transcriptome, proteome and the metabolome.

To perform such a study of the overall metabolic pathway changes LVH in man, we combined analysis of key parts of the metabolic transcriptome, the transcription of the master transcriptional regulators PGC-1α, PPARα and ERRα, the key fatty acid transport protein CPT1 and the glucose uptake transporter GLUT-4, and the enzyme controlling the crux of the glycolytic pathway via inhibition of PDH, the kinase PDK-4, with the resultant metabolome, and compared this to that of a non hypertrophied control.

There are a number of master transcriptional regulators. PGC1α was chosen because it is a transcriptional co activator that regulates the genes involved in energy metabolism, interacting with PPARα. It is also thought to be a master integrator of external signals and is known to be activated by a number of factors including reactive oxygen species, reactive nitrogen species, cAMP and the Akt pro-survival G-protein signals. PPARα was chosen as it is a controls the transcription of factors regulating cellular differentiation and metabolism, controlling carbohydrate, lipid and protein metabolism, and ERRα is a nuclear receptor widely expressed in tissue that

preferentially uses fatty acids as a predominant energy source. It is involved in regulating mitochondrial biogenesis, gluconeogenesis, oxidative phosphorylation and fatty acid metabolism.

There are a number of critical steps in fatty acid oxidation, however one of the crucial and rate limiting step is the transport of long chain acyl groups from fatty acids across the impermeable mitochondrial membrane by carnitine so that they can undergo β-oxidation [27]. On the outer mitochondrial membrane fatty acids are attached with a thioester bond to coenzyme A (CoA), a reaction catalysed by fatty-acyl-CoA synthetase [27]. Acyl CoA is conjugated to carnitine by carnitine palmitoyltransferase I (CPT-I) on the outer mitochondrial membrane and it is then shuttled inside by carnitine-acylcarnitine translocase. Acyl-carnitine is then converted to acyl-CoA by CPT-II on the inner mitochondrial membrane where it can then undergo β-oxidation [27]. Similarly, there are a number of crucial steps involved in glycolysis. The uptake of extracellular glucose is regulated by the transmembrane glucose gradient and the concentration and activity of glucose transporters including GLUT 4 [22, 23].

The metabolome represents the collection of all metabolites within the cell. These represent not just the end products of cellular metabolism but all the individual intermediate and by-products as well as all the proteins and enzymes. Using principal component analysis it is possible to possibly correlated variables into a smaller number of uncorrelated variables called principal components. The first principal component will account for as much of the variability in the data as possible. This means that in a complex system of interconnected reactions, small changes in the concentration of each individual intermediate, which individually may not be

significant, when analysed as a whole system can allow the differences to be identified.

12. Methods

12.1 Patients

Following local ethics approval, age and sex matched patients with LVH secondary to AS undergoing AVR with no significant coronary artery disease in the placebo group of the HINGE trial were compared to age and sex matched patients undergoing elective CABG without both echocardiographic evidence of hypertrophy and clinical evidence of hypertension, previous infarction or ongoing ischemia were studied as a control group.

12.2 Echocardiographic Assessment of LV Function

The LV end-diastolic dimensions (LVEDD/ mm), inter-ventricular septum thickness (IVS/ mm) posterior wall thickness (PW / mm) and LV ejection fraction (LVEF, %) were assessed using M-mode echocardiography as per the American Heart Association guidelines for reporting echocardiography [219].

12.3 Invasive Haemodynamic Studies

The pre-operative cardiac output was measured using a standard thermodilution technique at end-expiration with three successive injections of 10mls cold (6-10^{o}C)

5% dextrose. The recorded value was the mean of three individual measurements. Recorded values for pulmonary artery pressure, pulmonary artery wedge pressure (PAWP) and central venous pressure were obtained at end-expiration from graphic recordings.

12.4 Myocardial Biopsies

A single full thickness tru-cut biopsy was taken from the anterior wall of the left ventricle between the left anterior descending artery and its first diagonal branch immediately after instituition of cardiopulmonary bypass but prior to aortic cross-clamping. These were snap frozen and then stored at -80^{o}C until the analysis.

12.5 RT-PCr Analysis

Total RNA from LV biopsies was isolated using TRI Reagent (Ambion, UK). Following nano-drop spectrophotometer analysis for purity, cDNA was reversed transcribed using Taqman MultiScribe (Applied Biosystems, UK). Commercially available primers and probes were purchased (Applied Biosystems, UK) to amplify and quantify expression levels of target genes (PGC1 ,ERRα , PPARα , CPT1M, PDK4, SLC2A4) and of housekeeping gene 18s using an ABI 7500 real time PCR machine (Applied Biosystems, UK). PCR amplification was performed in duplicate and expression levels were normalised to 18s. Real time PCR data are presented as arbitrary units (AU) (calculated as AU=1000x2^(-ΔCt)). Expression levels of target genes were set as 1 in CABG patients (control) and compared to LVH using the Mann-Whitney-U test (statistical significance level was set as $p<0.05$).

12.6 Metabolomics

Metabolites were extracted from cardiac tissue biopsies using a two-step methanol:water:chloroform protocol [218]. The polar fraction from each biphasic mixture was removed, dried and then stored at -80°C until analysis. The samples were then resuspended in 80:20 methanol: water, containing 0.25% formic acid or 20 mM ammonium acetate for positive and negative ion analysis respectively. Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry-based metabolomics was conducted using a hybrid 7-T LTQ FT (Thermo Scientific, Bremen, Germany) equipped with a chip-based direct infusion nanoelectrospray ion source (Triversa, Advion Biosciences, Ithaca, NY).

Principal components analysis (PCA) was used to assess the metabolic differences between the sample groups confirmed using Student's t-tests. Taylor strategy was then employed to determine empirical formulae and putative metabolite identities of key peaks in the mass spectra [220], was followed, and Student's t-tests with appropriate correction for multiple hypothesis testing were conducted to assess the significance of these metabolic changes.

	LVH	Non-LVH	p value
Sample size	n=10	n=10	
Age	69 ± 2.1	67 ± 2.4	0.67
Gender M/F	5 / 2	5 / 2	0.45
Height (cm)	167.0 ± 1.98	166.3 ± 1.78	0.8
Weight (kg)	79.78 ± 2.95	82.1 ± 2.36	0.23
BSA (m^2)	1.89 ± 0.05	1.91 ± 0.05	0.1
CCS			
I	5	0	
II	5	9	
III	0	1	
IV	0	0	0.2
NYHA			
I	0	10	
II	4	0	
III	6	0	
IV	0	0	0.41
Hypercholesterolaemia	1	7	0.08
Hypertension	4	1	0.06
CAD	0	10	0.001
Pre-op Medication			
β -Blocker	0	10	0.001
ACEi	6	1	0.5
Statin	4	9	0.34

BSA – Body Surface Area; CCS – Canadian Cardiovascular Society; NYHA – New York Heart Association; CAD – Number of Coronary Arteries with flow limiting lesions

Table 4 Patient Demographics in the LVH and control groups

	LVH	Non-LVH	p value
IVSd (cm)	1.64 ± 0.04	1.06 ± 0.03	<0.0001
LVEDd (cm)	4.67 ± 0.13	4.97 ± 0.13	<0.0001
PWd (cm)	1.51 ± 0.04	0.97 ± 0.02	<0.0001
LVMI g/m^2	151.73 ± 24.23	109.35 ± 21.16	0.001
Peak Aortic velocity (ms^{-1})	4.78 ± 0.6	1.28 ± 0.2	<0.0001
Mean AV Gradient (mmHg)	56 ± 6.11	6.1 ± 0.03	<0.0001
Stroke Volume (mls)	46 ± 2.58	67 ± 3.46	<0.0001
Ejection Fraction (%)	57 ± 6.16	64 ± 7.23	0.01

IVSd – Interventricular septum in diastole; LVEDd – Left Ventricular End Diasolic dimension; PWd Posterior Wall in diastole; LVMI – Left Ventricular Mass Index

Table 5. Echocardiographic data from patient in the LVH and control groups

13. Results

13.1 Left Ventricular Hypertrophy and Diastolic Dysfunction in AS

In comparison to control patients, patients with aortic stenosis had significant left ventricular hypertrophy characterised by significantly increased LV septal and free wall thickness and calculated left ventricular mass index (LVMI) as detailed in Table 5. Although these patients exhibited preserved systolic function (EF 57% ± 6.16 vs 64% ± 7.23 in AS patients and controls respectively), the LVH patients had evidence of LV diastolic dysfunction manifested by an increase in PAWP (14 mmHg vs. 8

mmHg in LVH and non-LVH patients respectively, p=0.01) indicative of a raised left ventricular end diastolic pressure, and profoundly reduced stroke volumes (46 ml ± 2.58 vs 67 ml ± 3.46 in LVH and non-LVH patients respectively, p=0.01)

13.2 Down-regulation in the Cardiac Metabolic Transcriptome

Compared to non-LVH controls, the patients with LVH exhibited profound down-regulation of the mRNA transcript levels of the master transcriptional regulators PGC1α, PPARα, and ERRα. There was also a significant down-regulation of key metabolic enzymes involved with both free fatty acid metabolism and glucose transport (mCPT1 and GLUT4) (Fig 8).

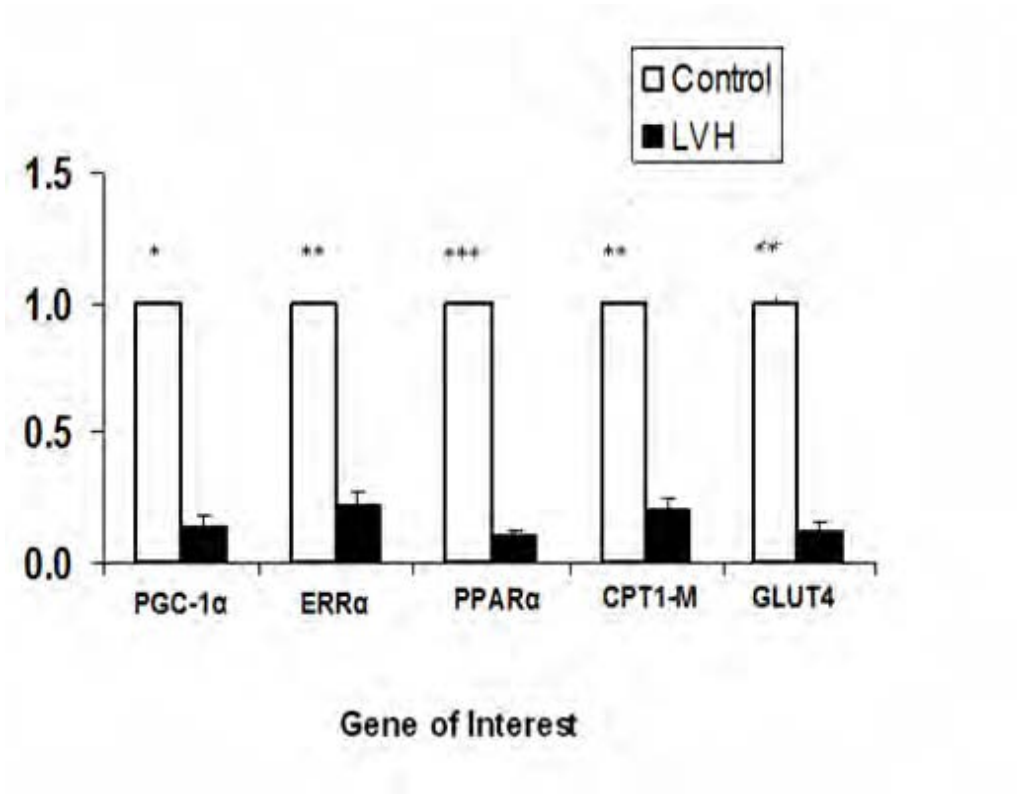

Figure 8*. Cardiac Metabolic Gene Expression Studies From Cardiac Biopsies of Patients with LVH and Non-LVH undergoing Cardiac Surgery. Non-LVH controls have been normalised to 1*
***p<0.05, **p<0.01, ***p<0.001**

13.3 Impaired Cardiac Energetics in LVH

Results were obtained on 9 patients with LVH in both positive and negative mode ionisation and on 7 non-LVH in positive and 9 non-LVH in negative mode. FT-ICR analysis of the cardiac extracts revealed 760 peaks in positive ion mode and 1464 peaks in negative ion mode common to all the samples. PCA was used to visualise any metabolic differences between the FT-ICR metabolic profiles of non-LVH and LVH tissue extracts. The PCA score plots for positive and negative ion modes (Fig 9) were consistent, showing a clear separation between the two groups along the PC2 axis. The p-values from t-tests conducted on the PC2 scores were 7.91×10^{-5} and 0.0053 for positive and negative ion modes, respectively, confirming that the overall metabolic profile changes between non-LVH and LVH tissue extracts were significant.

Peaks that contributed most to the separation of the metabolic profiles in multivariate space in both ionisation modes were identified, and the m/z values for these peaks were used to determine empirical formulae and putative metabolite identities. Alongside this approach, univariate statistical methods were applied to identify those metabolites that changed most significantly, and to assign statistical significance to the metabolites identified from the multivariate analysis; the significance of the p-values was adjusted for a False Discovery Rate (FDR) of 5. Table 6 lists selected metabolites associated with energy metabolism, as well as metabolites that were discovered to differentiate the two groups. Perturbations in energy-related metabolites included a 2-fold decrease in phosphocreatine and an almost 2-fold increase of both AMP and adenosine for LVH samples, with minimal change in ADP or ATP concentrations. Key metabolic changes from the positive ion data include

increases in glutamine, urocanate, phenylalanine, lysine and oxoproline in LVH, although only the latter two were significant after FDR correction. Changes linked to LVH in the negative ion data included increases in arginine, tyrosine, tryptophan and histidine, with a potential decrease in carnitine.

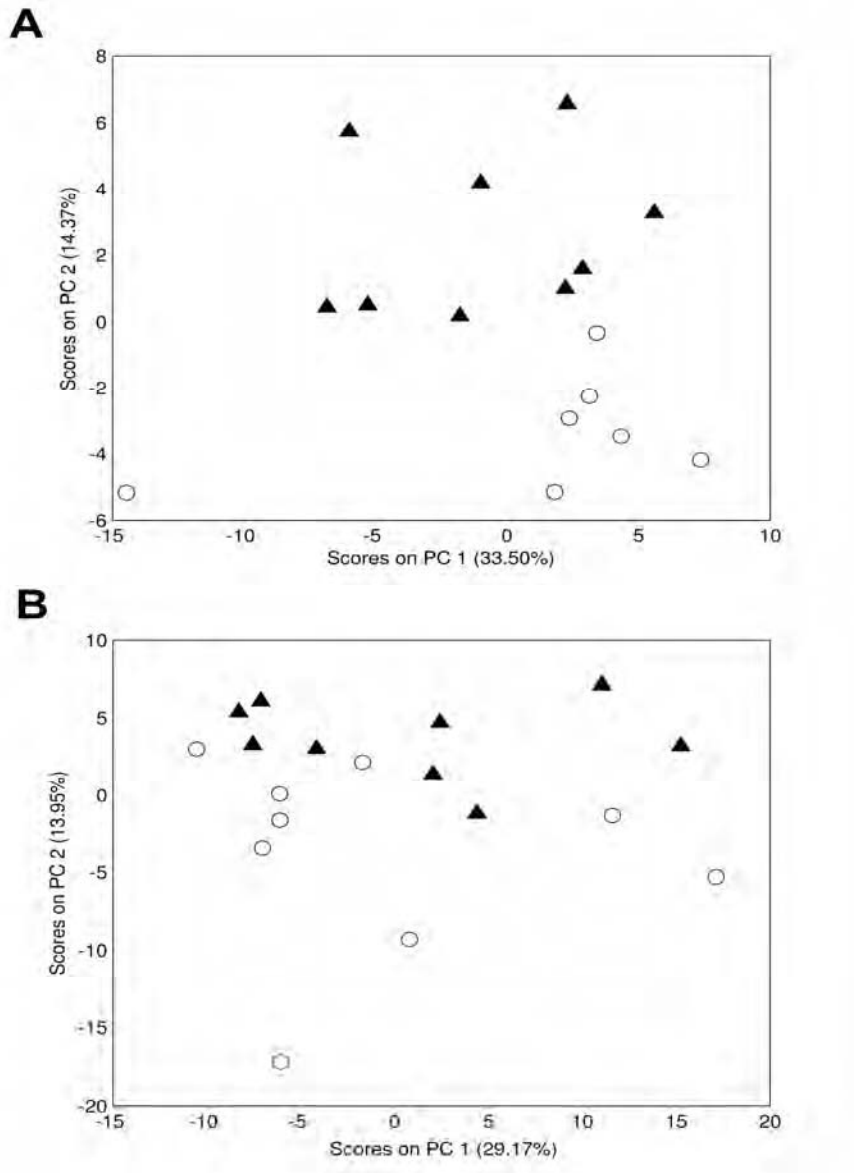

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Figure 9 PCA scores plots from analysis of (A) positive and (B) negative ion direct-infusion FT-ICR mass spectra of non-LVH (○) and LVH (▲)

Observed peak (m/z)	Potential empirical formula	Putative metabolite identification	Absolute mass error (ppm)	Ion form	Fold change[a]	*p*-value
Energy related metabolites						
382.0326	$C_{10}H_{14}N_5O_7P$	AMP	-0.289	$[M + {}^{35}Cl]^-$	1.71	0.014
302.0662	$C_{10}H_{13}N_5O_4$	adenosine	-0.077	$[M + {}^{35}Cl]^-$	1.84	0.065
210.0286	$C_4H_{10}N_3O_5P$	phosphocreatine	-0.078	$[M - H]^-$	0.52	0.128
450.0189	$C_{10}H_{15}N_5O_{10}P_2$	ADP	-0.461	$[M + Na]^+$	0.87	0.279
252.4907	$C_{10}H_{16}N_5O_{13}P_3$	ATP	0.202	$[2M - 2H]^{2-}$	0.96	0.922
Key metabolites from positive ion analysis[b]						
152.0318	$C_5H_7NO_3$	oxoproline	-0.030	$[M + Na]^+$	1.48	<0.0001*
147.1128	$C_6H_{14}N_2O_2$	lysine	-0.106	$[M + H]^+$	1.55	<0.001*
139.0502	$C_6H_6N_2O_2$	urocanate	-0.112	$[M + H]^+$	2.92	0.002
166.0863	$C_9H_{11}NO_2$	phenylalanine	0.033	$[M + H]^+$	1.61	0.003
147.0764	$C_5H_{10}N_2O_3$	glutamine	-0.072	$[M + H]^+$	1.32	0.043
Key metabolites from negative ion analysis[b]						
173.1044	$C_6H_{14}N_4O_2$	arginine	0.113	$[M - H]^-$	1.39	<0.001
180.0666	$C_9H_{11}NO_3$	tyrosine	0.098	$[M - H]^-$	1.52	<0.001
203.0826	$C_{11}H_{12}N_2O_2$	tryptophan	0.008	$[M - H]^-$	1.48	0.0015
154.0622	$C_6H_9N_3O_2$	histidine	0.134	$[M - H]^-$	1.24	0.0047
196.0746	$C_7H_{15}NO_3$	carnitine	-0.073	$[M + {}^{35}Cl]^-$	0.73	0.194

Table 6. Summary of key metabolic changes associated with LVH [a]

Fold change between the non-LVH and LVH samples, with positive value indicating an increase in LVH; [b] Metabolite selection based upon those with low *p*-values and high loadings, i.e., which defined the separation in the PCA scores plot; [*] *p*-value significant following FDR at <0.05

14. Discussion

The transcriptome data clearly demonstrated a significant down regulation of the master regulators PGC1α, PPARα and ERRα and their downstream metabolic pathways including both fatty acid and glucose metabolisms. Although the mechanisms underlying metabolic remodelling are poorly understood, there have been recent advances in defining the role of master transcriptional regulators such as PGC-1α, PPARα and ERRα in cardiac metabolism [221, 222]. PGC-1α is recognised to contribute to mitochondrial biogenesis and intermediary metabolism; its absence predisposes to HF. However, the role of PGC-1α in acquired heart disease, in both well-validated animal models of LVH and and in human disease has been less well investigated [223], although one study, suggested that in HF resulting from human mitochondrial cardiomyopathies, that maladaptive mitochondrial biogenesis was driven in part by PGC-1α down regulation. It has also been observed in both animal models and in patients with a more typical form of HF, that PGC-1α is progressively down regulated with worsening LV dysfunction [222]. This observation has led to the hypothesis that PGC-1α down-regulation contributes to the metabolic remodelling and energy deficiency of heart failure [74, 222]. We would also speculate that in human LVH, the metabolic remodelling would also appear to be associated with the down regulation of the other master transcriptional regulators PPARα and ERRα.

Additionally, we have demonstrated a significant down regulation of key steps of both the fatty acid and glycolytic pathways. Down regulation of CPT 1 may result in a decrease in the transport of free fatty acids across the mitochondrial membrane leading to a reduction in β–oxidation. This would correspond with the majority of the

animal data that suggest in LVH, there is a shift away from FFA utilisation [65]. It also corresponds with previous gene expression studies which have shown a shift towards a foetal pattern of myocardial gene expression with down regulation of genes involved in FFA metabolism [75]. Considerable debate however remains as to whether this shift in substrate utilisation is an adaptive response (to reduced tissue oxygen) or a maladaptive response, predisposing to the development of HF [65]. In addition to this, if cellular fatty acid uptake remains constant then reduced uptake and utilisation by mitochondria will result in an increased cytosolic LCFA concentration which may itself drive further uncoupling protein expression further aggravating the energetic deficit [83].

GLUT-4 transcription was also found to be significantly down regulated in LVH which corresponds with both the animal and human data in LVH that demonstrates cardiac insulin resistance. These results are in accordance with recent studies in animal models of LVH have shown that while glucose uptake and glycolysis are maintained or even increased, glucose oxidation is reduced [84]

In summary, our transcriptome data correlates with recent animal studies of LVH that have suggested a down regulation of both FFA and carbohydrate metabolic pathways. This maladaptive down regulation would render the heart energetically vulnerable. Furthermore if down regulation of FA β-oxidation is greater than down regulation of FA uptake, accumulation of LCFA's might increase uncoupling of the mitochondrial electron transport chain further aggravating energetic impairment [83].

The transition from compensated LVH to failure has previously been associated with, progressively compromised myocardial energetics [17, 58, 71, 74, 224]. Although there is consensus that this energetic compromise is attributable to maladaptive metabolic remodelling [65, 225], uncertainty remains regarding the precise changes that occur and their timing. For example, most studies suggest that in HF (including LVH+HF), inefficient FFA metabolism is diminished [65]. However studies in rodent LVH induced by supra-renal aortic constriction exhibit a preserved expression of genes involved in FFA metabolism [226]. Similarly, while a consensus exists about the adaptive up-regulation of carbohydrate metabolism in HF [65], the adequacy and beneficial effect of this substrate switch have been questioned [170, 227, 228, 229] It is likely that these discrepancies are attributable to the differing species, models/stages of LVH and the technologies with which metabolism has been studied [65].

This novel human LVH metabolomic study yielded a clear and significant separation between the LVH and non-LVH group. Whilst it is not possible at this stage to identify all the intermediates of carbohydrate and fatty acid metabolism within this analysis, we speculate that the complete separation we have demonstrated will reflect the highly abnormal concentrations of intermediate metabolites that will accumulate as a consequence of the transcriptional down regulation of significant metabolic pathways. This metabolomic analysis also corroborates the impairment of the metabolic transcriptome by demonstrating a decreased cellular energy charge (PCr/ATP), and a trend to reduced PCr, and elevated AMP levels perhaps as a manifestation of reduced energy reserve. These findings precisely replicate previous studies of patients with advanced AS assessed by ^{31}P MRS [230]. In addition to a trend towards decreased myocardial energy levels, Table 6 also demonstrates a

clear increase in myocardial levels of the keto- and glucogenic proteins lysine, tyrosine, tryptophan and phenylalanine. These changes are consistent with a derangement in glucose metabolism [231] and are consistent with existing small animal models of LVH and HF [232].

Although others have previously identified consistent metabolomic changes in human atrial tissue that plausibly contribute to disease pathogenesis (i.e. atrial fibrillation), the present study is the first to provide insights into the metabolome of the human left ventricle in disease. While these data are preliminary, they highlight the potential for metabolomic analysis to yield insights into the pathophysiology of LVH.

15. Conclusions

The principal finding of this integrative systems biology study is that in LVH, there is a significant down regulation of both the master transcriptional regulators and the key stages of both carbohydrate and fatty acid metabolism which results in an abnormal metabolome which is characterised by an impaired cardiac energetic status. This energy deficiency may contribute to the poor prognosis associated with untreated symptomatic AS. The significance of this energy deficiency with regards to myocardial protection is however less clear. A combination of inadequate delivery of cardioplegia through a less dense capillary bed, increased diffusion distance from oxygen and metabolites from capillary to myocyte and this impairment in myocardial energetics may contribute towards the findings that standard cardioplegic techniques may be inadequate in patients with LVH [165]. The resultant early post-ischaemic left

ventricular dysfunction may prejudice late reverse remodelling and reduce the prognostic benefits of surgery.

16. Limitations

The human non-LVH controls in this study were patients undergoing coronary artery bypass surgery and as such may not accurately reflect the metabolism of healthy hearts. The difficulty in obtaining LV tissue from healthy controls will remain a challenge to such studies as there is an increasing recognition that even “normal” brain stem dead human transplant donor tissue is a suboptimal control. Although this study is novel with respect to the metabolomics of human LVH tissue, it is preliminary and further metabolomic analysis of larger numbers of LVH tissue samples is required. Existing studies are inevitably biased towards the most abundant cellular metabolites that are preserved during tissue retrieval and storage and by the constraints of existing technology.

Chapter 4. A study of the effects of GIK on clinical outcomes following aortic valve surgery for aortic stenosis associated with left ventricular hypertrophy

17. Introduction

Patients undergoing aortic valve replacement for aortic stenosis often have significant left ventricular hypertrophy as an adaptive response to the increased pressure load of aortic stenosis. Whilst initially compensatory, this hypertrophy is associated with a significant down-regulation of metabolism that results in impaired cardiac energetics. In combination with the morphological changes in hypertrophy described earlier this renders the hypertrophied ventricle more susceptible to ischaemia-reperfusion injury during aortic valve surgery.

Approximately 30% of patients undergoing cardiac surgery develop significant ventricular dysfunction necessitating prolonged haemodynamic support. Patients requiring inotropic support have a 10-fold increase in morbidity and mortality, and myocardial injury is implicated in the 1500 patients that die within 30 days of cardiac surgery in the U.K. each year. Such patients consume 2.5 times more intensive care resources and in the long-term, early post-operative injury may prejudice late reverse remodelling and reduce the prognostic benefit of surgery.

Glucose-Insulin-Potassium (GIK) has been postulated to improve myocardial protection and reduce ischaemia-reperfusion injury through a variety of mechanisms including metabolic shift by promoting more efficient glucose metabolism and a direct cardioprotective effect of insulin through Akt and GSK-3B cell signalling [134, 140]. Previous trials GIK in patients undergoing coronary artery bypass grafting demonstrated a significant improvement in function with improved post-operative haemodynamics following surgery [198]. We hypothesised that the hypertrophied and

therefore energetically impaired ventricle of patients undergoing aortic valve replacement would benefit significantly from such metabolic support. A clinical trial was therefore designed randomising patients admitted for aortic valve replacement with echocardiographic evidence of left ventricular hypertrophy to be randomised in a 1:1 basis to either GIK or placebo in a double blind trial.

18. Methods

Following approval by the South Birmingham Research Ethics Committee, the HINGE Trial (Hypertrophy, Insulin, Glucose and Electrolytes) REC reference 04/Q2707/23 commenced recruitment on 1/10/04. This trial was registered with the International Standardised Randomised Controlled Trial Number Register (ISRCTN) Reference ISRCTN 05758301. For detailed materials and methods including intra and post operative management see Chapter 2: *Core Methodology*. 220 patients were randomised and trial recruitment was terminated on the 30.07.08. An end-points committee was appointed and the diagnosis of Low cardiac output episode (LCOE) was adjudicated. Finally, the trial was then unblinded and the data analysed.

19. Results

548 patients listed for aortic valve surgery were assessed for eligibility. 258 patients did not meet the entry criteria. 39 patients declined participation and 31 patients could not be recruited for logistical reasons. This left a total of 220 patients suitable for recruitment. 217 of these were consented and randomised. Patient recruitment and randomisation is detailed in the consort diagram (Fig 10).

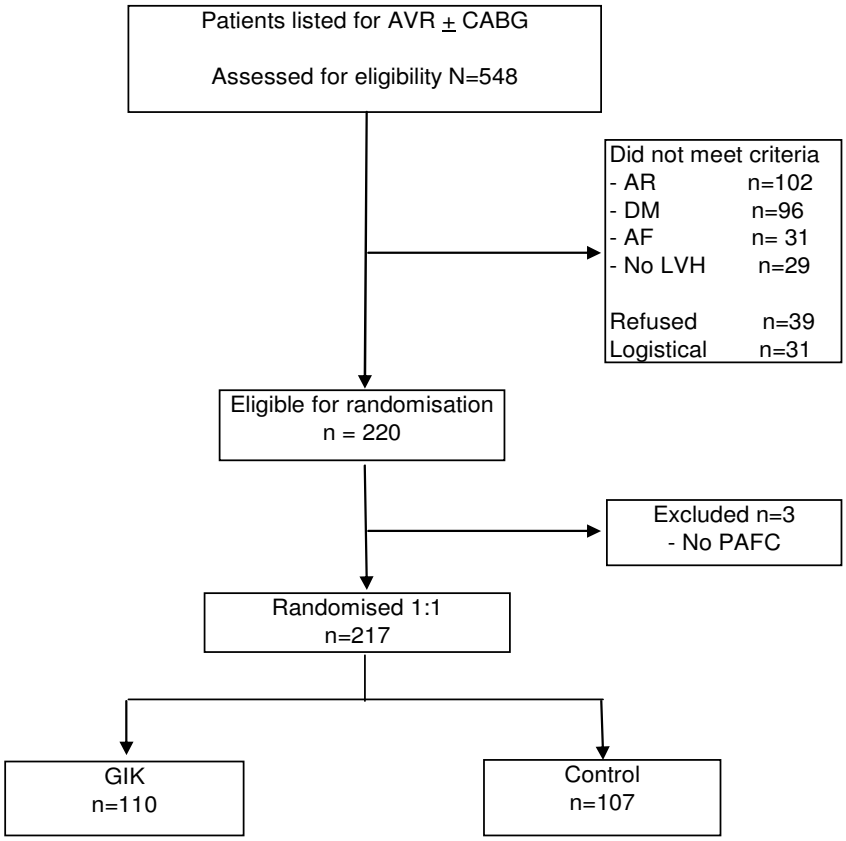

Figure 10 The HINGE Trial Consort Diagram

The demographics of the study population randomised to each group is presented in Table 7. There were no major differences in demographics between patients randomised to GIK or to placebo. The risk profile for the two groups was well matched. The mean EuroSCORE (logistic) in the GIK arm was 5.6(5.7) and 5.7 (5.8) in the control arm.

	GIK n=110	Control n=107
Age median, IQR	71.2 (63.4 to 76.5)	69.9 (65.0 to 74.4)
Female gender	43 (39.1%)	30 (27.5%)
CCS Class		
I	46 (41.8%)	42 (39.3%)
II	50 (45.5%)	53 (49.5%)
III	13 (11.8%)	12 (11.2%)
IV	1 (0.9%)	0
NYHA Class		
I	38 (34.5%)	37 (34.6%)
II	16 (14.5%)	15 (14%)
III	51 (46.4%)	51 (47.7%)
IV	5 (4.5%)	4 (3.7%)
Previous MI	5 (4.5%)	3 (2.8%)
Priority		
Elective	99 (90%)	95 (88.8%)
Urgent	11 (10%)	12 (11.2%)
PVD	2 (1.8%)	2 (18.3%)
TIA	2 (1.8%)	8 (7.3%)
CVA	2 (1.8%)	2 (18.3%)
Coronary artery disease		
None	82 (74.5%)	79 (73.8%)
Single vessel	11 (10%)	8 (7.5%)
Double vessel	12 (10.9%)	13 (12.1%)
Triple vessel	5 (4.5%)	7 (6.5%)
EuroSCORE median, IQR	6 (4 to 7)	6 (4 to 7)
Logistic EuroSCORE median, IQR	4.4 (2.7 to 7.1)	4.5 (2.8 to 7.7)

Table 7. Pre-operative demographics by patient group

191 patients had pure aortic stenosis and 26 had mixed aortic valve disease of which the predominant lesion was aortic stenosis. All patients had a pre-operative trans-thoracic echocardiogram to assess left ventricular hypertrophy. Echocardiographic data is detailed in Table 8.

Echocardiographic data	GIK	Control
n=	110	107
Peak Gradient (mmHg)	78±21	73±26
Mean Gradient (mmHg)	43±16	42±12
AVA cm^2	0.73±0.21	0.75±0.23
Aortic Regurgitation		
Mild or none	98 (89.1%)	97 (89.0%)
Moderate / Severe	12 (10.9%)	12 (11.0%)
EF (Simpsons)	58±11	60±4
EF Category		
Good (>50%)	94 (85.5%)	92 (86%)
Moderate (30-50%)	14 (12.7%)	10 (9.3%)
Poor (<30%)	2 (1.8%)	5 (4.7%)
IVSd (mm)	16.1±3.4	16.4±3.9
LVEDD (mm)	44.4±8.2	45.2±8.6
PWDd (mm)	15.3±3.6	15.1±3.2

Table 8. Echocardiographic data by randomisation group

Pre-operative medications were given as per protocol. Aspirin and clopidogrel were stopped at least 5 days prior to surgery; angiotensin converting enyzyme (ACE) inhibitors were withheld on the day of surgery. The pre-operative medications per trial group are detailed in Table 9.

	GIK	Control
n=	110	107
Aspirin	61 (55.5%)	62 (57.9%)
Clopidogrel	0	4 (3.7%)
ACEi	15 (13.6%)	19 (17.8%)
ACEii	12 (10.9%)	6 (5.6%)
Beta blocker	36 (32.7%)	33 (30.8%)
Ca^{2+} channel blocker	25 (23.7%)	22 (20.6%)
Statin	60 (54.5%)	52 (48.5%)
Diuretic	39 (35.5%)	36 (33.6%)
NSAID	2 (1.8%)	7 (6.5%)

ACE Angiotensin Converting Enzyme; NSAID Non-steroidal anti-inflammatory drug.

Table 9 Pre-operative medication by randomisation group

146 patients were listed for lone aortic valve replacement and 71 patients were listed for concomitant coronary artery bypass grafting. Of the 71 patients listed for grafting 2 did not have bypass grafts constructed because of surgeon choice. On peri-operative trans-oesophageal echocardiography, one patient was found to have severe functional mitral regurgitation in the context of impaired left ventricular function and a structurally normal mitral valve, and therefore underwent additional mitral valve repair with an annuloplasty ring only. One patient was found to have a structurally normal and non-calcified aortic valve and the outflow tract obstruction was instead due to a sub-aortic membrane which was resected.

5 patients underwent ascending aortic replacement due to post-stenotic dilatation. In all cases this was performed using a Gelweave™ graft (Vascutec Terumo, Scotland). There were no aortic root or arch replacements and circulatory arrest was not required. Surgical data is detailed in Tables 10 and 11. Mean bypass and cross clamp times were similar in both groups as were the number of bypass grafts required. Valve choice was at surgeon discretion and a wide variety of prosthesis were used.

	GIK	Control	p value
n=	74	72	
Time GIK-AXC	48±19	N/A	
CPB	93±46	92± 36	0.78
AXC	64±24	65±21	0.89
No. CABG	0	0	
Asc Aortic Replacement	1 (1.4%)	1 (1.4%)	0.91
Mitral Valve Repair	1 (1.4%)	0	
Resection sub-aortic membrane	1 (1.4%)	0	

Table 10. Surgical Data AVR only

	GIK	Control	p value
n=	36	35	n/a
Time GIK-AXC	53±48	n/a	
CPB	128±45	135±40	0.67
AXC	92±34	97±27	0.78
No. Of Grafts			
0	1(2.8%)	1 (2.6%)	1
1	16 (44.4%)	15 (39.5%)	0.92
2	10 (27.8%)	9 (23.7%)	0.89
3	9 (25.0%)	9 (23.7%)	0.93
4	0	4 (10.5%)	
Asc Aortic Replacement	0	1 (0.9%)	
Septal myectomy	1 (0.9%)	0	

Table 11. Surgical Data AVR + CABG

19.1 In-hospital Death

There were 2 in-hospital deaths in the study group. In the patients randomised to control there was one death secondary to respiratory failure and in patients randomised to GIK there was one death secondary to cardiac failure.

19.2 Low cardiac output episode

For the purpose of this study Low cardiac output episode (LCOE) was defined as cardiac index (CI) ≤2.2Lmin^{-1}m^{-2} in the presence of adequate filling pressure defined as CVP 8-12mmHg or PAWP 12-16 mmHg, and heart rate >75 bpm where systolic blood pressure <90mmHg and/or inotropic support and/or intra-aortic balloon pumping (IABP) for >60 minutes to maintain such a clinical picture. This was the primary endpoint for the trial therefore all patients who required inotropic support or had a recorded post-operative cardiac index ≤2.2 at any stage were presented to a blinded end-points committee and the endpoint adjudicated.

47 patients were diagnosed with a LCOE. The incidence in the GIK arm was 11/110 (10.0%) and in the Control arm was 36/107 (33%). This was analysed by in a pre-determined intention-to-treat non-linear mixed model accounting for surgeon and intention to perform CABG. The use of GIK was associated with a highly significant reduction in the incidence of low cardiac output episode (OR 0.219 95% CI 0.103 to 0.470, p=0.0001).

Study outcomes in Analyses Stratified According to CABG				
Outcome	**Control (n=107)**	**GIK (n=110)**	**Odds Ratio (95% CI)**	**P Value**
Primary Outcome Low cardiac output episode	**36 (33.6%)**	**11 (10.0%)**	**0.219 0.103 to 0.470)**	**0.0001**

Table 12 Primary outcome stratified according to intention to perform CABG

Further analysis was performed to assess the action of GIK in particular in patients with a higher (>6) logistic EuroSCORE, older patients (>70yrs) and in patients undergoing concomitant coronary artery bypass grafting. These pre-specified subgroup analyses for the primary end point revealed no heterogeneity in the effect of GIK as shown in the Forest plot below (Figure 11)

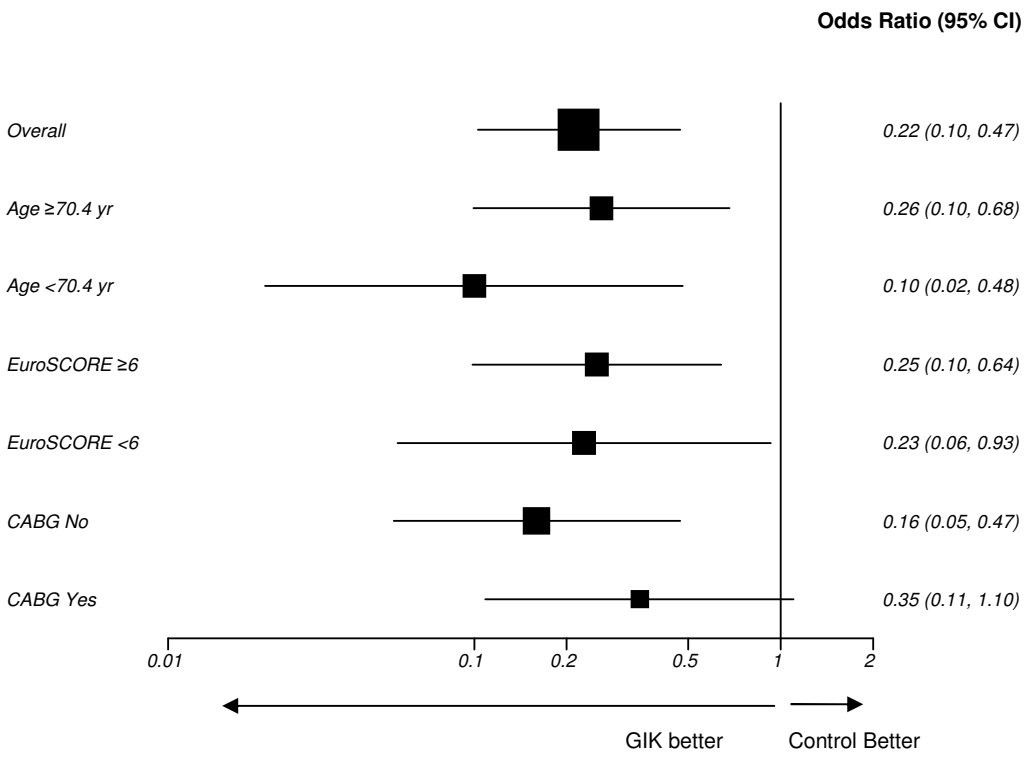

Figure 11 Sub-group analysis of primary outcome

19.3 Haemodynamic effects

All patients studied had a pulmonary artery flotation catheter floated. The rate, rhythm, mean arterial pressure (MAP), central venous pressure (CVP), pulmonary artery wedge pressure (PAWP) and cardiac output (CO) were recorded at baseline, immediately prior to administration of protamine, approximately 10 minutes post administration of protamine and then at 2, 4, 6, 9 and 12 hours post aortic cross clamp removal and a number of derived measures of cardiac performance were then calculated, the equations of which are listed in Appendix H.

19.3.1 Preload and Heart Rate

Throughout the study period (baseline to 12hrs) neither heart rate nor pre-load as measured by the pulmonary artery wedge pressure (PAWP) / mmHg differed significantly between groups. The post-operative goals were to achieve a heart rate between 80 and 110 and this normally required epicardial pacing at 90 beats per minute (bpm). The protocol also aimed for a post-operative filling pressure of a PAWP of 10-12mmHg and this is reflected in the PAWP in Figure 13.

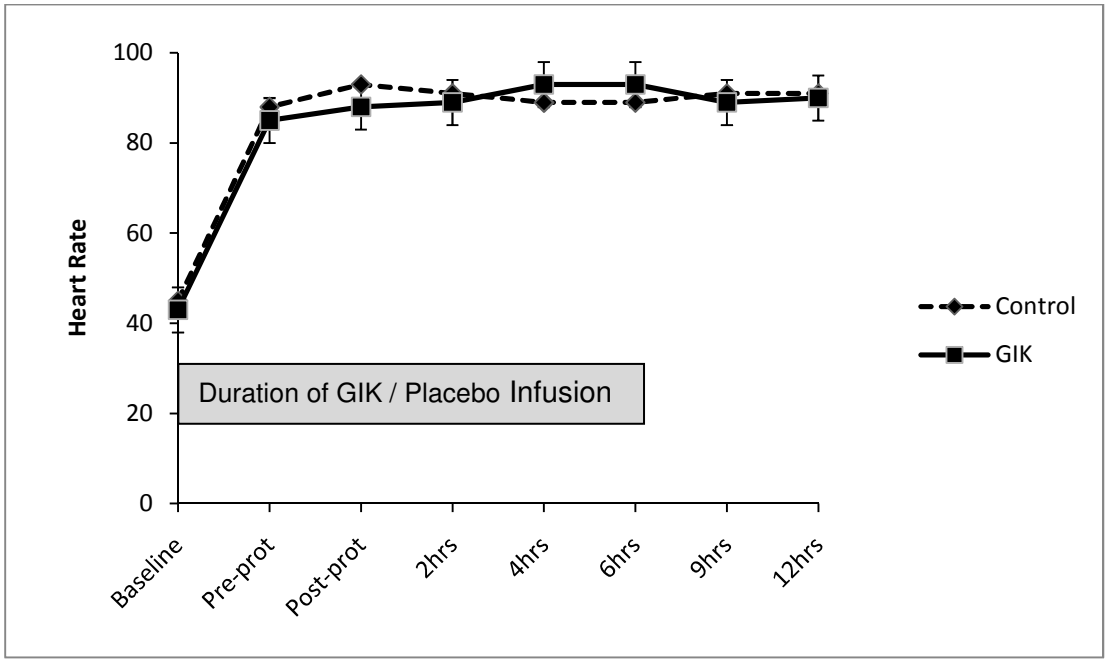

Figure 12 Mean heart rate / beats per minute (BPM) between treatment groups during period of haemodynamic monitoring. Error bars represent SEM.

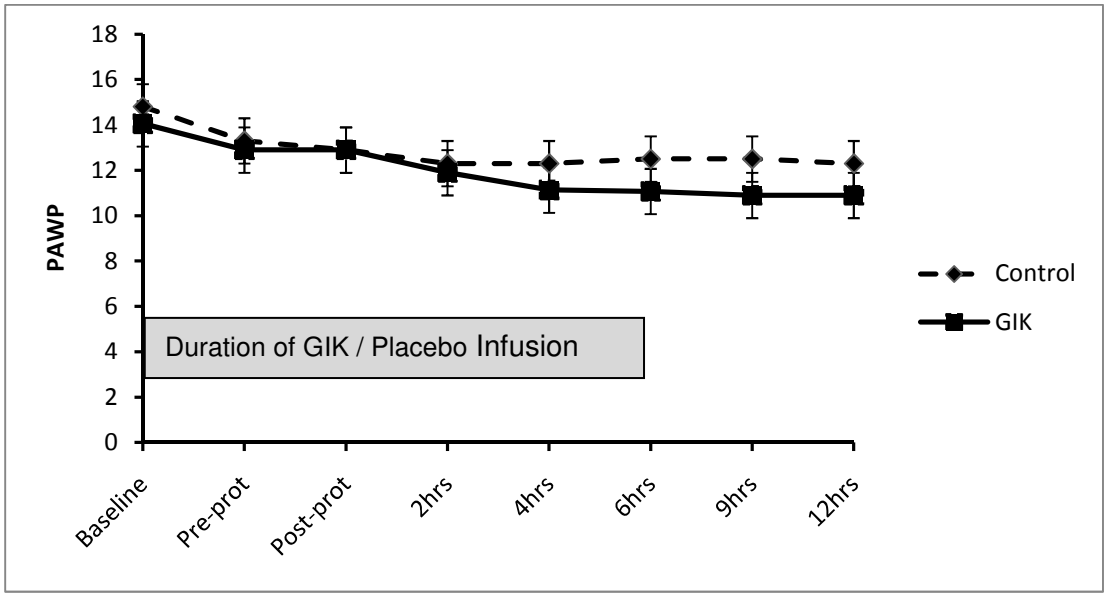

Figure 13. Mean pulmonary artery wedge pressure (PAWP) mmHg between treatment groups during period of haemodynamic monitoring. Error bars represent SEM, n=217

19.3.2 Cardiac Index

The baseline cardiac index (CI) was similar between GIK and control (2.12 vs. 2.27). Treatment with GIK was associated with a significant increase in CI throughout the treatment period (p=0.0001) as demonstrated in Figure 14.

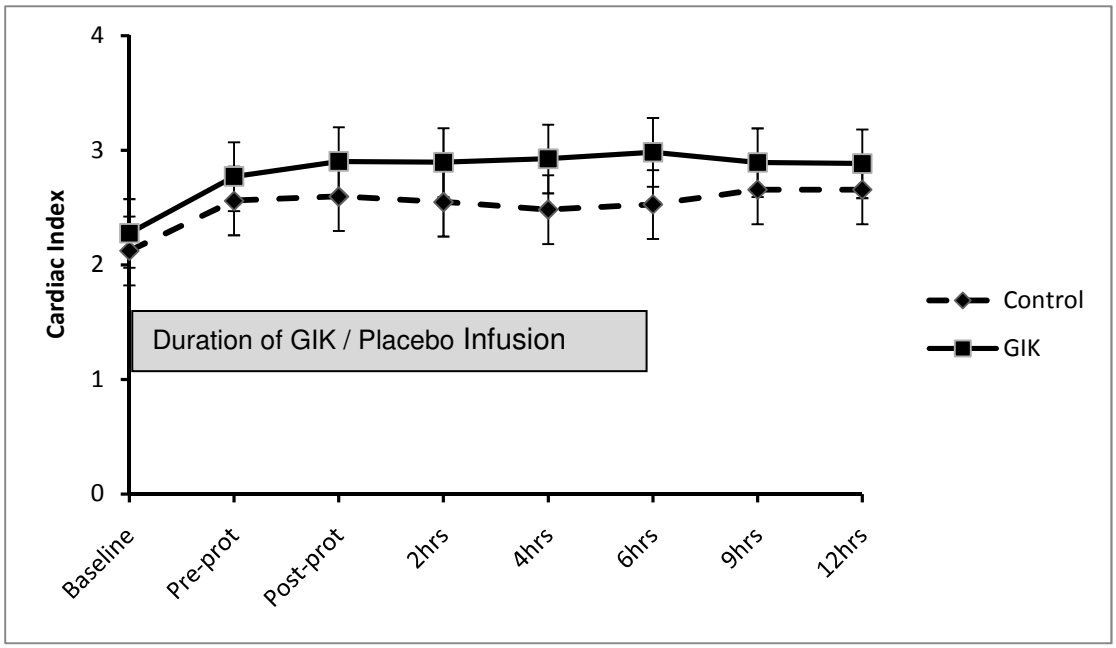

Figure 14. Mean cardiac index (CI) / lm^{-2} between treatment groups during period of haemodynamic monitoring. Error bars represent SEM, n=217

19.4.3.3 Systemic Vascular Resistance Index

Baseline systemic vascular resistance index (SVRI) was similar between GIK and control at baseline (2450 vs. 2630 dynes $s^{-1}cm^{-5}m^{-2}$). Treatment with GIK was associated with a reduction in SVRI in the 0-6 hour period during which the treatment was running, which became similar to control during the 6-12 hour study period. The mean SVRI between groups is shown in Figure 15.

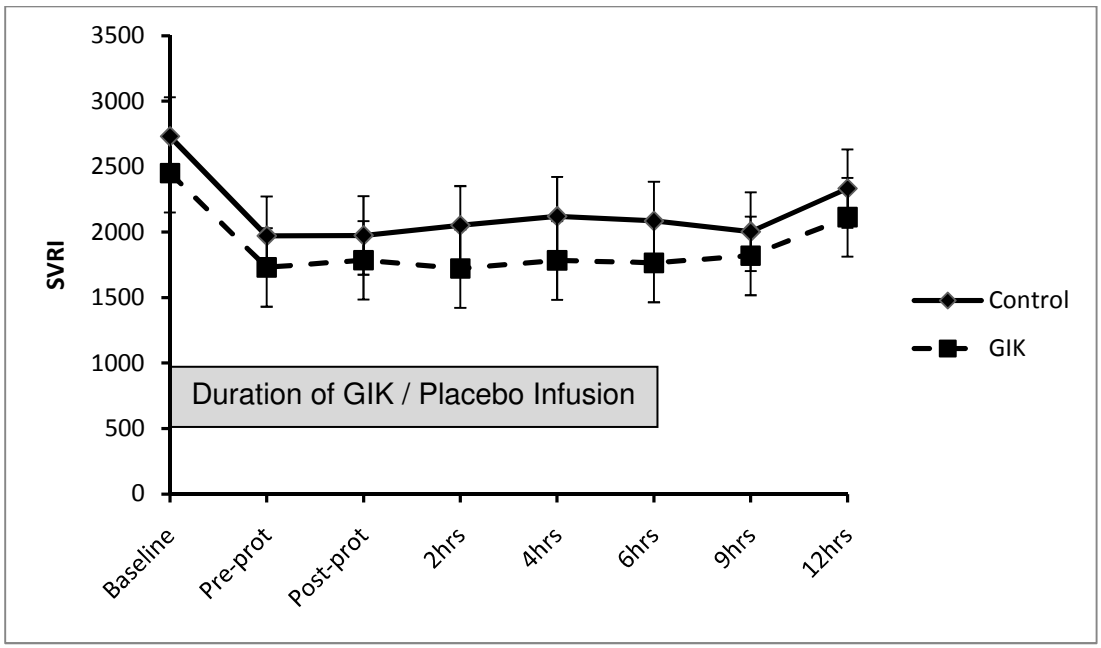

Figure 15. Mean systemic vascular resistance index (SVRI) / dynes $s^{-1}cm^{-5}m^{-2}$ between treatment groups during period of haemodynamic monitoring. Error bars represent SEM, n=217

19.3.4 Left Ventricular Stroke Work Index

The mean LVSWI was similar at baseline. Throughout the infusion period the LVSWI was higher in the GIK group with the greatest difference seen at six hours. Following cessation of therapy there was minimal difference between groups.

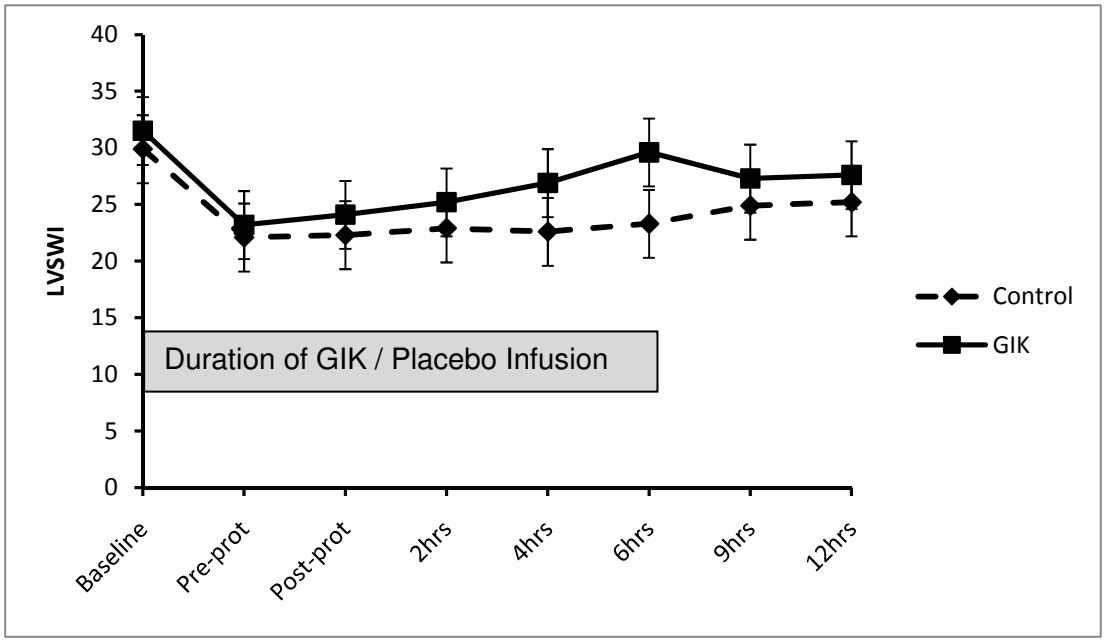

Figure 16. Mean left ventricular stroke work index (LVSWI)/ $gmm^{-2}beat^{-1}$ between treatment groups during period of haemodynamic monitoring. Error bars represent SEM n=217.

19.3.5 Cardiac Power Index

The mean cardiac power index was similar between groups at baseline. Throughout the infusion period the cardiac power index was higher in the GIK group with the greatest difference seen at six hours. Following cessation of therapy there was minimal difference between groups.

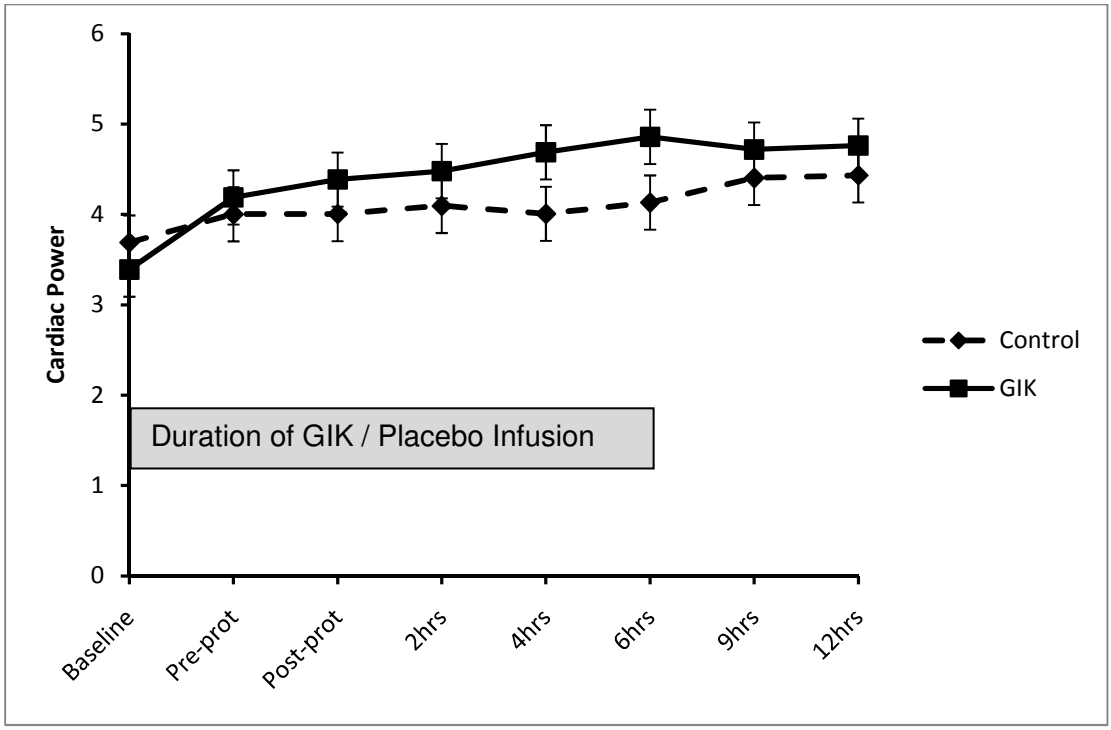

Figure 17. Mean cardiac power index/ Watts between treatment groups during period of haemodynamic monitoring. Error bars represent 95% CI for the mean, n=217

19.4 Use of Inotropes

In parallel with the reduction in the incidence of LCOE, the use of GIK was associated with a significant reduction in the prevalence of the use of inotropes in the study period baseline to 6 hours from 33% in the control group to 11.9% in the treatment group; OR 3.69 95%CI 1.77 to 7.69, p=0.0006. This difference continued from 6 – 12 hours reducing the prevalence of inotrope use from 34.9% in the control group to 13.8% in the GIK group OR 3.37 95% CI 1.68 – 6.77, p=0.0007. Estimates in differences between groups were adjusted for intention to perform CABG and Consultant.

The dose administered was calculated per kg per set time period. During the 0-6hour period GIK use was associated with a significant reduction in Dopamine (p<0.0001) and Epinephrine (p=0.001) requirements. Dose of Dopamine used per treatment group is shown in Figure18 and for Epinephrine in Figure 19.

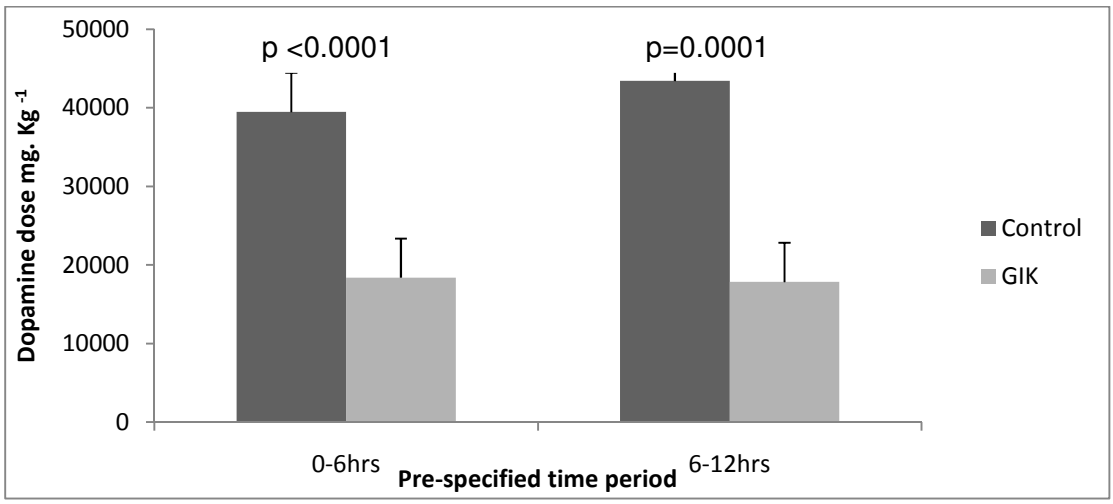

Figure 18 Total dose of Dopamine mg.kg^{-1} per pre-specified study period by treatment group, n=217

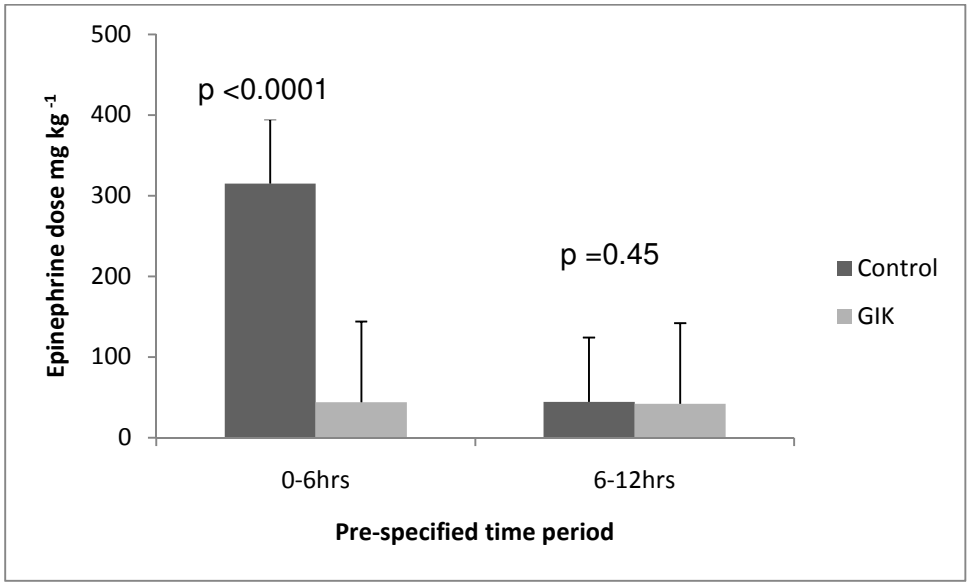

Figure 19 Total dose of Epinephrine mg.kg^{-1} per pre-specified study period by treatment group, n=217.

19.5 Use of Vasoconstriction

Bolus injections of Phenylephrine were titrated to blood pressure. If these requirements were considerable then an infusion was started (10mg Phenylephrine 50mls 5% dextrose). In cases of severe vasoplegia vasopressin was also commenced. Rarely (n=2), norepinephrine was used at medical staff request.

The use of GIK was associated with an increase in the prevalence of vasoconstrictor use in the baseline – 6 hour period from 45.3% in the control group to 64.2% in the GIK group p=0.001. The difference in prevalence of vasoconstrictor use was reduced in the 6 – 12 hour period but there was still a trend towards an increase in vasoconstrictor requirements from 40.6% in the control group to 56% in the GIK

group, p=0.04. The dose administered was calculated per kg per set time period. During the 0-6 hour period GIK use was associated with a significant increase in phenylephrine use (p=0.001). Dose of phenylephrine used per treatment group is shown in Figure 20.

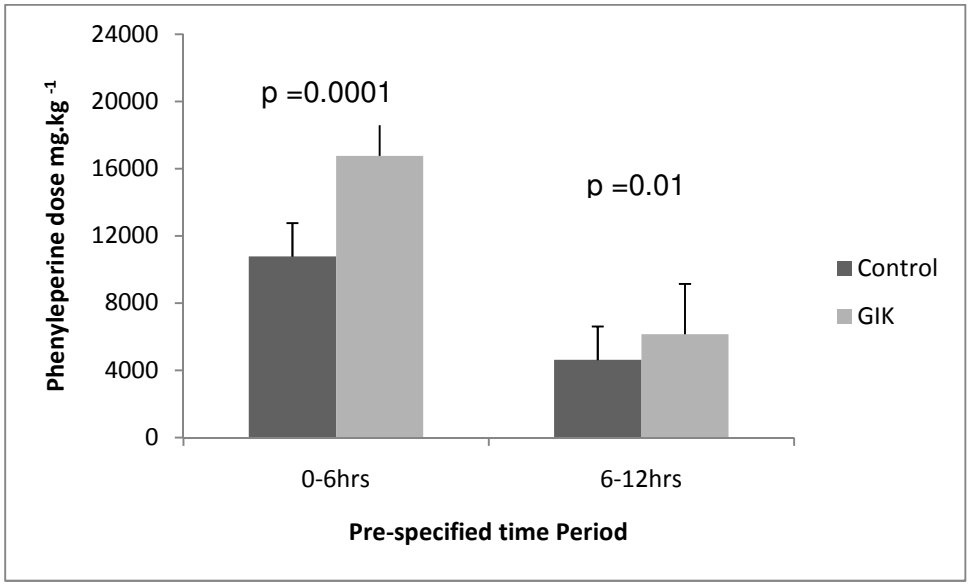

Figure 20. Total dose of Phenylephrine mg.kg^{-1} per pre-specified study period by treatment group, n=217.

19.6 Myocardial Injury

Serial ECGs were performed pre-operatively and on day 4 following surgery; where possible temporary pacing was halted to allow satisfactory ECG interpretation. The ECGs were collated and analysed by a blinded cardiologist at the end of the trial. Myocardial injury was defined as new Q waves of ≥2mm in 2 or more contiguous leads and/ or new left bundle branch block. 9 (8%) patients in the control group and 6

(5.5%) of patients in the GIK group met these diagnostic criteria, p=0.67. Whole blood samples were obtained from all trial subjects for analysis of Troponin T (TnT) at baseline, 6, 12 and 24 hours following cross clamp removal.

The primary endpoint for myocardial injury had been predetermined as the difference in means in the TnT levels at 6hrs when accounting for both consultant surgeon and intention to perform CABG using a continuous outcome. When accounting for these two variables, there was no significant difference in TnT levels between treatment groups (Table 13).

Continuous Outcome	**Control (n=106)**	**GIK (n=107)**	**Difference in Means (95% CI)**	**P Value**
TnT Mean (SD)	0.357 (0.32)	0.351 (0.25)	-0.00964 (-0.0869-0.067)	0.8059

Table 13 Difference in mean TnT ($\mu g.l^{-1}$) between groups at 6 hours

No patient was found to have had a significant rise in Troponin at baseline (max baseline TnT 0.02), and the mean Troponin rise at each time point was similar between groups.

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	106	0.357	0.296	0.418	0.030	2.460
12 TnT	106	0.512	0.332	0.692	0.130	9.600
24 TnT	101	0.567	0.426	0.708	0.040	6.800

Table 14 Mean TnT (µg.l^{-1}) in the control group at each pre-specified time point

GIK Group

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	107	0.351	0.304	0.399	0.110	2.130
12 TnT	106	0.497	0.432	0.563	0.100	2.130
24 TnT	105	0.588	0.507	0.668	0.110	2.120

Table 15 Mean TnT (µg.l^{-1}) in the GIK group at each pre-specified time point

In patients undergoing isolated AVR or combined AVR + CABG there was no significant difference in peri-operative infarction between patients randomised to GIK or control (Tables 14 and 15). In patients undergoing isolated AVR treated with GIK, 6.7% (5/74) had a significant TnT rise versus 14% (8/57) in the control group, p=0.42 (Tables 16 and 17). In patients undergoing combined AVR + CABG treated with GIK, 11% (3/28) had a significant TnT rise versus 11% (4/37) in the control group, p=0.57 (Tables 18 and 19). When examining the incidence of a significant TnT rise

in patients diagnosed with a low cardiac output episode, again there was no correlation. 8.5% (4/47) patients diagnosed with LCOE had a significant plasma TnT rise versus 9.2% (16/146) in patients who required no inotropic support. When the group was split into those undergoing isolated aortic valve (Tables 20 and 21) and those undergoing combined AVR + CABG (Tables 22 and 23) again there was no difference in mean plasma TnT levels between patients randomised to placebo and those randomised to GIK.

Isolated AVR

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	69	0.312	0.243	0.384	0.03	2.46
12 TnT	69	0.519	0.470	0.559	0.13	9.6
24 TnT	65	0.585	0.498	0.672	0.04	6.8

Table 16 Mean TnT ($\mu g.l^{-1}$) in the control group undergoing AVR only at each pre-specified time point

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	73	0.310	0.103	0.507	0.11	1.25
12 TnT	72	0.420	0.190	0.650	0.10	1.36
24 TnT	71	0.494	0.195	0.793	0.11	1.72

Table 17 Mean TnT ($\mu g.l^{-1}$) in the GIK group undergoing AVR only at each pre-specified time point

AVR + CABG

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	38	0.432	0.119	0.745	0.04	1.5
12 TnT	38	0.499	0.251	0.746	0.21	1.4
24 TnT	37	0.532	0.310	0.754	0.24	1.24

Table 18 Mean TnT ($\mu g.l^{-1}$) in the control group undergoing AVR + CABG at each pre-specified time point

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	35	0.441	0.112	0.770	0.14	2.13
12 TnT	35	0.549	0.094	1.003	0.13	2.13
24 TnT	35	0.669	0.209	1.214	0.18	2.12

Table 19 Mean TnT ($\mu g.l^{-1}$) in the GIK group undergoing AVR + CABG at each pre-specified time point,

Examining the relationship between clinically-defined low cardiac output episode and rise in TnT, there was no significant difference in TnT levels between patients treated for LCOE and those who required no inotropic support in either patients undergoing

isolated aortic valve replacement (Tables 20 and 21) or in those undergoing concomitant coronary revascularisation (Tables 22 and 23). The mean plasma TnT levels for each group are detailed below.

Isolated AVR

No Low cardiac output episode

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	115	0.323	0.05	0.594	0.12	2.46
12 TnT	114	0.501	0.03	1.42	0.14	9.6
24 TnT	110	0.548	0.04	1.24	0.04	6.8

Table 20 Mean TnT ($\mu g.l^{-1}$) in patients who developed no LCOE undergoing AVR only at each time point

Low cardiac output episode

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	27	0.252	0.110	0.399	0.03	0.72
12 TnT	27	0.333	0.269	0.497	0.10	0.92
24 TnT	26	0.495	0.163	0.894	0.92	1.91

Table 21 Mean TnT($\mu g.l^{-1}$) in patients who developed LCOE undergoing AVR only at each time point

AVR + CABG

No Low cardiac output episode

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	53	0.427	0.071	0.783	0.14	2.13
12 TnT	53	0.543	0.187	0.899	0.13	1.82
24 TnT	53	0.598	0.254	0.942	0.18	1.72

Table 22 Mean TnT ($\mu g.l^{-1}$) in patients who developed no LCOE undergoing AVR + CABG at each time point

Low cardiac output episode

Label	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Minimum	Maximum
6 TnT	20	0.460	0.264	0.656	0.04	0.79
12 TnT	20	0.642	0.188	1.096	0.21	2.13
24 TnT	20	0.779	0.203	1.366	0.26	2.12

Table 23 Mean TnT ($\mu g.l^{-1}$) in patients who developed no LCOE undergoing AVR + CABG at each time point

TnT release was also analysed by degree of LV hypertrophy as defined by the LVMI calculated from pre-operative echocardiographic measurements and indexed to body surface area. Extreme hypertrophy (LVMI >200 $g.m^{-2}$) was found to be associated with increased plasma TnT levels, p=0.01 (Fig 21).

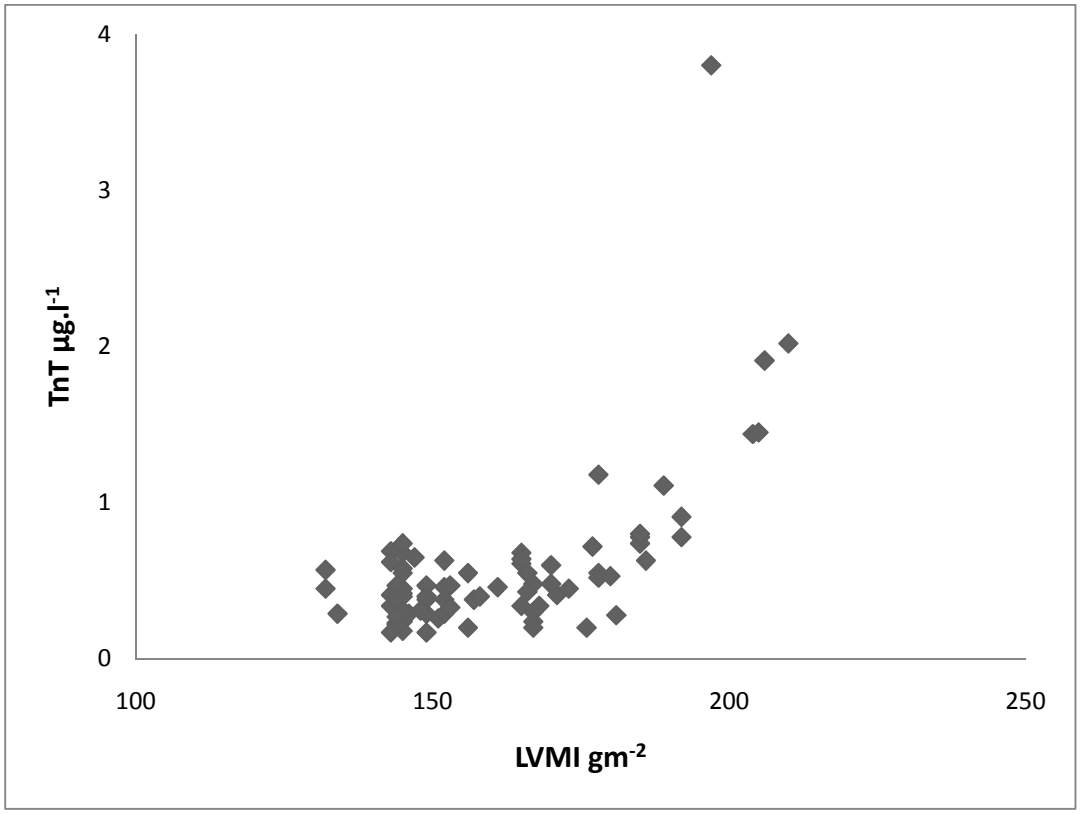

Figure 21 TnT ($\mu g.l^{-1}$) vs. LVMI ($g.m^{-2}$) n=217

19.7 Glucose, Insulin and Free fatty acids

GIK administration was associated with a rise in plasma glucose which was maintained throughout the infusion period (Figure 22). This was accompanied by similar rise in insulin levels to over four times baseline which normalised following

discontinuation of GIK therapy (Figure 23). The high level of insulin associated with GIK also lead to suppression of plasma free fatty acid levels throughout ischaemia and the six hour reperfusion during which the GIK was administered (Fig 24).

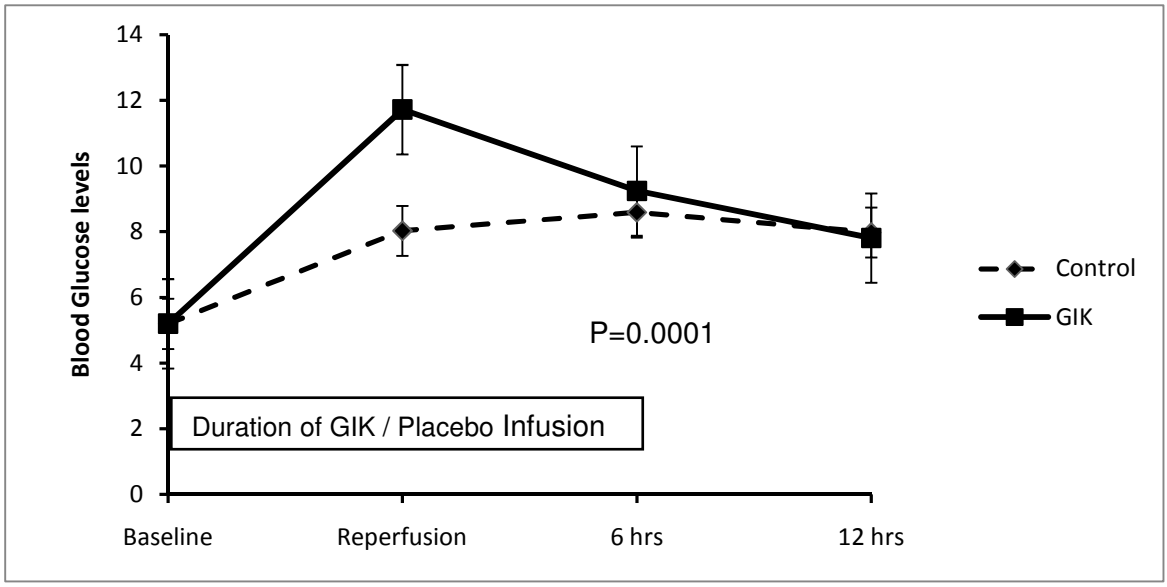

Figure 22. Mean blood glucose (mmol.l^{-1}) at each pre-specified time point by treatment group. Error bars represent SEM. n=150

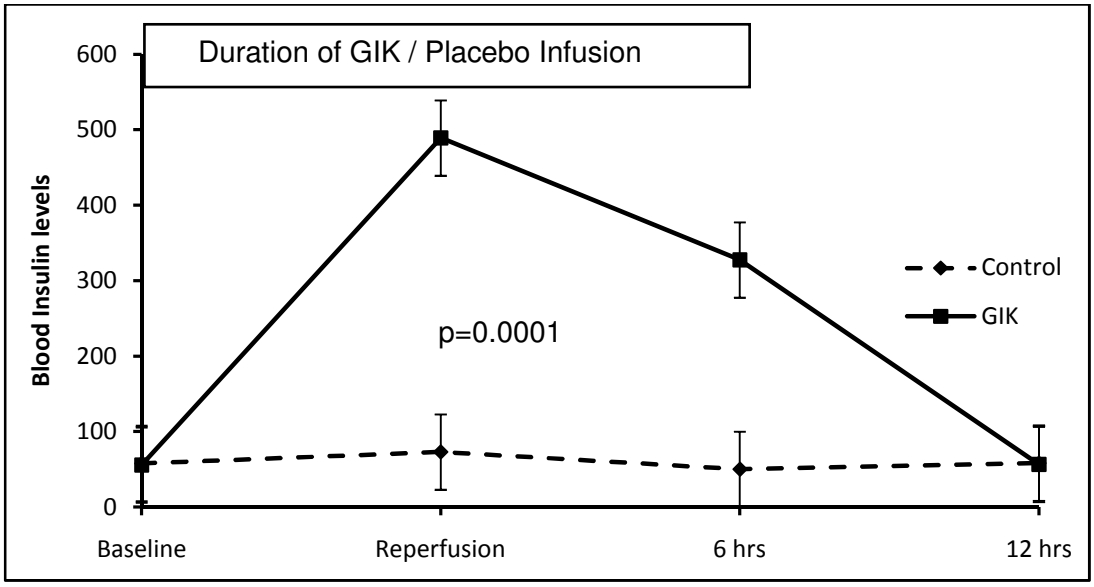

Figure 23 Mean blood insulin levels (µU.ml^{-1}) at each pre-specified time point by treatment group. Error bars represent SEM.n=150

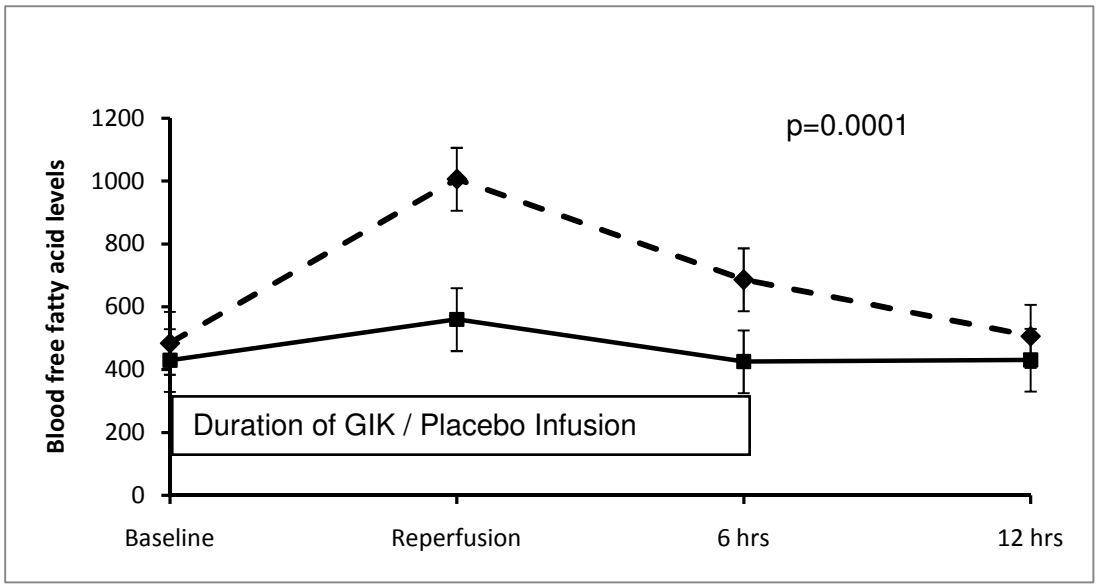

Figure 24 Mean blood free fatty acid levels (µmol.l^{-1}) at each pre-specified time point by treatment group. Error bars represent SEM. n=150

19.8 Insulin Requirements

Following commencement of the trial infusion, arterial blood gases were taken every 20 minutes to allow accurate titration of blood glucose levels. Additional insulin requirements were significantly higher in the GIK group versus the control group (91% vs 18%, p=0.01). The prevalence of insulin use fell to that of the control group following termination of GIK infusion, 45% GIK versus 39% control, p=0.89.

In parallel with the increase in incidence of additional insulin requirements, the total dose of insulin required was significantly higher in the GIK group both in the 0-6 hour study period (p<0.0001) and in the 6-12 hour period (p=0.001). The dose per kg is shown in Figure 25.

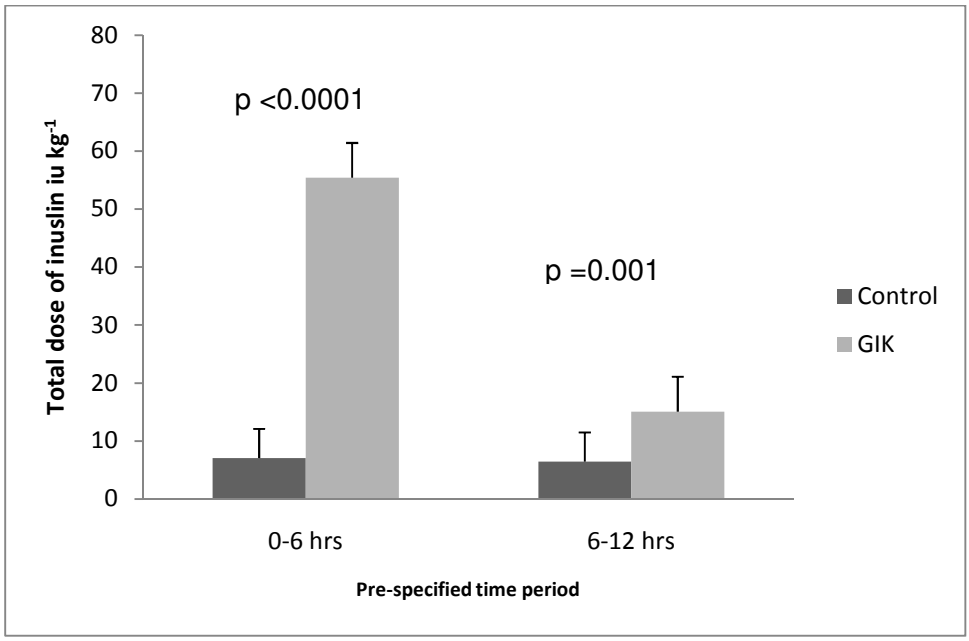

Figure 25. Total dose of Insulin(IU kg^{-1}) per pre-specified study period by treatment group. n=217

19.9 Use of Volume Expansion

Gelofusine was used as the first choice volume expander. 4.5% Human Albumin solution was used as second line volume expander when more than 2l of Gelofusine (1.5l if patient <70Kg) had been given. Volume expansion was indicated in patients with a MAP <60mm Hg or a CI <2.2 in the presence of a CVP or PAWP <15mmHg. There was no significant difference in the amount of Gelofusine or HAS transfused during any of the pre-specified time points (Figures 26 and 27).

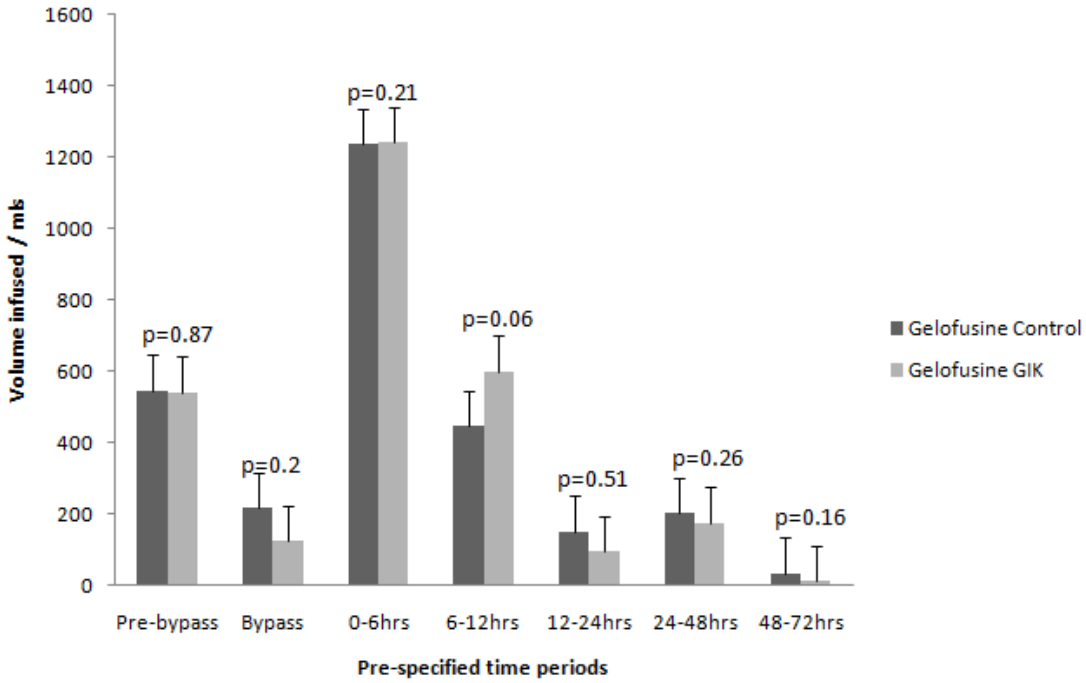

Figure 26. Mean volume of Gelofusine (mls) transfused by treatment group per pre-specified time period. Error bars represent the SEM. n=217

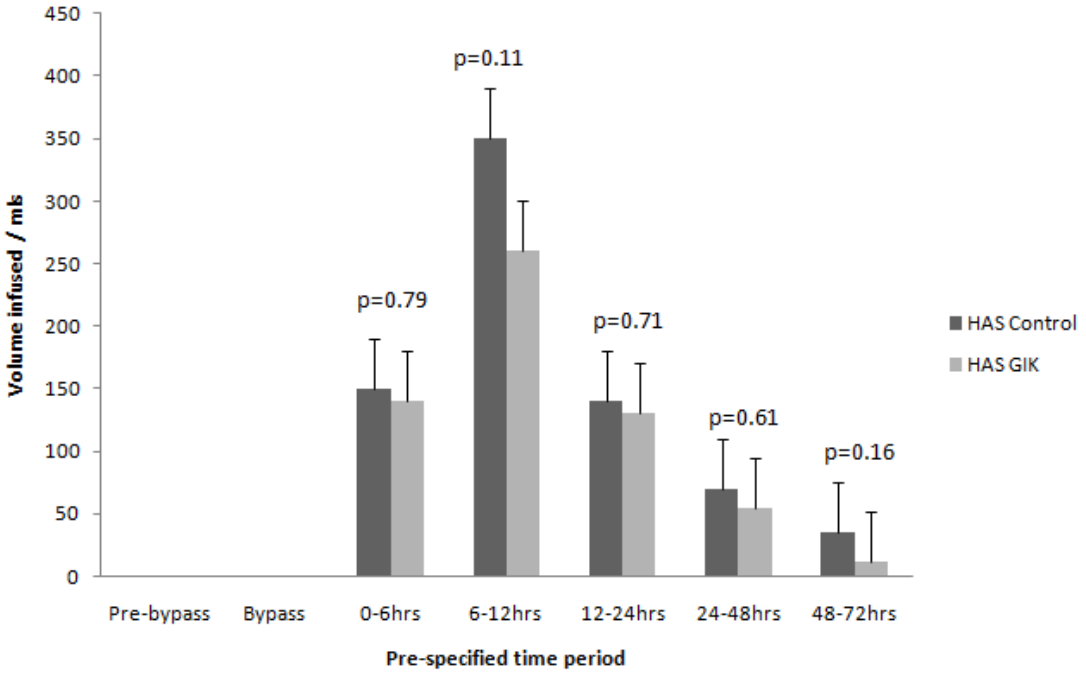

Figure 27. Mean volume of 4.5% HAS (mls) transfused by treatment group per pre-specified time period. Error bars represent the SEM. n=217

19.10 Use of blood and blood products

The indication for transfusion of blood and/ or blood products is detailed in the trial methodology. 50 (47%) patients in the control group and 57 (53%) patients in the GIK group required blood transfusion, p=0.07. 29 (27%) patients in the control arm and 29 patients (27%) required transfusion of blood products, p=0.97. The mean number of units of packed red cells per pre-specified time period is shown in Figure 28.

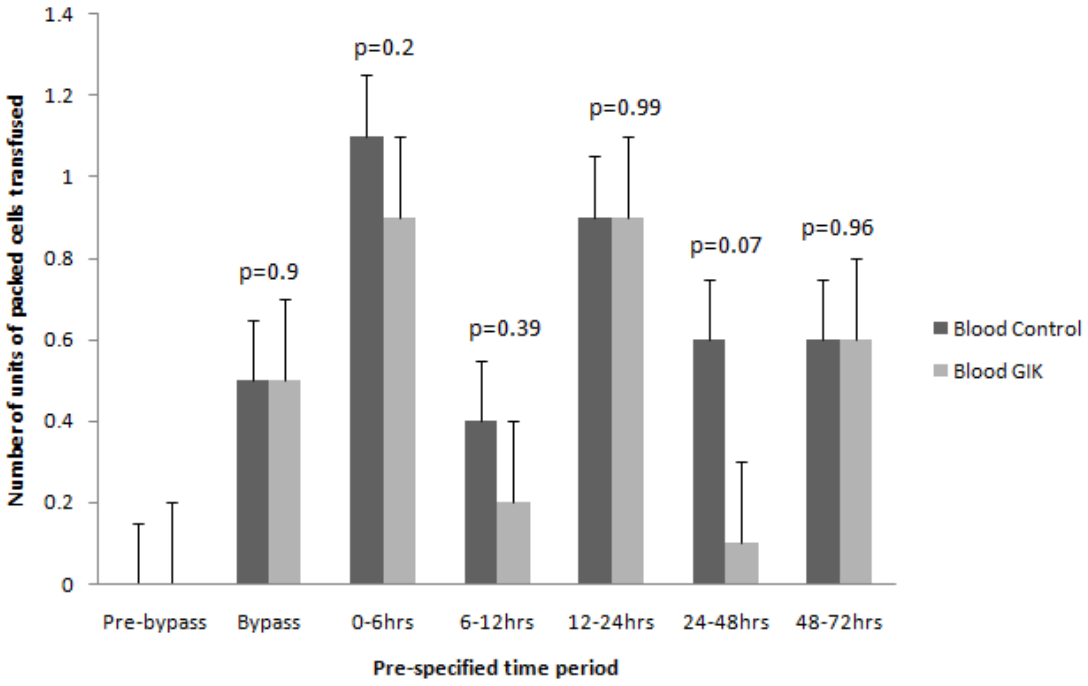

Figure 28. Mean number of units of packed red cells transfused per pre-specified time period. Error bars represent the SEM. n=217

19.11 New Arrhythmias and Need for Permanent Pacing

Data on post-operative atrial and ventricular arrhythmias were collected. Atrial fibrillation is one of the most common post-operative complications. Atrial fibrillation or flutter occurred post-operatively in 49 (45%) patients in the control arm and in 30 (27%) patients in the GIK arm, p=0.01. Ventricular fibrillation following cross clamp removal requiring DC cardioversion occurred in nine (8%) in the control group and in 10 (9%) patients in the GIK arm, p=0.85.

Three (2.7%) patients in the control group and two (1.8%) patients in the GIK group, p=0.87 required a permanent VVI pacemaker for sustained complete heart block post-operatively. No other type of pacemaker was implanted.

19.12 Length of Stay

The time from admission to the intensive care unit to warming to 36°C, extubation, step down from Level three (Intensive Care) to Level two (High Dependency) and then to Level one (Ward level care) to discharge was recorded. Mean times per group. The median length of time for each of these pre-specified time periods is detailed in Table 24

	Control Group	GIK Group	p value
Hours to 36°C	9.4 ± 2.4	8.7 ± 2.1	0.56
Hours to Extubation	15.3 ± 4.3	16.2 ± 4.9	0.78
Step-down to L2 / days	1 ± 0.5	1 ± 0.5	1
Step-down to L1 / days	2 ± 2.2	2 ± 2.8	
L1 to Discharge	3 ± 4.2	3 ± 3.6	

Table 24 Median length of stay at each level of care by treatment group n=217

19.13 Safety Outcomes

The incidence of standard post-operative complications including re-operations for bleeding/ tamponade or deep wound infections, pulmonary complications including tracheostomy, new stroke and need for new dialysis and early post-operative endocarditis were recorded. The criteria for meeting the above diagnoses is detailed

in Chapter 2. In addition, other pre-determined safety outcome measures included episodes of hypoglycaemia requiring treatment with 50% glucose, the bleeding rate at 12 hours following cross clamp removal and the total chest drainage at 12 hours.

5 (4.5%) patients in the control group and 6 (5.4%) patients required return to theatre for bleeding or suspected tamponade (p=0.9) and the mean drain loss at 12 hours following cross clamp removal was similar between groups, (876 ± 196 mls in the control group versus 986 ± 213 mls in the GIK group, p=0.76).

10 (9%) patients in the control group and 4 (3.6%) patients in the GIK group developed significant pulmonary complications, p=0.1, of which 3 (2.7%) patients in the control arm and 2 (1.7%) patients in the GIK arm required tracheostomy for ventilatory weaning, p=0.85.

2 (1.8%) in the control arm and 1(0.9%) required temporary haemofiltration (p=0.93). No patients required long term dialysis following surgery. There was 1 (0.9%) stroke in the control group and no new strokes in the GIK arm. No patients developed early infective endocarditis and at the time of writing no patient has been diagnosed with late endocarditis. No cases of significant mesenteric ischaemia or significant hepatic impairment were identified.

2 (1.8%) patients in the GIK group and no patients in the control group (p=0.12) required management of hypoglycaemia with 50% dextrose; both these events were without further complication.

20. Discussion

The key finding of this study is that infusion of GIK, initiated prior to aortic cross clamping and terminated 6 hours following reperfusion, results in a significant improvement in post-operative haemodynamic variables. There was a significant reduction in the incidence of post-operative low cardiac output episode and consequently a significant reduction in inotrope requirements.

The objective of every cardiac surgical operation is to produce a technically perfect result without producing myocardial damage. The consequences of inadequate myocardial protection range from subtle degrees of damage causing delayed myocardial fibrosis, to low cardiac output episode leading to multi-organ failure, increased mortality and prolonged hospital stay.

Myocardial stunning and cardiomyocyte necrosis associated with ischaemia-reperfusion injury results from the attenuation or cessation of coronary blood flow, such that oxygen delivery to the myocardium is insufficient to meet basal metabolic requirements to preserve cellular membrane integrity and viability. Recovery involves the resumption of normal oxidative metabolism and the restoration of myocardial energetics, the reversal of ischaemia-induced swelling, the loss of membrane ion gradients, and the repair of damaged cell organelles, such as the mitochondria and cytoplasmic reticulum.

After the aortic cross-clamp is removed, the cardiomyocyte may function normally, be stunned, or become dysfunctional from either necrosis or apoptosis. There is a

wealth of evidence that all patients undergoing cardiac surgery experience varying degrees of myocardial stunning. This is defined as a form of post-ischaemic dysfunction where there is normal myocardial perfusion, preserved contractile reserve and delayed but full recovery of function. This may require inotropic support, and resolves after hours or days leaving behind no objective evidence of myocardial infarction [233]. In this study LCOE, as a surrogate marker of myocardial stunning, as diagnosed by a blinded end-points committee, was reduced from 30% in the treatment arm to 10% in the GIK arm and there was consequently a similar reduction in the need for inotrope support.

The disadvantages of inotrope use in the early post-operative period is that increasing doses of catecholamines may cause cardiomyocyte necrosis as the myocardial oxygen consumption will exceed the hearts ability to increase coronary blood flow. There is recent evidence that therapeutic levels of inotropic support in the post-ischaemic heart increases intra-cellular calcium and subsequent apoptosis results in cell death [234]. We therefore hypothesise that a significant reduction in the incidence of myocardial stunning reflects improved myocardial protection and will result in less late fibrosis, improved reverse remodelling leading to improved exercise capacity, quality of life and ultimately increased late survival post surgery.

The incidence of low cardiac output episode in the control arm is reflective of the overall incidence of low cardiac output episode as defined by the need for inotropic support in the department Patient Analysis and Tracking Service (PATS) database of approximately 35%. The reduction in the incidence of low cardiac output episode associated with GIK therapy in this trial however was significantly greater than that

demonstrated in the MESSAGE trials [196, 198]. The trials were run with similar protocols for the diagnosis of low cardiac output episode and the institution of inotropic therapy.

Plasma Troponin levels are frequently elevated following cardiac surgery but multiple previous studies have demonstrated that low plasma levels should be considered innocuous caused simply by cardiac manipulation and surgical trauma to cardiac tissue and therefore of no prognostic significance. In this study, the mean plasma Troponin levels at baseline, 6, 12 and 24 hrs were similar between patients randomised to GIK and control. When accounting for intention to perform CABG and consultant surgeon, there was no significant difference between groups.

Increased levels of Troponin have however been shown to correlate with an increase incidence of adverse events. A prospective study of 1918 patients undergoing cardiac surgery in Canada demonstrated that significantly raised TnT levels were associated with a step-wise increase in MACCE, a composite end-point of all cause in-hospital death, LCOE (as defined by inotropic or IABP support to achieve a CI>2.0 $l.min^{-1}.m^{-2}$) and peri-operative MI (defined as new Q waves >0.04mm or R-wave reduction by >25% in at least 2 contiguous leads). The area under the receiver operating curve (AUROC) was used to determine significant TnT values by procedure type. A TnT value of 0.8 $\mu g.l^{-1}$ was discriminatory for patients undergoing isolated CABG or valve surgery, and a TNT value of 1.3 $\mu g.l^{-1}$ for patients undergoing combined procedures [235].

In this study, the values of 0.8 $\mu g.l^{-1}$ and 1.3 $\mu g.l^{-1}$ for AVR only and AVR + CABG corresponded to the top decile of plasma TnT levels. There was no significant difference between the incidence of a significant rise in TnT between treatment groups. Examining the relationship between a clinical diagnosis of low cardiac output episode requiring inotropic support and plasma TnT levels demonstrated no differences in mean levels at the pre-specified 6, 12 and 24 hr time period, and again when accounting for intention to perform CABG and consultant surgeon, there was no significant difference between groups. There was no significant difference in the incidence of a TnT ≥0.8 $\mu g.l^{-1}$ or 1.3 $\mu g.l^{-1}$ between patients diagnosed with low cardiac output episode and those requiring no inotropic support. The mean peak TnT in patients diagnosed with LCOE was 0.49 $\mu g.l^{-1}$ and only 8.5% (4/47) patients diagnosed with LCOE had a significant plasma TnT rise.

The clinical interpretation of plasma TnT levels following cardiac surgery remains controversial, and whilst there is no doubt that significant myocardial necrosis resulting in high plasma TnT levels is associated with poor outcome, the absolute levels used to define a significant TnT release remain somewhat arbitrary. The study by Nesher et al defining the predictive power of a peak TnT rise as ≥0.8 μgl^{-1} or 1.3 μgl^{-1} is highly dependent on the diagnosis of LCOE as this was the overwhelming component of MACCE [235]. However in this study there was no protocol directing the use of inotropes and the patients' volume status, right heart catheterisation results and amount of inotropic support is unknown [235].

This trial used strict criteria for inotrope support dependent upon cardiac output studies and only after pre-specified measures to address inadequate output and the

diagnosis of LCOE was only made following detailed discussion by a blinded end-points committee. In addition, the mean peak TnT following isolated AVR was 0.6 $\mu g.l^{-1}$ and it is therefore unsurprising that a peak TnT of ≥0.8 $\mu g.l^{-1}$ was not associated with significantly worse MACCE outcomes.

Ischaemia-reperfusion results in a vast number of cellular and metabolic changes. Initially these are only minor and reversible. Ongoing injury will lead to loss of contractile function as ATP and creatine phosphate stores are depleted and there is an increase in cytoplasmic ionised calcium but at this stage cellular ultra-structural changes appear only minor with mitochondrial swelling and contracture formation. Only continued injury will lead to irreversible cellular changes with activation of lipoprotein lipases, increasing cellular oedema, loss of mitochondrial respiratory control, major ultra-structural changes in mitochondria and myofibrils and finally cell death and tissue necrosis resulting in cellular autolysis and leakage of macromolecules such as Troponin into the interstitial space.

This pattern of ischaemia injury justifies both the rationale for metabolic therapy in improving myocardial function and reducing post ischaemic stunning following cardiac surgery and explains the failure to identify a reduction in TnT release with this therapy. Initial reduction of oxidative metabolism reduces myocardial contractility and therefore therapies to improve ATP and phosphocreatine during early reperfusion should improve myocardial contractility and therefore reduce the incidence of low cardiac output episode.

There are a number of potential reasons to explain the increased effectiveness of the use of GIK in patients with left ventricular hypertrophy. It has been shown that in patients with significant left ventricular hypertrophy undergoing aortic valve replacement standard myocardial protection strategies are not always adequate [165]. Patients with LVH have a global down-regulation in the metabolism and indeed have a significantly different metabolome characterised by a reduced ATP: PCr ratio [58]. This impairment in energetics may increase the vulnerability of the myocardium to ischaemia-reperfusion, and therefore metabolic manipulation with GIK suppressing free fatty acids and stimulating more efficient carbohydrate metabolism may lead to significant metabolic improvement. In addition, compared to the non hypertrophied heart, LVH secondary to AS has been shown to demonstrate insulin resistance with a significant decrease in the GLU4-GLUT1 ratio. GIK infusion during critical reperfusion with high circulating levels of both insulin and glucose may therefore be beneficial in overcoming this resistance [86].

In conjunction with a sustained increase in cardiac index patients treated with GIK had significantly higher requirements for vasoconstriction with Phenylephrine as demonstrated by the higher incidence of the use of Phenylephrine in this group and also by the increase in total dose administered. This well recognised vasodilatation associated with high dose insulin infusion with the associated need for vasoconstriction has meant that the overall intensive care length of stay and length of stay overall is similar between groups. It has also lead to controversy over whether or not the increase in cardiac output we have demonstrated is the result primarily of improved myocardial protection and increased cardiac function following ischaemia-reperfusion or a manifestation of the low systemic vascular resistance. It was

attempted to address this issue within the trial methodology with all patients managed with vasoactive drugs to achieve a target SVR of 800 – 1400. However, analysis of SVRI demonstrated a reduction in patients treated with GIK during the infusion period and therefore the vasoactive properties of insulin cannot simply be discounted.

To attempt to account for the impact of the differing load conditions, cardiac power and LVSWI were also calculated and compared between patient groups. CP and LVSWI both attempt to account for both the flow generated, and the perfusion pressure maintained. The two measurements do however differ. LVSWI is energy output per stroke whereas cardiac power is energy per unit time. For this reason recent analysis of the SHOCK registry has noted that cardiac power is the strongest predictor of mortality with cardiogenic shock [236]. In this trial however, the results of these two calculations were clearly similar as patients were all paced to achieve a target HR of 90-110 bpm.

This study has demonstrated that GIK is a safe agent to use with a satisfactory side-effect profile. Only 2 patients in the treatment group required treatment with 50% dextrose for hypo-glycaemic episodes and there were no gross neurological sequelae in either patient. Hyperglycaemia (as defined as blood glucose >10mmol/l) has been associated with poor outcomes in patients in intensive care but this is clearly not in the context of infusing high levels of glucose and insulin and we demonstrated no increased incidence of either superficial or deep wound infections.

Data from previous studies had suggested a trend to increased blood and blood product usage, although no increased rate of re-operation for bleeding was identified. In this study, we demonstrated no increased requirements for transfusion, or coagulopathy as defined by the need for transfusion of blood products. The re-exploration rate in both the treatment and control group was similar suggesting no increased risk of bleeding with GIK therapy, although during the time period of this study Aprotinin was used in all patients as part of a blood conservation strategy.

21. Conclusion

The use of GIK in patients with LVH secondary to aortic stenosis undergoing AVR was associated with a significant reduction in the incidence of LCOE, a reduction in the requirements for inotropic support and a significant reduction in the incidence of post-operative AF, but GIK was also associated with a significant increase in the need for vasoconstriction. The reduction in LCOE in this trial however was not associated with either a reduction in TnT release or a reduction in the length of stay on intensive care.

GIK is a safe adjunct with no significant increase in the incidence of bleeding, as defined by a return to theatre for bleeding or the need for transfusions of blood or blood products, and no increase the rate of infections or stroke despite higher levels of blood glucose.

22. Study Limitations

The main limitation of the haemodynamic data from this trial is that a load dependent measure (cardiac index) was used. Whilst every attempt was through strict adherence to trial protocols to try and obtain consistent loading conditions and heart rates, it is not feasible to ensure that this occurred on an individual patient basis, although comparison of the two treatment arms reveals similar loading conditions, heart rates and volume expansion. Although we have attempted to perform calculations to assess left ventricular work these data need to be interpreted with caution as they remain load dependent.

The main assessment of cardiac performance during this trial was of systolic function. This was assessed by haemodynamic measurement with a PAFC, the results of which are dependent upon loading parameters. As well as improving myocardial systolic function, GIK has also been hypothesised to improve diastolic function, which is now well recognised to be an active, energy dependent process. There are a number of ways to assess diastolic function using Doppler echocardiography. This can be used to measure the peak early filling (E Wave caused by differences in atrium-ventricle pressure) and atrial filling (A wave caused by atrial contraction). During the design phase of this trial, early post operative assessment of diastolic function utilising this methodology was suggested. However, all these patients had severe LV hypertrophy and therefore by definition, some degree of diastolic dysfunction. This in combination with cold blood cardioplegic arrest, topical hypothermia and subsequent requirements for sequential pacing at 90 – 110 bpm because of AV conduction delay, meant that all patients manifested severe diastolic dysfunction, with almost no recordable A wave. This unfortunately meant that tissue Doppler echocardiography could not be employed to compare diastolic function in the immediate post-operative phase.

Chapter 5. A study of the cardioprotective mechanism of GIK during aortic valve surgery.

23. Introduction

Although the cardioprotective properties of glucose-insulin-potassium (GIK) remain controversial, in part due to the different contexts, doses, timing and protocol of GIK employed in different studies, there is a growing consensus supporting GIK's utility in cardiac surgery [190, 196]. Multiple complimentary mechanisms may contribute to GIK related myocardial protection: (i) suppression of lipolysis resulting in a reduction of circulating levels of free fatty acids available for cardiac metabolism [93, 104, 107, 175] ; (ii) increasing insulin-related glucose flux and increasing myocardial oxygen efficiency, increasing cardiac energy provision (i.e. glycolytic ATP) [174] which may augment myocardial glycogen levels [40, 173, 176] and mitigate the consequences of low myocardial pH [99]; (iii) beneficial augmentation of TCA intermediates (anaplerosis) [178]; and (iv) through insulin's pleotropic signalling properties including its anti-apoptotic properties acting via phosphatidylinositol 3-kinase (PI-3-K) which may directly reduce myocardial injury [180]. Activation of a cascade of what have been termed "Reperfusion Injury Salvage Kinases" such as PI-3K/protein kinase B (Akt) and AMP-activated protein kinase, which have an established role in insulin signalling[237], may have a role in cardioprotection[238]. In addition to these established post-translational modifications (e.g. phosphorylation), insulin may promote modification of serine and threonine residues in proteins by O-linked beta-N-acetylglucosamine (O-GlcNAc)[157]. However the pertinence and contribution of these mechanisms in clinical context, especially in human LVH, remains speculative and unclear.

In Chapter 4, it was demonstrated that this regimen of GIK is highly efficient in suppressing FFA levels and gives rise to sustained increased levels of insulin and

glucose which will facilitate increasing cardiac energy provision via glycolytic ATP production [174] mitigating the consequences of low myocardial pH [99] and also augmenting TCA intermediates (anaplerosis). We now proposed to examine the potential beneficial effect of Reperfusion Injury Salvage Kinases" such as PI-3K/protein kinase B (Akt) and AMP-activated protein kinase, which have an established role in insulin signalling[237], may have a role in cardioprotection[238], and also to attempt a novel exmaination of the effect of insulin on O-linked beta-N-acetylglucosamination of myocardial proteins.

Cardiomyocyte survival after reperfusion has been negatively correlated to the fraction of cellular mitochondria that undergo mPTP induction [122]. Activation of a number of complex sets of cell signalling pathways which increase the mPTP-ROS threshold can provide the cardiomyocyte with increased protection. These signalling pathways include the Akt signalling pathway and it was therefore elected to study iys phosphorylation during reperfusion.

Activation at a variety of points along the various signalling pathways will result in comparable levels of protection of cardiomyocytes from reperfusion injury and it has therefore been hypothesised that there exists a common final mechanism to integrate these diverse signals to the end effecter, the mPTP [122]. It has been proposed that GSK-3β is the most likely candidate for this point of convergence [122]. Unlike most kinases, GSK-3 is highly active in the basal state and exerts a tonic-negative inhibitory effect on down-stream pathways. Phosphorylation results in enzyme inactivation and therefore relief of its tonic inhibition and activation of downstream targets [131]

Extensive evidence has now accumulated that GSK-3β is critical to the role of cardioprotection [133-136], it has been proposed that cardioprotective signalling deactivates GSK-3β by phosphorylation and leads either directly or indirectly by involving de-phosphorylation of VDAC and the release of VDAC from the GSK-3β-bound pool of ANT, to a shift in balance within the Bcl-2 family of proteins. The resultant de-phosphorylation of VDAC may then increase the ROS threshold for mPTP induction [141] thus improving myocardial protection

AMPK activation itself has also been shown to induce preconditioning in isolated cardiomyocytes and to prevent hypoxic injury [116], although the degree to which AMPK activation is either required or sufficient to induce preconditioning is uncertain. The molecular mechanism behind which AMPK activation might induce preconditioning are complex but may include activation of ATP-sensitive potassium channels [116]. Activation stimulates the movement of these channels from storage membranes to cell surface membranes where they are physiologically active and can shorten the action potential and therefore potentially reduce calcium overload during reperfusion [116]. In addition to this preconditioning effect, AMPK also plays a number of key metabolic roles. It increases cardiac glucose utilisation by a number of different mechanisms including promoting glucose uptake [112], phosphorylates and therefore activates (PFK-2) a potent promoter of glycolysis, and therefore its activation during ischaemia may be beneficial to the heart in terms of increasing glucose utilisation and subsequent anaerobic ATP production. A second major metabolic consequence of AMPK activation during ischaemia and reperfusion, is a stimulation of fatty acid oxidation [61] via phosphorylated and therefore inhibition of

Acetyl-CoA Carboxylase (ACC), the enzyme that converts acetyl CoA to malonyl-CoA, an inhibitor of CPT-1 [62]. Inactivation therefore of ACC results in increase in fatty acid transport into the mitochondria and subsequent oxidation. The question therefore of whether or not AMPK activation is beneficial during reperfusion remains to be fully elucidated. [113]

Increased O-GlcNAc levels have been proposed to increase tolerance to ischaemic stress via a number of different mechanisms [146]. Increasing O-GlcNAc levels have been associated with increased transcription of heat shock proteins (HSP), especially HSP70 [147],[150]. Activation of O-GlcNAc formation has also been shown to attenuate mPTP opening, a critical step in oxygen free radical-induced apoptosis and necrosis [151, 152]. One of the O-GlcNAc modified proteins identified as a potential O-GlcNAc target is VDAC, and it has been hypothesised that VDAC may preserve mitochondrial integrity by interfering with mPTP formation [151]. Increased O-GlcNAc levels with glucosamine attenuated the ischaemia-induced p38 MAPK phosphorylation, decreased ischaemic contracture and reduced the incidence of reperfusion arrhythmias [146, 148]. Increased levels of O-GlcNAc have also been reported to inhibit protein degradation [147] most likely due to inhibition of the proteasome [147] and this could also contribute to improved cell survival. In addition there is also some experimental evidence that increasing O-GlcNAc levels inhibit Ca^{2+} overload during reperfusion [155].

24. Methods

24.1 Myocardial biopsies

Ventricular biopsies were taken from age- and sex-matched patients enrolled in the HINGE trial undergoing isolated AVR with no significant coronary artery disease and no previous percutaneous coronary artery intervention. These biopsies were taken from the left ventricular wall between the left anterior descending artery and its first diagonal branch and were immediately snap frozen in liquid nitrogen at -80^{o}c until the analysis. Pre-operative demographics are detailed in Table 25. Biopsies were taken at baseline; after institution of bypass but before aortic cross clamping, and then at reperfusion, 10 minutes after release of the aortic cross clamp.

24.2 Protein extraction

Biopsies were initially homogenised using Ultraturax homogenizer at 10,000 rpm for 30-60 seconds in the presence of 1ml Tri Reagent©. The samples were allowed to stand at room temperature for 5 minutes. 0.2ml chloroform per ml of TRI Reagent© was added and thoroughly mixed. This was then allowed to stand for a further 10 minutes at room temperature.

Samples were centrifuged at 12000g for 15 minutes at 4 ^{o}C separating into an upper colourless aqueous phase containing RNA, a middle layer of protein and a lower layer of DNA. Following removal of the aqueous phase containing RNA, the DNA was precipitated with 0.3ml of 100% ethanol per 1ml TRI Reagent© used for the initial homogenisation. The samples were thoroughly mixed and incubated at room

temperature for 2-3 minutes. The sample was centrifuged at 2000g for 5 minutes at 4 ^{o}C to sediment the DNA.

300 µl of the supernatant (phenol/ethanol phase) was transferred to a clean eppendorf and the DNA destroyed. Proteins were then precipitated by adding three volumes of acetone. The sample was thoroughly mixed for 10-15 seconds to obtain a homogenous solution and stored at room temperature for 10 minutes at room temperature before being centrifuged at 2000g for 10 minutes at 4 ^{o}C to sediment the protein.

The supernatant was discarded and the protein pellet re-suspended in 0.5mk of 0.3M guanidine HCl in 95% ethanol 2.5% glycerol (1:1). Following dispersion a further 0.5ml of guanidine HCL in 95% ethanol 2.5% glycerol (1:1) wash solution was added and the sample incubated at room temperature for a further 10 minutes. The protein was sedimented by centrifugation at 8000g for 5 minutes at room temperature.

The wash solution was decanted and two further washes with incubations and spins in 1 ml of guanidine HCL in 95% ethanol 2.5%glycerol (1:1) were made. After each wash the sample was vortexed to disperse the pellet and remove residual phenol.

The final wash was made in 1ml ethanol containing 2.5% glycerol (v:v) and the sample was incubated for 10 minutes at room temperature. The proteins were sedimented by centrifuging at 8000g for 5 minutes at room temperature. The alcohol was decanted and the samples air dried for 30 minutes at room temperature. The proteins were then solubilised by adding 200µl 1% SDS and gently dispersed and

supplemented with 0.025% Tween 80. The proteins were stored at -80°C until required.

22.3 Western blotting

A stacking gel of 5% acylamide, 0.2M Tris-HCl pH 6.87, 7mM SDS, 0.2% v/v TEMED and 8.8mM APS was then prepared and layered on top of the resolving gel. Protein samples were loaded alongside Bio-rad Precision Plus Protein Prestained Dual Colour standards. Gels were run using the Mini-Protean III electrophoresis cell (Bio-Rad) and electrophoresed at 200 V through the resolving gel in SDS running buffer (25mM Trizma base, 192 mM glycine, 3.5 mM SDS) for 50 minutes.

Two pieces of 3mm filter paper (Whatmann, Kent, UK), a 10 cm x 8 cm sheet of PVDF membrane (Hybond-P, Amersham Biosciences, Amersham, UK pre-soaked in 100% methanol), and two fibre pads were equilibrated in transfer buffer (25mM Trizma base, 192mM glycine, 20% methanol), and the electrophoresed SDS-PAGE gel was sandwiched in a transfer cassette of the Mini Protean III transfer apparatus (Bio-Rad, Hemel Hempstead, UK).

Protein was transferred onto the PVDF membrane at 250 mAmp for ninety minutes. Following transfer, the membrane was blocked with 5% (w/v) Marvel dried skimmed milk in TBS-T (Tris-buffered saline containing 20mM Tris-HCl pH 7.6, 140mM NaCl and supplemental with 0.025% Tween 80) for at least one hour on a rocker bed at room temperature. Membranes were probed overnight with the primary antibodies diluted in 5% (w/v) milk-TBS-T at 4 °C on a rocker bed. Primary antibodies are

detailed in Appendix F. Membranes were then washed four times in TBS-T with fifteen minute incubations on a rocker bed between each wash.

The membrane was incubated with the appropriate secondary antibody diluted in 5% milk TBS-T for one hour on a rocker bed. The membranes were further washed for one hour with TBS-T at room temperature changing the wash solution every ten minutes. Excess wash buffer was then discarded and the membranes incubated with ECL (Enhanced Chemiluminesence) Plus (Amersham Biosciences) at 5 minutes at room temperature. The excess detection reagent was then drained off and the blots covered and then placed protein side up in an X-Ray film cassette. A sheet of autoradiography film (Hyperfilm© ECL, Amersham Biosciences) was then placed on top of the membrane. The cassette was then closed and exposed for 10 seconds and the developed. On the basis of the appearance of this film, the exposure time for further films was adjusted to give optimal results.

25. Results

Baseline biopsies demonstrated no evidence of AMPK or Akt phosphorylation, and insignificant levels of protein O-GlcNAcylation (data not shown). The reperfusion biopsies however demonstrated significant post-translational change. Phosphorylation and GlyNAcylation was compared between GIK and control.

Akt, GSK-3β and AMPK signalling was studied in 16 patients (8 control and 8 GIK) undergoing isolated AVR. There were no significant differences in the pre-operative demographics or in the echocardiographic markers of function between groups (Tables 25 and 26). Immunoblotting demonstrated a ~2.5 fold increase in the phosphor-Akt:pan-Akt ratio (p=0.03) (Fig. 29), a ~2.2 fold increase in the phosphor-GSK-3β: pan- GSK-3β ratio (p=0.001) (Fig. 30), and a ~1.7 fold increase in the phospho-AMPK:AMPK ratio (p=0.0004) (Fig 31)

Protein O-GlcNAcylation was studied in a further 16 (8 GIK 8 control) undergoing isolated AVR. Again there were no significant differences in the pre-operative demographics or in the echocardiographic markers of function between groups (Tables 31 and 32). O-GlcNAcylation as assessed by visual inspection suggested that the GIK group were more prominently O-GlcNAcylated. Accordingly, individual band analysis suggested that a band of approximately ~60 kDa in the GIK group was 46% more O-GlcNAcylated when than controls, p=0.01 (Fig.33). Protein loading was assessed by GADPH and β-tubulin. Formal band densitometry was performed using the Quantity One software package.

25.1 The effect of GIK on Akt, GSK-3β and AMPK signalling

	GIK	Control	p value
Sample size	n=8	n=8	
Age	67 ± 2.2	68 ± 2.3	0.63
Gender M/F	3 / 0	3 / 0	0.45
CCS			
I	0	0	
II	3	3	
III	0	0	
IV	0	0	1.0
NYHA			
I	0	0	
II	3	3	
III	0	0	
IV	0	0	0.41
Hypercholesterolaemia	0	0	
Hypertension	2	2	
CAD	0	0	
Pre-op Medication			
β -Blocker	0	0	
ACEi	7	7	
Statin	7	7	

CCS – Canadian Cardiovascular Society; NYHA – New York Heart Association; CAD – Number of Coronary Arteries with flow limiting lesions; ACEi – Angiotensin Converting Enzyme Inhibitor

Table 25 Demographics of patients undergoing analysis of Akt and AMPK signalling pathways.

	GIK	Control	p value
Sample size	n=8	n=8	
IVSd (cm)	1.47 ± 0.14	1.41 ± 0.13	0.78
LVEDd (cm)	4.67 ± 0.15	4.47 ± 0.21	0.81
PWd (cm)	1.45 ± 0.09	1.37 ± 0.12	0.83
LVMI $g.m^{-2}$	147.81 ± 27.23	143.35 ± 31.61	0.67
Peak Aortic velocity ($m.s^{-1}$)	4.698 ± 0.5	4.65 ± 0.4	0.8
Mean AV Gradient (mmHg)	52 ± 7.23	53 ± 8.12	0.85
Stroke Volume (mls)	49 ± 3.83	47 ± 4.46	0.77
Ejection Fraction (%)	55 ± 7.6	54 ± 6.11	0.9

IVSd – Interventricular septum in diastole; LVEDd – Left Ventricular End Diastolic dimension; PWd Posterior Wall in diastole; LVMI – Left Ventricular Mass Index

Table 26 Echocardiographic data of patients undergoing analysis of Akt, GSK-3β and AMPK signalling pathways

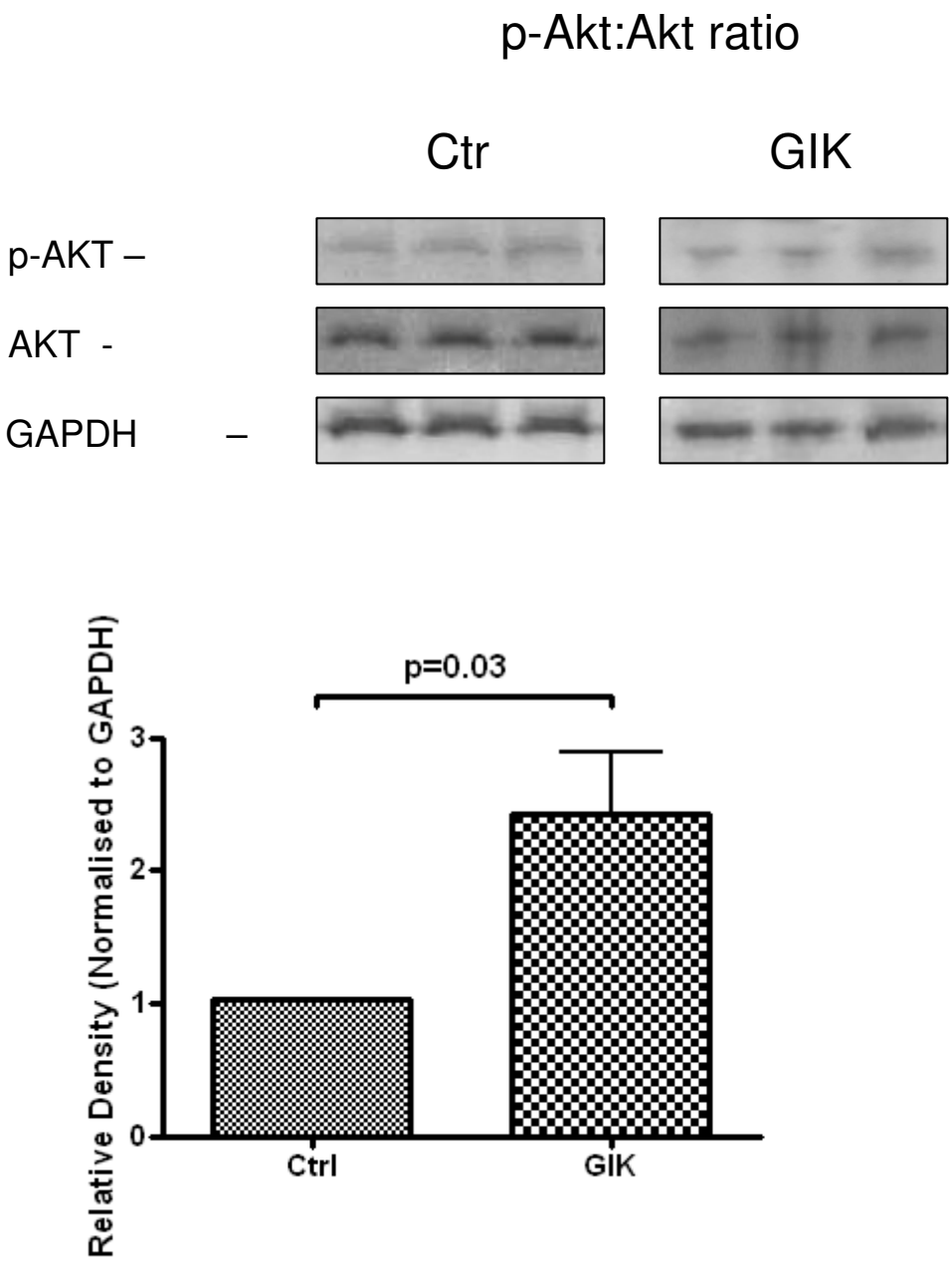

Figure 29 Western blotting for phospho-Akt and pan-Akt in patients randomised to GIK and control (.n=8).

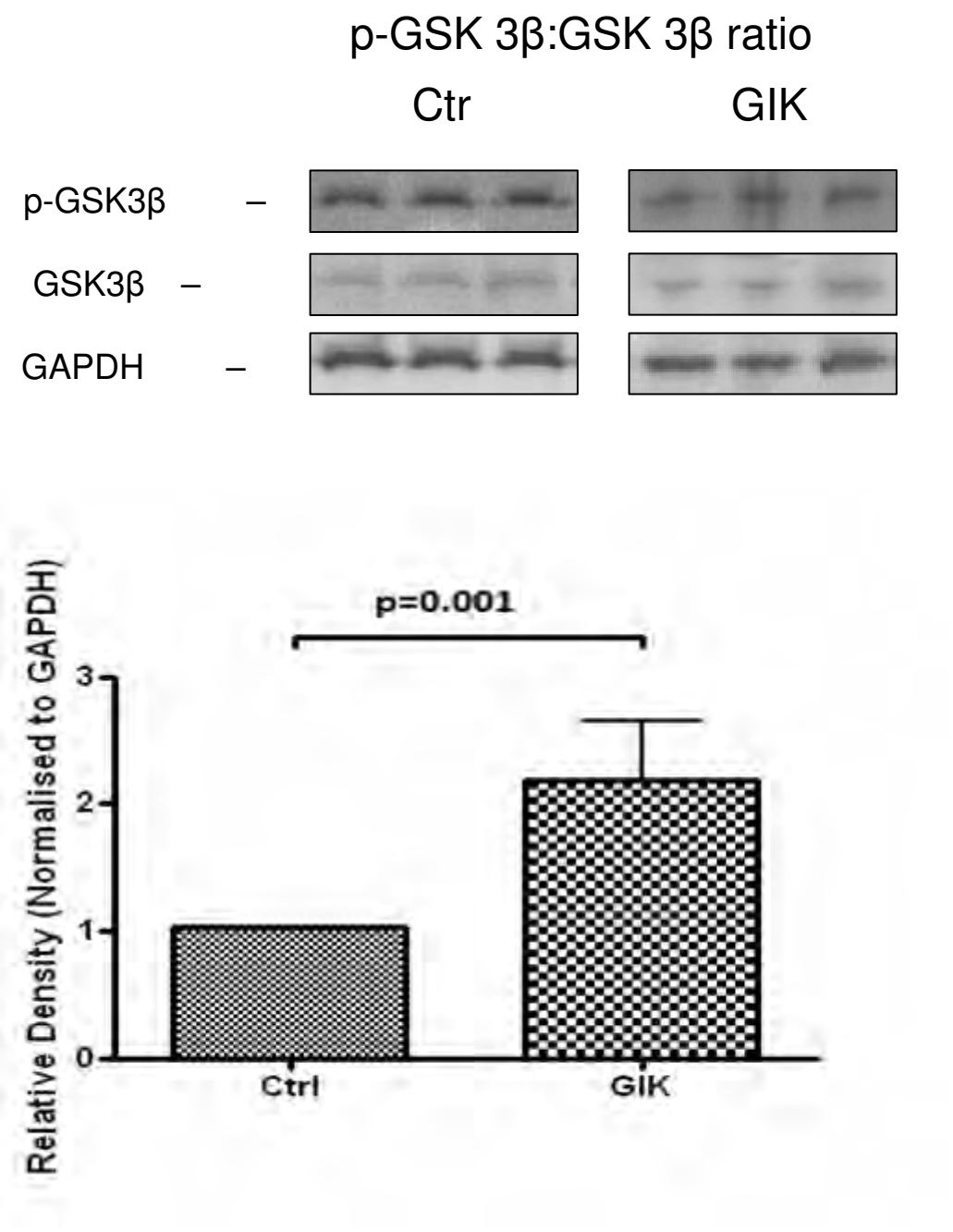

Figure 30 Western blotting for phospho-GSK-3β and pan- GSK-3β in patients randomised to GIK and control (n=8)

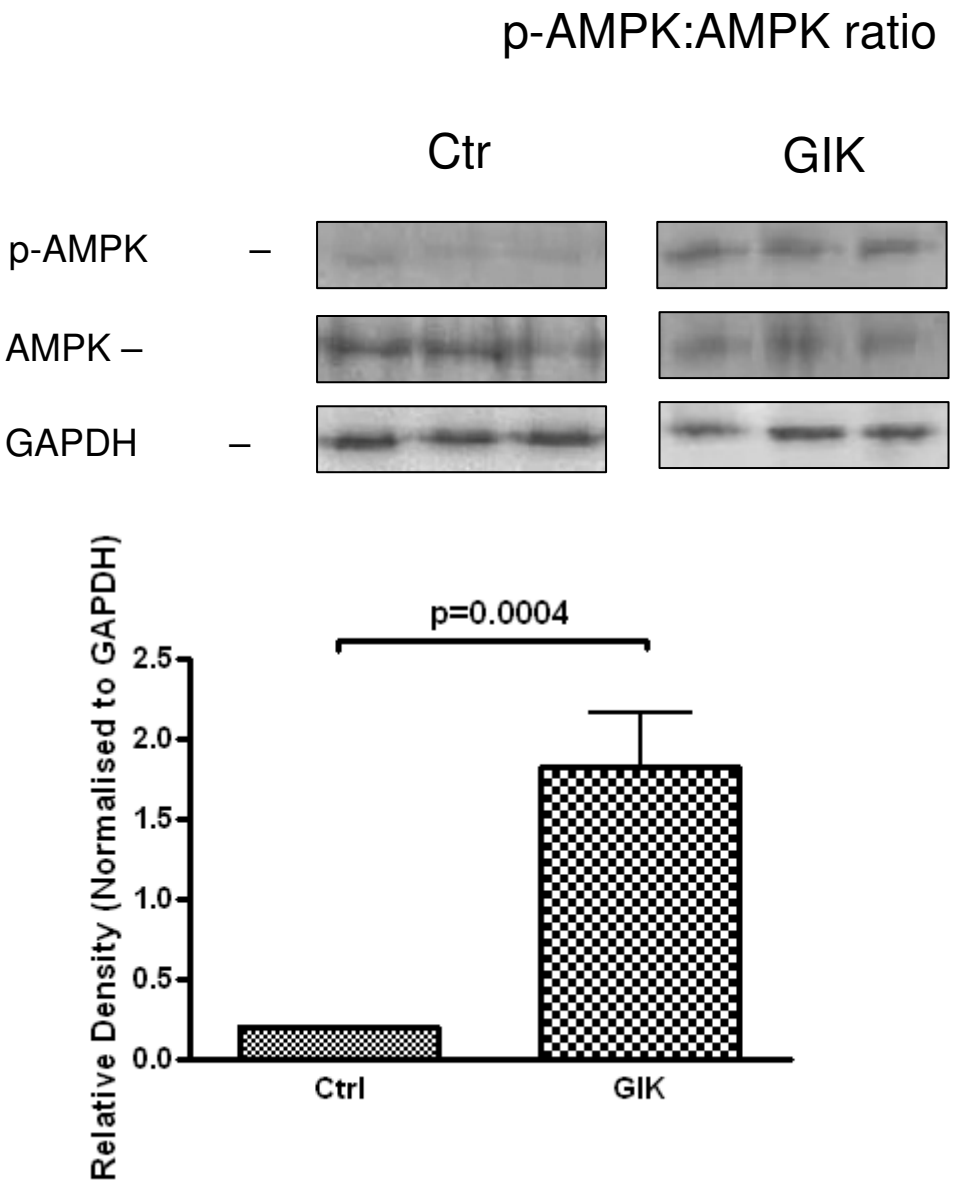

Figure 31 Western blotting for phospho-AMPK and pan- AMPK in patients randomised to GIK and control (n=16)

25.2 The effect of GIK on Protein O-GlcNAcylation

	GIK	Control	p value
Sample size	n=8	n=8	
Age	69 ± 1.8	68 ± 2.1	0.63
Gender M/F	7 / 1	6 / 2	0.45
CCS			
I	1	2	
II	4	3	
III	3	3	
IV	0	0	1.0
NYHA			
I	0	0	
II	5	5	
III	3	3	
IV	0	0	0.41
Hypercholesterolaemia	0	0	
Hypertension	4	3	
CAD	0	0	
Pre-op Medication			
β -Blocker	0	1	
ACEi	7	6	
Statin	6	6	

CCS – Canadian Cardiovascular Society; NYHA – New York Heart Association; CAD – Number of Coronary Arteries with flow limiting lesions; ACEi – Angiotensin Converting Enzyme Inhibitor

Table 27 Demographics of patients undergoing analysis of Protein O-GlcNAcylation

	GIK	Control	p value
IVSd (cm)	1.48 ± 0.24	1.43 ± 0.19	0.82
LVEDd (cm)	4.58 ± 0.19	4.41 ± 0.20	0.81
PWd (cm)	1.41 ± 0.09	1.42 ± 0.12	0.73
LVMI $g.m^{-2}$	145.81 ± 25.3	142.41 ± 29.82	0.72
Peak Aortic velocity ($m.s^{-1}$)	4.72 ± 0.4	4.68 ± 0.5	0.80
Mean AV Gradient (mmHg)	53 ± 6.89	54 ± 7.99	0.85
Stroke Volume (mls)	48 ± 2.9	49 ± 546	0.80
Ejection Fraction (%)	57 ± 8.6	55 ± 6.5	0.92

IVSd – Interventricular septum in diastole; LVEDd – Left Ventricular End Diastolic dimension; PWd Posterior Wall in diastole; LVMI – Left Ventricular Mass Index

Table 28. Echocardiographic data of patients undergoing analysis of Protein O-GlcNAcylation

Western blot analysis of global *O*-GlcNAc of total human left ventricular protein. Immunoblotting was performed using the CTD 110.6 antibody, and normalised against loading control, β-tubulin, in control and GIK samples, n=8 respectively. Arrows indicate the molecular weight of bands analysed for *O*-GlcNAc (dashed: ~80 kDa; solid: ~60 kDa; dotted ~40 kDa) (Fig 32).

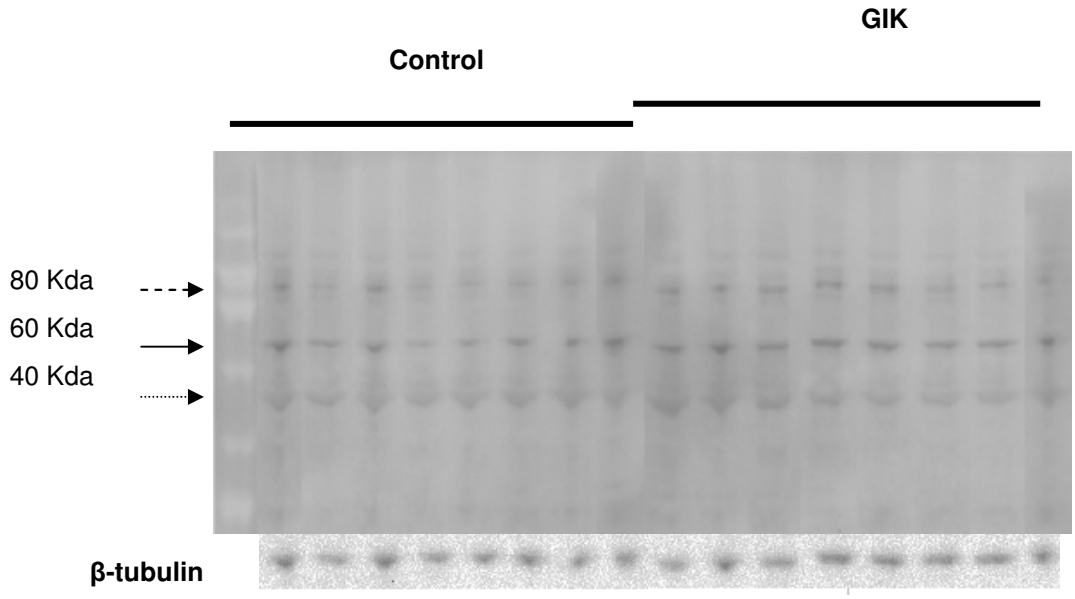

Figure 32 Western blot Analysis of *O-GlcNAcylation* of human left ventricular biopsy derived protein in control and GIK treated samples (n=16)

Whole lane analysis showed a tendency towards an increase in the levels of O-GlcNAcylation in the GIK group (control 1.00±0.09 SEM, n=8; GIK 1.09±0.13 SEM, n=8). A significant increase in *O*-GlcNAcylation was seen in a band of approximately 60kDa (control 1.00±0.13 SEM, n=8; GIK 1.46±0.10 SEM, n=8). In contrast, no significant changes were evident for the 40 kDa bands (control 1.00±0.11 SEM, n=8; GIK 1.02±0.18 SEM, n=8) or the ~80kDa bands (control 1.00±0.53 SEM, n=8; GIK 1.30±0.38 SEM, n=8) (Fig 33).

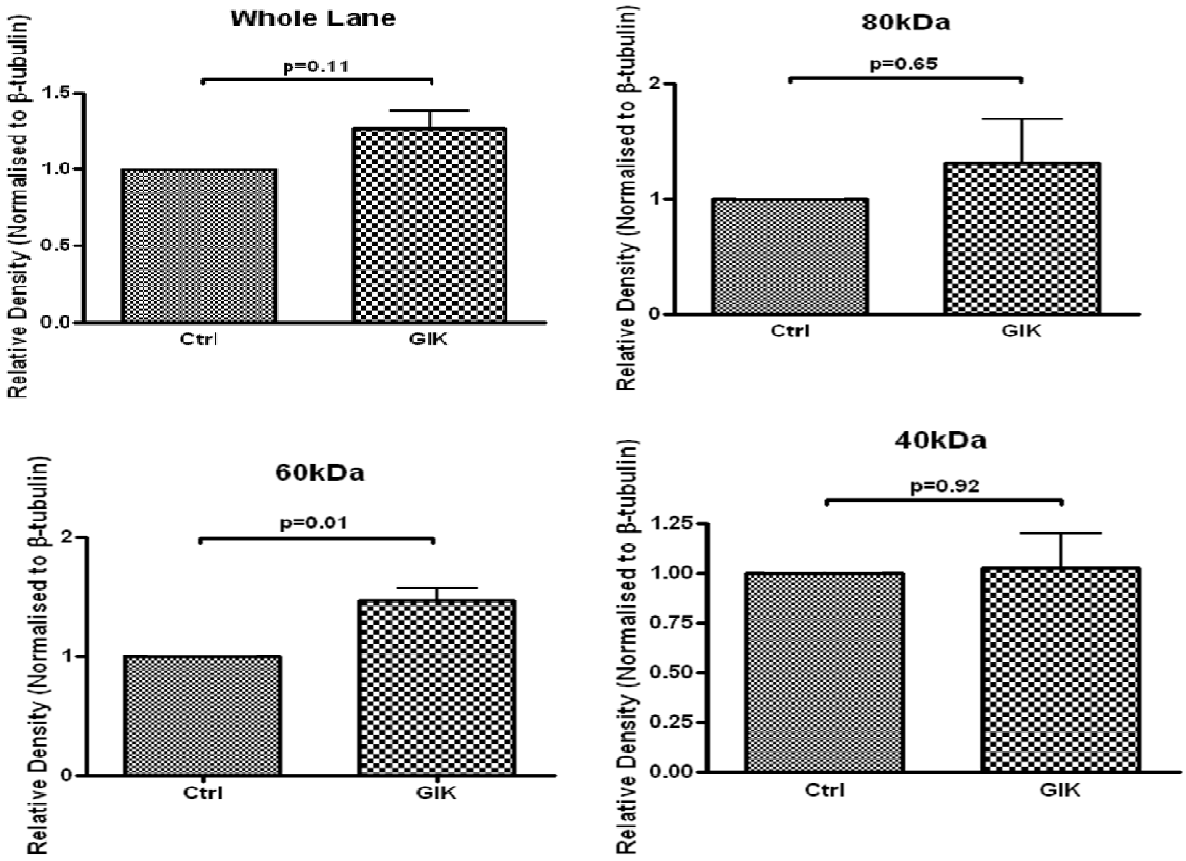

Figure 33 Bar charts describing results of normalised optical densitometry analysis. (n=16)

26. Discussion

The clinical trial presented in Chapter 4 demonstrated that GIK therapy was associated with a significant improvement in haemodynamics and a reduction in low cardiac output episode suggestive of improved myocardial contractility following aortic valve replacement. Recovery from peri-operative myocardial stunning involves normalisation of intermediary and oxidative metabolism resulting in restitution of myocardial energy reserves, reversal of cell swelling, and gradual accumulation of the total adenine nucleotide pool. The more established role for insulin in cardioprotection is through amelioration of myocardial metabolism including a switch from deleterious free fatty acid metabolism to more efficient glucose metabolism. Such a role for GIK has been extensively established in experimental studies [178], however, in addition to the metabolic benefits conferred by GIK, recent studies have adduced a role for pH homeostasis [239], and insulin mediated anti-apoptotic and cardioprotective signalling pathway activation[180]. Thus while it is likely that the main cardioprotective action of GIK is through a shift to more efficient glucose metabolism, we investigated whether myocardial signalling events contributed towards GIK-related myocardial protection.

26.1 The role of insulin on Akt/ GSK 3β signalling with GIK therapy

Insulin, acting through the insulin receptor's tyrosine kinase activity, phosphorylates and activates insulin receptor substrate–1/2, which in turn activate PI3K. PI3K activation generates phosphoinositide-3,4,5 triphosphate, which mediates the phosphorylation and activation of Akt. In cardiomyocytes, Akt has been shown to

protect against apoptosis after ischemia/reperfusion injury [180], and as a corollary, pharmacological inhibition of Akt has been shown to abolish the cardioprotective effect of insulin [180]. To the best of our knowledge, the present study is one of the first to confirm, in accordance with the extensive animal model literature associating Akt /GSK 3β phosphorylation with insulin treatment, that GIK activates this pathway. This in part is likely to confer some of the myocardial protection suggested in Chapter 4.

26.2 AMPK Activation by GIK during reperfusion

In this study we have demonstrated a significant increase in AMPK phosphorylation. AMPK. AMPK activation is known to play a crucial role in the regulation of cardiac energy metabolism, and is thought to be an adaptive mechanism in cardiac ischemia [240]. The question however of whether or not AMPK activation is beneficial during reperfusion remains to be completely elucidated, and the finding that AMPK phosphorylation is enhanced in the context of GIK, may be considered paradoxical [241] This apparent "paradox" is predicated on the observation that insulin, via activation of Akt, inhibits AMPK by phosphorylation of Ser 485/491 of α-AMPK[242, 243]. Reconciling these observations, it appears that while high insulin concentrations, especially in the absence of lipid do indeed inhibit AMPK activity, the inhibitory effect of insulin on AMPK activity is relieved when insulin and lipid concentrations are more physiological (palmitate 0.2 - 1.2 mmol/L) [244]. Accordingly, we propose that GIK in this study was able to activate Akt with its attendant benefits, but suppressed systemic lipid levels to such a permissive level that the beneficial effects of AMPK activation were manifest during ischemia-reperfusion [245].

Recently it has been shown that activation of the survival pathways associated with ischaemic preconditioning is also associated with significant activation of AMPK [114]. This activation is associated with up-regulation of GLUT-4 expression, which occurs in a protein kinase C-dependent manner [115]. The activation of H11 kinase that occurs in preconditioned hearts is also associated with activation of AMPK, therefore resulting in an increase in the transcription of metabolic enzymes such as GLUT-4. Although the activation of AMPK and increases in glucose uptake has the potential to benefit the heart, it has yet to be directly determined whether this activation of AMPK is associated with cardioprotection in the setting of ischaemic preconditioning.

AMPK activation itself has also been shown to induce preconditioning in isolated cardiomyocytes and to protect against hypoxic injury [116], although the degree to which AMPK activation is either required or sufficient to induce conditioning is uncertain. The molecular mechanism behind which AMPK activation might induce preconditioning are complex but may include activation of ATP-sensitive potassium channels [116]. Activation stimulates the movement of these channels from storage membranes to cell surface membranes where they are physiologically active and can shorten the action potential thereby preventing calcium overload during reperfusion.

26.3 Increased Protein O-GlyNAcylation with GIK

We also observed a selective increase in protein *O*-GlcNAcylation. This is the first evidence of *O*-GlcNAcylation in humans and its potential interaction with insulin.

Protein *O*-GlcNAcylation refers to the post-translational modification of serine and threonine residues of nuclear and cytoplasmic proteins by the O-linked attachment of the monosaccharide β-N-acetylglucosamine (*O*-GlcNAc). This modification is emerging as a key regulator of a number of critical biological processes including nuclear transport, translation and transcription, signal transduction and apoptosis and in the settings, activation appears to be an endogenous stress response designed to enhance cell survival [146]. In the isolated perfused heart, ischemia has been shown to increase overall O-GlcNAc levels [148] suggesting that this endogenous stress-activated pathway is active in the heart, and that increased O-GlcNAc levels, improved contractile function and decreased tissue injury after reperfusion [149, 155].

Several mechanisms have been suggested to explain the increased tolerance to stress associated with increased O-GlcNAc levels. Increasing O-GlcNAc levels have been associated with increased transcription of heat shock proteins including HSP40 and HSP70 [147] HSP70 in particular has been identified as a target for GlcNAcylation [150], and activation of O-GlcNAc formation has also been shown to attenuate mPTP opening. There is also some experimental evidence that increasing O-GlcNAc levels inhibit Ca^{2+} overload during reperfusion [155], and that increase in ROS production leads to an increase in O-GlcNAc synthesis [156] via the Hexosamine biosynthesis pathway (HBP), which in turn leads to increase in O-GlcNAcylation of mitochondrial proteins and increased tolerance of mitochondria to stress [151]. Furthermore, there is some experimental data that suggests that inhibition of GADPH by mitochondrial superoxide is an important factor leading to increased O-GlcNAcylation [156] and taken together these studies suggest that ROS and mitochondria may contribute towards

the regulation of O-GlcNAc synthesis which could in turn modulate the response to oxidative stress.

A number of components of the insulin signalling pathway including IR-β, IRS-1/2, PI3K, Akt and GSK-3β have all been reported to be O-GlcNAcylated resulting in reduced activity[148]. Insulin signalling is also regulated by nitric oxide (NO), the production of which could also be regulated by direct O-GlcNAcylation of NO synthetase (NOS). Increasing flux through the HPB pathway is therefore envisaged to decrease NO production not only by direct modification of NOS but also by reducing NOS phosphorylation via O-GlcNAcylation of the IR complex. We therefore speculate that insulin increases GlcNACation by increasing intracellular glucose flux through the HPB pathway thus improving myocardial protection. We speculate that insulin increases *O*-GlcNAcylation by increasing intracellular glucose flux and hence delivers cardioprotection. Which proteins are modified and the specificity of this process remains the subject of investigation.

27. Conclusion

While we present novel human data supporting the role of metabolic modulation, insulin signalling pathway activation (e.g. via traditional pathways such Akt and AMPK activation), and protein O-GlcNAcylation in insulin mediated cardioprotection, this data is preliminary and necessarily only hypothesis generating. Further evidence will be required to ascertain whether this protective pathway is responsible for improved myocardial protection in the context of GIK therapy. It is likely that the main beneficial effect of GIK during ischaemia-reperfusion is to drive more efficient

carbohydrate metabolism. Insulin may exert an additional cardioprotective effect on the cardiomyocyte and the evidence that we present is that this is most likely through increased protein O-GlcNAcylation.

28. Study Limitations

The data on myocardial protein phosphorylation is based upon small (<30mg) biopsies. Although the biopsies are all taken from full thickness of the left ventricular anterior wall, concerns exist that differences in perfusion, particularly in the sub-endocardium could affect the results.

Chapter 6. Study Conclusions and Direction of Future Work

29. Study Conclusions

Over the last decade, the department of cardiac surgery at University Hospital Birmingham has conducted 3 trials of the cardioprotective effect of GIK in patients undergoing cardiac surgery. The first 2 trials were conducted in patients undergoing CABG. These studies demonstrated a significant improvement in haemodynamics as assessed by PAFC thermodilution studies but although there was a trend towards a reduction in the incidence of LCOE, these studies were underpowered to demonstrate this end-point. This HINGE trial was the first trial to demonstrate a significant reduction in the incidence of LCOE. This was not due to LCOE being more frequently diagnosed, indeed the incidence of 30-35% LCOE would appear to be consistent throughout the range of elective adult cardiac surgical procedures in our department, and instead the use of GIK in this group of patients was associated with a significant reduction in this endpoint (10%).

Although patients undergoing both CABG and AVR are subject to almost identical ischemia-reperfusion injury, the two disease processes result in very different pathological states. It has been clearly demonstrated that the hypertrophied ventricle is energetically impaired as demonstrated by a significant reduction in the ATP: PCr ratio. In Chapter 3, we have demonstrated that this energetic abnormality however is the result of significant changes in both the master transcriptional regulators of energy pathway control which results in a degree of change of metabolism that principal component analysis results in complete chemotatic separation of hypertrophied and non-hypertrophied hearts.

There are a number of speculative reasons to account for the improved cardioprotection afforded by GIK in patients with LVH. The mechanisms through which GIK has been hypothesised to work are many and varied including as discussed before, the suppression of lipolysis resulting in a reduction of circulating levels of free fatty acids available for cardiac metabolism, increasing insulin-related glucose flux and increasing myocardial oxygen efficiency, increasing cardiac energy provision through the generation of glycolytic ATP, mitigating the consequences of low myocardial pH, augmenting TCA intermediates (anaplerosis) as well as through insulin's pleotropic signalling properties including its anti-apoptotic properties acting via phosphatidylinositol 3-kinase (PI-3-K) which may directly reduce myocardial injury.

When we examine these potential beneficial actions of GIK in association with the metabolic changes demonstrated in Chapter 3, it becomes apparent that GIK may potentially offer more cardioprotective benefits in the energetically impaired hypertrophied heart than in the heart without limitations in resting coronary flow undergoing CABG. Hypertrophied hearts have been shown *in vivo* to demonstrate insulin resistance, and in Chapter 3 we demonstrate a significant reduction in GLUT-4 the key glucose transport transcription mechanism. GIK may therefore play a substantial role in increasing glucose flux resulting in increased glycolytic ATP production via both a direct action of insulin and an indirect action on lipolysis. The abnormal metabolome we have shown may also derive greater benefit from increased anapleurosis which may well reduce the impairment in energy production during the key period of reperfusion.

The role of insulin's pleotropic signalling and anti-apoptotic properties acting via phosphatidylinositol 3-kinase (PI-3-K) have also been hypothesised to reduce myocardial injury. We have demonstrated a significant increase in AMPK and Akt / GSK-3β signalling. Presumably the activation of this cardio-protective signalling pathway is equally prevalent in both hypertrophied and non-hypertrophied hearts. It may therefore be speculated that whilst GIK has a direct and beneficial cardioprotective effect on hearts undergoing ischemia-reperfusion, energetically impaired hearts may derive additional metabolic benefit. This may explain the difference in magnitude of results obtained in our series of GIK trials.

This speculation raises a number of further questions which future work will attempt to address. If GIK has a significant effect on glucose flux, glycolytic ATP production and anaplerosis then these changes may be measured by further metabolomic analysis. Even small insignificant changes in metabolite concentration, if present throughout the numerous metabolic pathways, may result in a significant change in metabolome on principal component analysis.

If the metabolome of the hypertrophied cell can be acutely manipulated by GIK, it may be possible to chronically improve metabolism over a number of weeks or months prior to surgery. This would have several potential advantages. Chronic improvements in metabolism over such a time period could potentially near-normalise cardiac energetics prior to surgery, rather than simply attempting to acutely improve metabolism during the time of ischemia-reperfusion. Employing GIK as part of a standard cardioprotective strategy is labour intensive. It is associated with

vasodilatation, increased vasoconstrictor requirements and hyperglycaemia. Although in our trials these were not associated with adverse outcomes they were intensively managed by research fellows aware of these potential complications. The use of such a metabolic agent rather than a metabolic cocktail that contains agents such as insulin which itself has been demonstrated to have a cardioprotective effect is also useful from a scientific point of view. It may allow comparison of the effect of one particular mode of action; metabolic manipulation, rather than attempting to speculate upon which action or combination of actions has produced the desired effect. It may also demonstrate that a cardio-protective from metabolic manipulation is minimal, and that the other direct actions of insulin are of greater importance.

There are a number of pharmacological agents, including Perhexiline and Trimetazidine, which may exert a similar glucose sparing metabolic effect, without these other manifestations. Perhexiline has a license from use in the UK and is currently being trialled in the context of heart failure in this department and it was therefore elected to examine its use in a further trial.

30. Limitations of the GIK studies

The main problem we have encountered in running these studies is the validity of using a diagnosis of LCOE as a marker for myocardial stunning. The exact mechanism behind myocardial stunning remains unclear as is the reason why some patients with very similar pathologies and very similar ischaemia-reperfusion injuries manifest such different requirements for inotropic support following surgery. It has been hypothesised that fundamental genetic traits determine our own individual

response to such stresses but pilot work by our group and others have failed to take this concept forward.

The diagnosis of LCOE and all the haemodynamic data are derived from thermodilution measurements of cardiac output with a pulmonary artery catheter. Although this is a well validated technique, it remains both dependent on preload and afterload. Whilst in our trials, every effort was made to try and achieve similar filling pressures and resistance with targeted and protocol driven filling and vasoconstriction, in all our trials we see a reduction in the SVRI, and a mode of action dependent on vasodilatation cannot therefore be excluded.

Ultimately, to demonstrate a beneficial effect of GIK as an adjunct to myocardial protection a reduction in mortality will have to be demonstrated, either peri-operative mortality with large multi-centre trials or improved late outcome by following this cohort of patients up. Whilst the logistical difficulties associated with GIK mean a multi-centre trial may not be feasible, late follow up of this cohort may further our understanding of its potential benefits.

31. Metabolome changes in ischaemia-reperfusion

Just as LV hypertrophy results in an abnormal metabolome we hypothesise that ischaemia-reperfusion will result in similar changes in intermediate metabolites and high energy phosphates. The use of FT-ICR may therefore allow us to quantify these changes and to evaluate the effects of GIK on myocardial protection in patients undergoing cardiac surgery.

Before this study can take place a number of steps are required to optimise the methodology. These were designed as:

Phase 2A - dilution optimisation

Phase 2B - confirmation that mass spectrometric analysis can be conducted across multiple measurement days

Phase 2C – study of cardiac samples

We have subsequently carried out Phase 2A but not without difficulty. Although in principle this was an easy experiment to conduct, it was dependent upon there being a clear metabolic difference between the biopsies at the set time-points, and more importantly that this difference was statistically significantly when using a small number of cardiac samples. Unfortunately the study we planned and conducted comprised too few biological replicates per dilution. Although this gave us a little insight into optimal dilution, it was not successful at answering the question with sufficient rigour. Consequently, phase 2A was redesigned to include more biological replicates for each of only two dilutions. This experiment proved successful, and we have concluded that a 1:5 dilution of the samples would yield high quality mass spectra, with considerable number of peaks/metabolites detected, but with low technical variation. This phase is now complete.

The planned analysis of multiple cardiac biopsies will analyse more samples than has ever been analysed in a single metabolomics study. That number of samples cannot be measured in one "run", so Phase 2B has been designed to evaluated how

best to collect the data such that it can be properly combined and then subject to statistical analysis. Although we are in principle ready to proceed with phase 2B, we have secured support from the University to upgrade the FT-ICR mass spectrometer FT-ICR Ultra. This new spectrometer has higher sensitivity, mass accuray and resolution but this upgrade will delay the start of this phase of the experiment until the end of 2010 with analysis of cardiac biopsies following completion of this work.

32. Defining myocardial metabolome in patients with LVH

FT-ICR spectroscopy with the ability to measure thousands of individual metabolites based upon the charge to mass ratio was used in this research to identify the abnormal metabolome in patients with left ventricular hypertrophy, consisting of changes in both high energy phosphates and metabolite pathway intermediates. Having demonstrated the ability of metabolomics to discover such perturbed molecular pathways, one of the next steps is to construct a more extensive library of metabolic pathways. Mining that more comprehensive dataset could then provide a definitive series of metabolic pathways. Rapid targeted analyses using multiplexed LC-MS approaches could then be used to screen thousands of chemical for disruption of these multiple pre-specified pathways.

33. Improving the myocardial metabolome in patients with LVH

It has been hypothesised that the energetic impairment associated with LV hypertrophy and failure predisposes the myocardium to injury during cardiac surgery.

Work presented in this thesis correlates severe hypertrophy with raised plasma Troponin levels following surgery and impaired contractility. Chronic metabolic therapy in the weeks before surgery may therefore improve the metabolome of patients with LV hypertrophy and decrease the vulnerability of the myocardium to injury.

There are a number of metabolic agents that have been shown in small series to improve myocardial metabolism in patients with hypertrophy and failure. Perhexiline maleate is an anti-anginal drug that inhibits the enzymes carnitine-palmitoyl-transferase-1 and -2 (CPT 1 and 2), shifting substrate utilisation from FFA towards glucose. Perhexiline has no direct negative inotropic effects and does not alter systemic vascular resistance at plasma levels within the therapeutic range. Randomised controlled trials have demonstrated that perhexiline exerts marked and incremental anti-anginal effects in patients receiving β–blockers or "triple" anti-anginal therapy. A recently completed study in patients with severe chronic heart failure, demonstrated that the addition of perhexiline to standard medical treatment was associated with substantial improvements in VO_2 max, quality of life and LV function, particularly in patients with both ischaemic and non-ischaemic heart failure.

The potential side effects of long term perhexiline therapy (neuropathy and hepatotoxicity) occurring in slow metabolisers can be prevented by monitoring and maintaining the plasma levels within the therapeutic range (0.15 - 0.60 $mg.L^{-1}$) and the safety of the drug has been demonstrated in a randomised, double blind crossover study.

We therefore hypothesise that pre-operative treatment of patients with LV hypertrophy secondary to aortic stenosis undergoing planned AVR with Perhexiline maleate would improve myocardial energetics as defined by the metabolome assessed by FT-ICR spectroscopy toward that of the non-hypertrophied ventricle and improve myocardial protection.

Authorisation to conduct this trial was obtained by the Medicines and Healthcare Regulatory authority and from the Central Office for NHS Research Ethics, UK. An application made to the British Heart Foundation for a 3 year project grant to conduct this trial was successful. A research fellow has been appointed and trial recruitment began in January 2010.

Chapter 7. Appendixes

34. Appendix A - The AHA Indications for Aortic Valve Replacement

Class I

AVR is indicated for symptomatic patients with severe AS (Level of Evidence B)

AVR is indicated for patients with severe AS undergoing coronary artery bypass graft surgery (CABG). (Level of Evidence C).

AVR is indicated for patients with severe AS undergoing surgery on the aorta or orther heart valves. (Level of Evidence C).

AVR is recommended for patients with severe AS and LV systolic dysfunction (EF <0.50) (Level of Evidence C).

Class IIa

AVR is reasonable for patients with moderate AS undergoing CABG or surgery on the aorta or other heart valves. (Level of Evidence B)

Class IIb

AVR may be considered for asymptomatic patients with severe AS and abnormal response to exercise. (Level of Evidence C)

AVR may be considered for adults with severe asymptomatic AS if there is a high likelihood of rapid progression (age, calcification, and CAD) or if surgery might be delayed at the time of symptom onset. (Level of Evidence C)

AVR may be considered in patients undergoing CABG who have mild AS when there is evidence such as moderate to severe calcification that progression may be rapid. (Level of Evidence C).

AVR may be considered for asymptomatic patients with extremely severe AS (aortic valve area less than 0.6cm^2, mean gradient >60mmHg and jet velocity >5.0ms^{-1}) when the operative mortality is 1% or less. (Level of Evidence C)

Class III

AVR is not useful for the prevention of sudden death in asymptomatic patients with AS who have none of the findings listed under the class IIa/IIb recommendations. (Level of Evidence B)

35. Appendix B Patient Information

PATIENT INFORMATION SHEET

Metabolic and Substrate Support in Left Ventricular Hypertrophy – The HINGE Trial

An invitation to participate in research.

The heart surgery team at the Queen Elizabeth Medical Centre is inviting patients to participate in research aimed at making aortic valve surgery safer. We would like to recruit patients undergoing this kind of surgery into a clinical trial where potentially advantageous methods of altering the way we treat patients are used to see what differences they actually make to the patient's progress. We include a simple and non-technical summary of the reasons for the trial and what it involves for patients over and above their routine treatment if they take part. If you are being approached in the pre-admission clinic we would like you to take these information sheets provided home with you to read and consider participating in this trial between now and your admission date at which time a heart surgical research doctor will be available to discuss the trial further and answer any questions. You can only be included in this clinical trial if you give your express permission in the form of signed consent. If you are already an inpatient on the ward then a member of the heart surgical team will be happy to discuss the trial with you once you have had at least 24 hours to read through these information sheets.

PATIENT INFORMATION SHEET

Metabolic and Substrate Support in Left Ventricular Hypertrophy – The HINGE Trial

What is the study about?

This study is about trying to improve the treatment of patients undergoing surgery on their aortic valve. A heart operation to replace the aortic valve requires the help of a heart-lung machine. This supports your body's circulation whilst the surgeon replaces the defective aortic valve. During your surgery the heart has to be protected so that it is not injured by the strain of the operation. Despite using standard techniques to protect the heart some temporary injury still occurs from which the heart gradually recovers during the first few hours and days following operation. The aim of this study is to see whether by the use of a special sugar drip we can improve the protection of the heart during the valve surgery.

How has this clinical trial been designed?

This study is what is called a double-blind randomized placebo-controlled trial. This means that if you agree to take part you would be allocated by chance to receive either a sugar solution containing glucose, insulin and potassium (GIK) or a placebo – a standard weak sugary drip. The code is broken once all the patients in the trial have been completed.

How will we measure the effects of this treatment?

To detect changes in heart function we need to take measurements of performance before, during and after surgery to show that the treatments that are used have made a difference. To do this we will make use of small monitoring catheters (plastic tubes)

inserted in blood vessels that are routinely used in many heart surgical procedures. These catheters are placed in position in blood vessels whilst you are under anaesthetic and are usually removed on the first or second post operative day. We will record measurements from these catheters throughout this period at specific time points. We will insert an additional sampling catheter during your operation to monitor blood in the veins of your heart. This will be removed at the end of the use of the heart lung machine. Again such catheters or plastic tubes are in routine use in many heart operations. In your case we will insert these catheters to measure heart function and take samples from them to provide the research team with additional information on how your heart is working.

Measuring the possible beneficial effects on the heart's metabolism

The sampling of additional blood tests: the blood tests performed on these samples can tell us how well the heart is tolerating the surgery. These blood samples are removed through the same pressure measuring lines already mentioned. The total amount of additional blood taken for this study is about a cupful.

Imaging the heart using a transthoracic echocardiogram: An Echocardiogram is a scan that uses ultrasound (sound waves) to produce pictures of the heart. The test is painless and without side effects. An ultrasound probe and a small amount of gel are gently placed on your chest and will be moved to different positions. The scan will take approximately 20 –40 minutes to complete and will be performed once before surgery and once after.

The removal of tiny samples of the heart muscle known as biopsies: This will help tell us how the treatment improves the way the heart works. We would aim to obtain 3 heart muscle biopsies all whilst you are asleep under the anaesthesia, one at the beginning, middle and end of the bypass. They will be performed by the operating surgeon and are very small, about so long and thin (==). A suture is placed in the tiny defect left behind and their removal incurs no increased risk during the surgery nor has any effect on the strength of the heartbeat. We have performed on more than 1500 biopsies in patients at the time of writing of this information leaflet with no complications.

What will I have to do?

If you decide to participate then there are a number of stages to the study. You will be guided through these stages by us (the research doctors looking after you).

You will by now have received the written relevant information and asked to read this at your leisure at home or on the ward if you are already an inpatient. On the day prior to your surgery a member of the research team will visit you so you can ask any questions. If you give signed consent to participate then we will take the opportunity to perform a transthoracic echocardiogram (20 minutes) on the day before surgery.

In the anaesthetic room of the operating theatre the anaesthetist will give you a general anaesthetic. Once you are asleep the anaesthetists will insert the pressure measuring catheters normally inserted at this stage. The operation will be conducted in a normal way under the care of the consultant cardiac surgeon, who is in charge of your case. The blood samples and biopsies will be performed while you are asleep

and you will be unaware of them. The sampling of blood from the veins of the heart will be performed whilst you are asleep during the use of the heart-lung machine only.

When you awake you will be on the intensive care unit as routine. Only some further blood tests and monitoring tests will remain. The last blood test will be performed 48 hours after the operation.

In summary we will be taking measurements from pressure measuring catheters that are routinely used in heart surgery. The entire sampling of blood for the research will total 160 ml and will not affect your recovery and, because we can use intravenous pressure measuring lines, there will be no additional need for needles. The biopsies that we take are removed painlessly during the operation and have no effect on the heartbeat.

What are the benefits?

We do not know whether the treatment will benefit you as an individual. This study is investigating that very question. It is hoped that this study will provide information that will benefit future populations of patients undergoing cardiac surgical procedures. In particular it will help us design more suitable treatments for patients who have a very weak heartbeat before surgery and are at considerable risk of complications after surgery. In the future and after more research the use of this tablet could make all the difference in their recovery.

What are the risks?

All heart operations carry some risk and these will have already been discussed with you. For this study we insert routinely used monitoring lines to measure heart function. The risks of this are minimal and the possibility of a severe complication is in the region of 1 in 15000. The only additional invasive procedure performed on you is the biopsy.

What are the alternatives?

If you do not wish to take part in the study, your surgery will be undertaken in the standard manner without any additional measurements, treatments or tests. Your surgeon and anaesthetist may still use the monitoring lines that we have described if they feel that their use is in your best interest.

What happens to the information?

The information from the study will be analysed. The information will be presented at scientific meetings and published in scientific journals to inform other doctors and health professionals of our findings. All data is confidential and stored by code on computer. This ensures that your identity will not be revealed at any time.

Who else is taking part?

We will recruit 220 patients to this study.

What if something goes wrong?

The standard care of patients undergoing heart surgery involves intensive monitoring. This monitoring allows us to detect any problems early in their development. We do not expect the study itself to cause any problems, however as

for all heart surgery we are in an ideal position to deal with any untoward events during your operation and these will be treated in the normal manner, regardless of the research study. At the time of all the measurements you will be in the theatre or I.T.U. where trained staff is at hand at all times. Your safety during and after surgery is paramount, and takes precedence over any research.

What happens at the end of the study?

At the end of the study all test infusion will stop and treatment will continue as would that of a patient who had not been involved with the study.

What if I have more questions or do not understand something?

Please feel free to ask one of the investigators about any questions or worries that you may have so that any points can be clarified. You should feel free to ask questions at any time you like. Contact number 0121 472 1311 ask QE switch board to page Mr Neil Howell or if on the ward ask the nurses to contact either Mr Howell for you.

What happens now if I decide to take part?

We will take some details and ask you to sign a consent form that documents your willingness to participate. You will be listed for surgery as normal. You are free to withdraw from this study after initially consenting without prejudice to your continuing care.

36. Appendix C Consent Form

Metabolic and Substrate Support in Left Ventricular Hypertrophy

- The HINGE Trial

This is a consent form that documents your agreement to participate in this particular research. Before signing this form please tick the boxes below.

I have read the invitation form	☐
I have read the information sheet	☐
I have understood the nature of the research	☐
I have had time to consider my response/reply	☐
I have been able to ask any questions and am satisfied with the answers	☐
I understand that I can withdraw at any time without giving explanation	☐
The participation or lack of it in this research will not affect my clinical care in any way	☐

Please remember that there is no obligation to participate in this study and that even if you do you can withdraw at any time. If you do not consent to this study your operation and care will proceed as planned. There are no rewards or penalties for participating in research although we are grateful for your efforts and inconvenience. If after reading this consent form and the patient information sheet you agree to participate please sign the form below and return to **Mr Neil Howell**. If you have any further questions about any aspect of the research feel free to ask **Mr Neil Howell**.

The study has been explained by:

Print Name:

Signature: Date:

Please sign below if you agree to participate in this study

I have read and understand the above, and I am happy to take part in the above study.

Signed Name (Print) Date

37. Appendix D Management of Glucose

Target glucose range of 4.0-10.0mmol.l^{-1}

Insulin Sliding scale: 50IU of insulin actrapid in 50ml normal saline

Sliding scale for use in operating theatre, prior to removal of the AXC

Blood glucose	Units/hr
0-10.0	0
10.1-15.0	5 (plus additional 4 unit bolus if required)
≥ 15.1	10 plus additional 8 unit bolus if required)

Sliding scale for use in operating theatre and on ICU in the first six hours following removal of the AXC

Blood glucose	Units/hr
0-10.0	0
10.1-15.0	5
≥ 15.1	10

Sliding scale for use in the six to 12 hour period following removal of the AXC

Blood glucose	Units/hr
0-10.0	0
10.1-14.0	2-4

14.1-18.0	6
18.1–22.0	8
22.1–26.0	12
≥ 26.1	14

Management of hypoglycaemia

Hypoglycaemia for the purposes of this study was defined as a blood glucose level less than 4.0mmol.l^{-1}. This requires a bolus of 25mls of 50 % dextrose. Blood glucose measurement should be repeated after 30 minutes.

38. Appendix E - Guidelines for extubation and discharge criteria

Guidelines for Weaning from the Ventilator

Adequate spontaneous respiratory rate of 8-18 breaths per minute

Core temperature > 35.5°C (nasopharyngeal probe)

PaO_2> 10kPa on an FiO_2 of ≤ 0.5

$PaCO_2$ < 7kPa

pH> 7.30

Haemodynamic parameters met as set by patient's clinical team

Acceptable safe limits of chest tube drainage < 100ml.h^{-1} blood for two hours.

Stable rhythm

Criteria for Extubation

Co-operative

Adequate spontaneous respiratory rate of 8-18 breaths per minute

Core temperature > 35.5°C (nasopharyngeal probe)

PaO_2 > 10kPa on an FiO_2 of ≤ 0.5

$PaCO_2$ < 7kPa

pH > 7.3

Haemodynamic parameters met as set by patient's clinical team

Acceptable safe limits of chest tube drainage < 100ml.h^{-1} blood for two hours.

Stable rhythm

Criteria for discharge from ITU

- Good pain control at least 60 minutes after extubation
- Acceptable safe limits of chest tube drainage < 100ml.h^{-1} blood for two hours.
- Cardiac index ≥ 2.2l.min^{-1}.m^{-2} on ≤ 10µg.kg^{-1}.min^{-1} dopamine
- Adequate ventilation
- Adequate urinary output > 0.5ml.kg.h^{-1}

Criteria for discharge from the ward

- Alert
- Orientated
- Temperature < 37.5 °C for 24 hours
- Walk for 30 m and up one flight of stairs
- Wounds manageable at home

40. Appendix F - Stacking Gel Mixes & Buffers

	6%	7.5%	9%	10.5%	12%	15%
30% Acrylamide 0.8% Bisacylamide	1.0	1.25	1.5	1.75	2.0	2.5
2x TRIS HCl/SDS pH 8.8	2.5	2.5	2.5	2.5	2.5	2.5
Water	1.5	1.25	1.0	0.75	0.5	0

Then add 10 µl TEMED + 30 µL Ammonium Persulphate (APS)

SDS-PAGE Separating Gel Buffer

45.5g Tris-Base in 350ml dH_20 adjusted to pH 8.8

Made up to 490 ml with dH_20. Filtered. Add 10ml 20% SDS.

SDS-PAGE Stacking Gel Buffer

19.75g Tris-HCl in 200 ml dH_20 adjusted to pH 6.8

Made up to 500 ml with dH_20. Filtered. Add 10ml 10% SDS.

41. Appendix G - Primary Antibodies

Antibodies	Source	Dilution	Supplier	Cat. No.
O-GlcNAc				
CTD 110.6	Mouse	1:2000	Covance	MMS-248R
Anti-mouse IgM	Goat	1:5000	Sigma	A8786
β-tubulin	Rabbit	1:2000	Abcam	ab6046
Anti-rabbit IgG	Donkey	1:2000	GE Healthcare	NA934
Kinases				
AKT	Rabbit	1:1000	Abcam	ab8805
Phospho-AKT (Ser473)	Rabbit	1:1000	Abcam	ab28821
AMPKα1/2	Rabbit	1:1000	Santa-Cruz	sc-25792
Phospho-AMPKα (Thr172)	Rabbit	1:1000	Santa-Cruz	sc-33524
GSK-3β	Mouse	1:1000	Sanat-Cruz	sc-56911
Phospho- GSK-3β	Mouse	1:1000	Sanat-Cruz	sc-81494
GAPDH	Rabbit	1:1000	Abcam	ab9485
Anti-rabbit IgG	Goat	1:4000	Abcam	ab6721
Anti-mouse IgG	Goat	1:4000	Abcam	ab20043

42. Appendix H - Equations for calculation of derived variables

Systemic vascular resistance indexed

$$SVRI = \frac{(MAP-CVP)}{CI} * 80$$

Left ventricular stroke work indexed

$$LVSWI = \frac{(MAP-PAWP)*CI}{HR} * 0.0136 * 1000$$

Cardiac power output

$$CPO = CO*MAP*0.00222$$

Systemic oxygen delivery indexed

$$O2\ delivery = 0.134*CI*Hb*SaO_2$$

Systemic oxygen consumption indexed

$$O2\ consumption = 0.134*CI((Hb*SaO2)-(Hb*MvO2))$$

43. Appendix I Acknowledgements

Principle Investigator

This work was supervised by Mr Domenico Pagano, Consultant and Reader in Cardiothoracic Surgery

Consultant Surgeons

Mr Domenico Pagano

Mr Jorge Mascaro

Mr Timothy Graham

Mr Robert Bonser

Mr Stephen Rooney

Mr Ian Wilson

Consultant Anaethetists

Dr Mark Wilkes

Dr Deborah Turfrey

Dr David Green

Dr Peter Townsend

Dr Mustafa Faroqui

Dr Tessa Oelefsa

Dr Peter Lilley

Dr David Riddington

Additional Patient Recruitment

Mr Nigel Drury

ECG & Echocardiographic Analysis

Dr Lynne Williams, Clinical Lecturer Cardiovascular Medicine, University of Birmingham

Genomic and Proteomic Analysis

This was performed at the Wellcome Centre for Human Genetics under the supervision of Dr Houman Ashrafian and Professor Hugh Watkins

Metabolomic Analysis

This was performed in the School of Biosciences, University of Birmingham under the Supervision of Dr Mark Viant. Dr Lisa Bishop performed the spectral processing.

Statistical Analysis

Pre-determined primary and secondary end-point analysis was performed by Dr Melanie Calvert and Professor Nick Freemantle in the department of School of Health and Population Sciences, University of Birmingham

44. Appendix J - Glucose–Insulin–Potassium Reduces the Incidence of Low Cardiac Output Episode Following Aortic Valve Replacement for Aortic Stenosis in patients with Left Ventricular Hypertrophy. Results from the HINGE (Hypertrophy, Insulin, Glucose & Electrolytes) Trial

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Circulation; In press

Abstract

Background

Patients undergoing aortic valve replacement (AVR) for critical aortic stenosis (AS) often have significant left ventricular hypertrophy (LVH). LVH has been identified as an independent predictor for poor outcome following AVR, due to a combination of maladaptive myocardial changes and inadequate myocardial protection at the time of surgery. Glucose-insulin-potassium (GIK) is a potentially useful adjunct to myocardial protection, this study was designed to evaluate the effects of GIK infusion in patients undergoing AVR surgery.

Methods and Results

Patients undergoing AVR for AS with evidence of LVH were randomly assigned to GIK or placebo. The trial was double blind and conducted at a single centre. The primary outcome was the incidence of low cardiac output syndrome. Left ventricular biopsies were analysed to assess changes in 5' adenosine monophosphate-activated protein kinase (AMPK) and Akt phosphorylation; and protein O-linked beta N-acetylglucosamination (O-GlcNAcylation). 217 patients were randomised over a four year period (107 control; 110 GIK). GIK treatment was associated with a significant reduction in the incidence of low cardiac output state (OR 0.22 95% CI 0.10 to 0.47, p= 0.0001), and a significant reduction in inotrope use 6 to 12 hours postoperatively, (OR 0.30 95% CI 0.15 to 0.60) p=0.0007. These changes were associated with a substantial increase in AMPK and Akt phosphorylation and a significant increase in the *O*-GlcNAcylation of selected protein bands.

Conclusions

Peri-operative treatment with GIK was associated with a significant reduction in the incidence of low cardiac output state and the need for inotropic support. This benefit was associated with increased signalling protein phosphorylation and O-GlcNAcylation. Multi-centre studies and late follow up will determine if routine use of GIK improves patient prognosis.

Clinical Trial Registration Information

This trial was registered with the International Standardised Randomised Controlled Trial Number Register (ISRCTN) Reference ISRCTN 05758301.

http://www.controlled-trials.com/ISRCTN05758301/05758301

Key Words

Aortic valve replacement; Hypertrophy; Glucose; Insulin

Introduction

The natural history of aortic stenosis (AS) is characterized by a protracted latent period accompanied by the development of left ventricular hypertrophy LVH as a compensatory mechanism to reduce wall stress. Although the operative mortality for AVR in the UK has remained approximately 3% despite an increasing elderly population with a greater risk profile [208], the presence of LVH is associated with an increased risk and severity of post-ischemic myocardial injury and contractile dysfunction post AVR [246], of post-surgical complications including respiratory failure, renal failure, heart failure and arrhythmias [247], and has been identified as an independent predictor of in-hospital mortality following AVR [248]. The increased risk associated with LVH is, at least in part, attributable to inadequate myocardial protection due to a combination of inadequate delivery of cardioplegia through a less dense capillary bed [249], increased diffusion distance for oxygen / metabolites from capillary to myocytes [250] impaired myocardial metabolism characterised by a shift away from free fatty acid utilisation [65], uncoupling of glycolysis and glucose oxidation [84] and an increase in expression of mitochondrial uncoupling proteins [251] resulting in a reduction in the PCr:ATP ratio [58]. The resultant early post-ischemic left ventricular dysfunction may not only prejudice late reverse remodelling of LVH following AVR and reduce the prognostic benefits of surgery, but also predicts the potentially important role of metabolic interventions.

Although the cardioprotective properties of glucose-insulin-potassium (GIK) remain controversial, in part due to the different contexts, doses, timing and protocol of GIK employed in different studies, there is a growing consensus supporting GIK's utility in cardiac surgery [190, 196]. Multiple complimentary mechanisms may contribute to GIK related myocardial protection: (i) suppression of lipolysis resulting in a reduction of circulating levels of free fatty acids available for cardiac metabolism [93, 104, 107, 175] ; (ii) increasing insulin-related glucose flux and increasing myocardial oxygen efficiency, increasing cardiac energy provision (i.e. glycolytic ATP) [174] which may augment myocardial glycogen levels [40, 173, 176] and mitigate the consequences of low myocardial pH [99]; (iii) beneficial augmentation of TCA intermediates (anaplerosis) [178]; and (iv) through insulin's pleotropic signalling properties including its anti-apoptotic properties acting via phosphatidylinositol 3-kinase (PI-3-K) which may directly reduce myocardial injury [180]. Activation of a cascade of what have been termed "Reperfusion Injury Salvage Kinases" such as PI-3K/protein kinase B (Akt) and AMP-activated protein kinase, which have an established role in insulin signalling[237], may have a role in cardioprotection[238]. In addition to these established post-translational

modifications (e.g. phosphorylation), insulin may promote modification of serine and threonine residues in proteins by O-linked beta-N-acetylglucosamine (O-GlcNAc)[157]. However the pertinence and contribution of these mechanisms in clinical context, especially in human LVH, remains speculative and unclear.

Although the results of AVR continue to improve, some patients exhibit a low cardiac output episode (LCOE) characterised by a period of left ventricular dysfunction requiring inotropic support. In some patients, this dysfunction will be severe and prolonged, and the morbidity and mortality associated with LCOE continues to be substantial [252]. In patients undergoing AVR with LVH, LCOE has been identified as the most common cause of death [248]. In addition to increasing resource allocation, in the long-term, myocardial injury sustained during surgery may result in heart failure. The need to improve myocardial protection in patients with LVH is well recognised and may reduce mortality, morbidity and the resource implications of heart surgery. We report herein, a single-centre prospective double-blind randomised controlled trial to investigate the effect of GIK on the incidence of LCOE in patients with aortic stenosis and LVH undergoing aortic valve replacement (AVR), either in isolation or in combination with coronary artery bypass grafting (CABG). To test the hypothesis that any cardioprotection delivered by GIK is contributed to by rapid and diverse post-translational modifications, we assessed any changes in established insulin-related signalling molecules Akt and AMPK, and any increase in protein O-GlcNAcylation in left ventricular biopsies from GIK treated patients in relation to controls.

Methods

Study Design

We performed a prospective, single centre, double blind, randomised placebo controlled trial of GIK in patients undergoing isolated AVR ± CABG with echocardiographic evidence of LVH as defined by a left ventricular mass index greater than 134 $g.m^{-2}$ for men, or 100 $g.m^{-2}$ for women[205, 206]. The study was approved by the South Birmingham Research Ethics Committee (REC reference 04/Q2707/23), and the Hospital Trust Board of Research. The trial was registered with the International Standardised Randomised Controlled Trial Number Register (ISRCTN) Reference ISRCTN 05758301. Patients were enrolled between October 2004 and June 2008. All research was performed in accordance with the Declaration of Helsinki within a Research Governance framework.

Surgery, Anaesthesia, Cardiopulmonary Bypass and Myocardial Protection

Anaesthesia, cardiopulmonary bypass (CPB) and myocardial protection utilizing intermittent antegrade cardioplegia using St Thomas' solution buffered in cold blood, were all standardised as previously described [196], except that Aprotinin (Bayer) was used in all patients as per departmental blood conservation strategy during this time, Phenylepherine was used as the first line vasoconstrictor, and patients were cooled to 32^{o}C on bypass. Surgical technique was also standardised. In patients undergoing concomitant revascularisation, the distal anastomoses of all free grafts were first performed. Proximal graft anastomoses were performed during a period of partial aortic clamping. If the ascending aorta was dilated then it was replaced with a Gelweave™ interposition graft (Vascutec Terumo, Scotland).

End-points

The primary outcome was the incidence of low cardiac output state (LCOE). This was defined *a priori* as CI of less than 2.2l.min^{-1}.m^{-2} refractory to appropriate intra-vascular volume expansion following correction or attempted correction of any dysrhythmias [253]. In the case of inotropic support being instituted due to a LCOE, a blinded end-points committee assessed all data. In order to qualify as a LCOE episode a unanimous verdict was sought but in the event of disagreement, contentious cases were discussed and a consensus opinion agreed. Secondary end-points included comparison of cardiac index (CI) and the use of inotropes and vasoconstrictors. Peri-operative myocardial infarction (PMI), assessed by an independent blinded cardiologist, was defined by the presence of new Q-waves ≥2mm in ≥2 contiguous leads by postoperative day 4.

Statistical Analysis

All pre-specified analyses were conducted according to the intention-to-treat principle. The study had a statistical power of 80% to identify a relative risk of 0.50 statistically significant given an incidence of LCOE in the control group of 0.37 and a conventional one-sided α value of 0.025. Analyses were conducted with the use of SAS software (version 9.1, SAS Institute). P values other than for the primary end point are nominal. Dichotomous outcomes were analysed with the use of nonlinear mixed models, which included CABG as a patient-level covariate and surgeons as random effects. Continuous data were analysed with the use of mixed models, which included CABG as a patient-level covariate and

surgeons as random effects. [215] The authors had full access to the data and take responsibility for their integrity. All authors have read and agree the manuscript as written.

Results

Study population

A total of 220 patients were eligible for randomisation, however, in three patients, a pulmonary artery flotation catheter could not be placed, so they were excluded from the study. In total 107 patients were randomised and received the control infusion and 110 were randomised and received the GIK infusion (Figure 1). Baseline pre-operative characteristics were similar between groups (Table 1). There were no differences in demographics and risk profile between the groups with a median EuroSCORE in the GIK arm of 4.4 (interquartile range 2.7 to 7.1) and 4.5 (IQR 2.9 to 7.7) in the control arm. 191 patients had lone AS and 29 had mixed aortic valve disease of which the predominant lesion was AS. All patients had pre-operative transthoracic echocardiography (Table 1). 146 patients were scheduled for isolated AVR and 75 patients were scheduled for concomitant CABG. Of the 75 patients listed for additional CABG, 2 did not receive this additional procedure due to small coronary artery size. Seven patients underwent additional procedures. One underwent mitral valve annuloplasty. One patient was found to have a structurally normal and non-calcified aortic valve and the outflow tract obstruction was due to an undiagnosed sub-aortic membrane, which was resected. Five patients underwent ascending aortic replacement due to post-stenotic dilatation. Median bypass and cross clamp times were similar in both groups as were the number of bypass grafts required. Valve choice was at surgeon discretion and a wide variety of prostheses were used.

GIK administration is associated with hyperglycaemia, therefore in this trial all patients were closely monitored with hourly blood glucose measurements throughout the study period. Patients with hyperglycaemia were treated aggressively with supplemental insulin aiming for a blood glucose <10mml.$l^{-1.}$ Despite this approach patients randomised to GIK demonstrated a rise in blood glucose which was maintained throughout the infusion period (mean reperfusion glucose 11.8 mmol.l^{-1} GIK vs. 7.3 mmol.l^{-1} control, p=0.01). Once the period of GIK infusion had terminated blood glucose levels were similar between groups (mean glucose 7.3 mmol l^{-1} GIK vs. 7.7 mmol.l^{-1} control, p=0.78). (Figure 2). GIK infusion was accompanied by a similar rise in insulin levels to over four times baseline (mean

reperfusion 478 $\mu U.ml^{-1}$ GIK vs. 66 $\mu U.ml^{-1}$, p=0.0001) which returned to baseline following discontinuation of GIK therapy. The high level of insulin associated with GIK also lead to suppression of plasma free fatty acids throughout ischemia and the six hour period of reperfusion during which GIK was infused (mean reperfusion 483 $\mu U.ml^{-1}$ GIK vs. 892 . $\mu U.ml^{-1}$ control, p=0.0001).

Primary Outcome

A Low Cardiac Output Episode was diagnosed in 47 patients. The incidence in the GIK group was 11/110 (10.0%) and in the Control group was 36/107 (33.6%). The use of GIK was associated with a significant reduction in the incidence of low cardiac output state (OR 0.22 95% CI 0.10 to 0.47, p= 0.0001) (Table 1). Pre-specified subgroup analyses revealed no heterogeneity in the effect of GIK on LCOE (Figure 3).

Secondary endpoints

Haemodynamic Data

Cardiac index was higher in the GIK group from starting the treatment until 12 hours after removal of cross clamp (p=0.0001) (Figure 2). Throughout the study heart rate, central venous pressure and pulmonary artery wedge pressure were similar between groups.

Inotrope and Vasoconstrictor Use

The use of GIK was associated with a significant reduction in the use of inotropes during the infusion period (n=35 patients (33.0%) in the control group *vs.* 13 patients (11.9%) in the treatment group; OR 0.27 (95% CI 0.13 to 0.57; p=0.0006). This difference continued from 6 to 12 hours (Table 2). The use of a GIK infusion was also associated with an increase in the prevalence of vasoconstrictor use in the period between baseline to 6 hour after removal of aortic cross clamp (48 patients (45.3%) in the control group vs. 70 patients (64.2%) in the GIK group p=0.005). The difference in vasoconstrictor use was reduced in the 6 to 12 hour period but evidence of increased vasoconstrictor requirements remained (Table 2; p=0.02).

Myocardial Injury

ECG evidence of myocardial injury occurred in 9 (8%) patients in the control group and 6 (5.5%) patients in the GIK group (p=0.67). When accounting for both surgeon and intention to perform CABG using a continuous outcome, there was no significant difference in plasma Troponin levels between treatment groups, at 6hrs the difference in means was -0.01 (95% CI -0.09 to 0.07, p=0.81).

Akt and AMPK signalling and Protein *O*-GlcNAcylation

Akt and AMPK signalling was studied in 16 patients (8 control and 8 GIK) and protein O-GlcNAcylation was studied in 16 (8 GIK 8 control) undergoing isolated AVR. There were no significant differences in the pre-operative demographics or in the echocardiographic markers of function between groups. Immunoblotting demonstrated a ~2.5 fold increase in the phosphor-Akt:pan-Akt ratio (p=0.03) and a ~1.7 fold increase in the phospho-AMPK:AMPK ratio (p=0.0004) (Fig. 4a and b respectively). Visual inspection suggested that there was more prominent O-GlcNAcylation in the GIK group .Individual band analysis suggested that in the GIK group a band of approximately ~60 kDa manifested ~1.85 fold more O-GlcNAcylation than controls, p=0.004. Protein loading was assessed by GADPH and β-tubulin. Band densitometry was performed using the Quantity One package.

Discussion

In this trial of GIK in addition to standard myocardial protection during AVR and combined AVR and CABG, GIK treatment resulted in a significant reduction in LCOE. There was an associated significant reduction in the use of inotropic support but an increase in the need for vasoconstrictor therapy. The addition of GIK however had no effect on the incidence of postoperative MI on ECG criteria or on serial plasma troponin release. We also report that GIK therapy was associated with an increase in dynamic post translational protein modification including AMPK and Akt phosphorylation and selective protein O-GlcNAcylation that may have contributed to GIK's beneficial cardioprotective effect

The results of this trial are likely to correspond to real-world clinical practice, the median logistic EuroSCORE of the patients not recruited during the study period was 5.1 (IQR range 2.3 to 8.2), compared to 4.5 (IQR 2.8 to 7.1) for patients enrolled, suggesting a non-likelihood of bias. Additionally, the incidence of inotrope usage in patients randomised to control is similar to that of patient's not enrolled (data not shown).

We previously studied the use of GIK in patients undergoing isolated CABG and demonstrated a similar improvement in cardiac haemodynamic performance and a reduction in the need for inotropic support [196], however, the magnitude of effect seen in relation to GIK therapy in this study was significantly greater than that which we have previously reported. All the patients randomised in this trial manifested significant LV hypertrophy secondary to aortic stenosis. LVH has been shown to be associated with an impairment in cardiac energetics manifested by a reduction in PCr:ATP ratio on MRS and a down regulation in metabolism [58, 76]. This energetically impaired myocardium may be more vulnerable to ischemia-reperfusion injury and may thus derive greater benefit from improved metabolism.

In both these studies, although hemodynamic indices were improved, no significant effect on plasma TnT levels was demonstrated. The discrepancy between improved haemodynamic performance and TnT release has led to speculation that rather than improving myocardial protection, GIK increases cardiac output simply by vasodilatation [254]. Consistent with this hypothesis, we have detected an increase in the need for vasoconstriction in patients treated with GIK. While there is evidence to suggest that TnT release has modest prognostic value in cardiac surgery[235], myocardial protection in cardiac surgery is highly efficacious and the majority of patients undergoing surgery have only modest TnT rise of insignificant value. Myocardial necrosis with TnT release is the end stage of a series of reversible and finally irreversible steps the myocytes undergoes during ischemia-reperfusion, and significant TnT release only occurs in a minority of patients undergoing cardiac surgery. Myocardial stunning however is more common following cardiac surgery, is likely to significantly contribute to LCOE, is not necessarily associated with TnT release [255] and may be responsive to GIK therapy. The dissociation of the influence of GIK on LCOE and TnT release may thus be explained by the impact of GIK on myocardial stunning rather than necrosis *per se*.

Recovery from peri-operative myocardial stunning involves normalisation of intermediary and oxidative metabolism resulting in restitution of myocardial energy reserves, reversal of cell swelling, and gradual accumulation of the total adenine nucleotide pool. The more established role for insulin in cardioprotection is through amelioration of myocardial metabolism including a switch from deleterious free fatty acid metabolism to more efficient glucose metabolism. Such a role for GIK has been extensively established in experimental studies [178], however, in addition to the metabolic benefits

conferred by GIK, recent studies have adduced a role for pH homeostasis [239], and insulin mediated anti-apoptotic and cardioprotective signalling pathway activation[180]. Thus while it is likely that the main cardioprotective action of GIK is through a shift to more efficient metabolism, we investigated whether myocardial signalling events contributed towards GIK-related myocardial protection.

Insulin, acting through the insulin receptor's tyrosine kinase activity, phosphorylates and activates insulin receptor substrate–1/2, which in turn activate PI3K. PI3K activation generates phosphoinositide-3,4,5 triphosphate, which mediates the phosphorylation and activation of Akt. In cardiomyocytes, Akt has been shown to protect against apoptosis after ischemia/reperfusion injury [180], and as a corollary, pharmacological inhibition of Akt has been shown to abolish the cardioprotective effect of insulin [180]. To the best of our knowledge, the present study is one of the first to confirm, in accordance with the extensive animal model literature associating Akt phosphorylation with insulin treatment, that GIK activates Akt, which is likely to contribute to the benefits noted herein. We also demonstrate a significant increase in AMPK phosphorylation. AMPK activation is known to play a crucial role in the regulation of cardiac energy metabolism, and is thought to be an adaptive mechanism in cardiac ischemia [240]. However the finding that AMPK phosphorylation is enhanced in the context of GIK, may be considered paradoxical [241]. This apparent "paradox" is predicated on the observation that insulin, via activation of Akt, inhibits AMPK by phosphorylation of Ser 485/491 of □-AMPK[242, 243]. Reconciling these observations, it appears that while high insulin concentrations, especially in the absence of lipid do indeed inhibit AMPK activity, the inhibitory effect of insulin on AMPK activity is relieved when insulin and lipid concentrations are more physiological (palmitate 0.2 - 1.2 mmol/L) [244]. Accordingly, we speculate that GIK in this study was able to activate Akt with its attendant benefits, but suppressed systemic lipid levels to such a permissive level that the beneficial effects of AMPK activation were manifest during ischemia-reperfusion [245]. The magnitude of kinase activation in this study may however have been mitigated by hyperglycemia secondary to glucose infusion. There is a compelling association between hyperglycemia and increased mortality from myocardial ischemia, whether in the context of myocardial infarction [256, 257] or coronary surgery [258]. This effect of hyperglycemia is, at least in part, due to the hyperglycemia-induced decrease of myocardial Akt activation and may have been relevant to the present GIK study [259]

We also observed a selective increase in protein *O*-GlcNAcylation. This is the first evidence of *O*-GlcNAcylation in humans and its potential interaction with insulin. Protein *O*-GlcNAcylation refers to the post-translational modification of serine and threonine residues of nuclear and cytoplasmic proteins by the O-linked attachment of the monosaccharide β-N-acetylglucosamine (*O*-GlcNAc). This modification is emerging as a key regulator of a number of critical biological processes including nuclear transport, translation and transcription, signal transduction and apoptosis and in the settings, activation appears to be an endogenous stress response designed to enhance cell survival [146]. In the isolated perfused heart, ischemia has been shown to increase overall O-GlcNAc levels [148] suggesting that this endogenous stress-activated pathway is active in the heart, and that increased O-GlcNAc levels, improved contractile function and decreased tissue injury after reperfusion [149, 155]. We speculate that insulin increases *O*-GlcNAcylation by increasing intracellular glucose flux and hence delivers cardioprotection. Which proteins are modified and the specificity of this process remains the subject of intensive investigation.

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Conclusion

In patients with significant left ventricular hypertrophy, the addition of peri-operative GIK therapy to standard myocardial protective techniques resulted in a significant reduction in LCOE. We observed a significant reduction in inotrope usage but a corresponding rise in vasoconstrictor usage, secondary to the known vasodilatation associated with insulin, consequently resulted in no reduction in post-operative resource utilisation. Although the mortality in this trial was significantly lower than predicted, this study was not powered to detect a difference in mortality. While we present novel human data supporting the role of metabolic modulation, insulin signalling pathway activation (e.g. via traditional pathways such Akt and AMPK activation), and protein O-GlcNAcylation in insulin mediated cardioprotection, this data is preliminary and necessarily only hypothesis generating. Further evidence will be required to ascertain whether this protective pathway is responsible for improved myocardial protection in the context of GIK therapy.

Study Group

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Conflict of Interest Disclosures

None.

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